BIODIVERSITY OF ANAEROBIC CELLULOLYTIC BACTERIA IN LANDFILL SITES

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Dissertation submitted in compliance with the requirements for the Master's Degree in Technology in the Department of Biotechnology, Technikon Natal, Durban.

I hereby declare that the dissertation represents my own work.

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I hereby approve the final submission of the following dissertation

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this 11th day of June 2001 at Technikon Natal.

DEDICATION

To my parents, Denzil and Sheila

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ABSTRACT

Landfills play an important role in the removal of waste from the surroundings. There is a limit to the types of waste that can be recycled and the landfill becomes the final method of waste disposal. Because waste constitutes a wide variety of materials, the microbial consortia that develop within a landfill will be equally varied, depending on the type of waste deposited, the temperature of the landfill and moisture content of the waste. The metabolism of these microbial consortia can result in products that are either harmful or beneficial. In order to increase the pool of knowledge on landfill microbiology, it is important to study the various consortia that inhabit the landfill to determine the various microbial interactions that occur and subsequently to manipulate these interactions to enhance the benefits of a landfill site and reduce the harmful effects. In this research, an attempt was made to isolate anaerobic cellulolytic bacteria from a landfill site. Six waste samples, varying in age were obtained over a period of two years. Samples were excavated from a maximum depth of 4m. Samples are processed in anaerobic, phosphate buffer and cultivated in various pre-reduced anaerobic media and incubated under anaerobic conditions. Samples were also collected from other potential anaerobic sites namely, anaerobic sludge, decomposing bagasse, compost, manure, rumen and pond sediment. Results of degradation of the cellulose source (Whatman No. 1 filter paper) indicated that it was possible to cultivate cellulose-degrading microorganisms from the landfill. Zones of clearing around colonies, which would be indicative of cellulose degradation on solid media, were not obtained. Samples from the anaerobic sludge, compost and rumen showed degradation of cellulose in liquid media but not on solid media. It is concluded that the solid media used was unsuitable for the cultivation of anaerobic, cellulolytic bacteria or that the anaerobic conditions employed were not adequate to initiate the growth of the anaerobic cellulolytic bacteria.

PREFACE

Some of the material presented in this dissertation has been presented and published elsewhere.

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CHAPTER 1

1.0. GENERAL INTRODUCTION

1.1 Solid waste management in South Africa

Historically, waste management in South Africa has operated with minimal legislative control. Until the promulgation of the Environment Conservation Act, No 73 of 1989, which specifically required that no waste disposal site should be established or operated without a permit and that waste should only be discarded on a site that has been issued with a permit, there was no framework of standards, procedures, or policies in which to execute effective control over waste disposal (Department of Water Affairs and Forestry, 1998).

In order to provide the standards and procedures necessary for the enforcement of the waste disposal site permitting system, the Department of Water Affairs and Forestry embarked on the Minimum Requirements project in 1994. The objectives of the Minimum Requirements for Waste Disposal by landfill are:

- To improve the standard of waste disposal in South Africa.
- To provide guidelines for environmentally acceptable waste disposal for a spectrum of landfill sizes and types.
- To provide a framework of minimum waste disposal standards within which to work and upon which to build (DWAF, 1998).

1.2 The role of the landfill in the waste management system

Waste has been disposed of on land, as this is the cheapest and most convenient method of waste disposal. It is estimated that in excess of 95% of the waste generated in South Africa is disposed of in landfills, while the world figure is believed to be in excess of 85%. (DWAF, 1998).

Regardless of the waste minimisation technologies implemented whether for volume reduction or resource recovery, some form of residue will always remain and waste will continue to be generated (DWAF, 1998).

1.3 Development of a landfill

In the past, a landfill represented an open hole or marsh where waste was dumped. Often, the waste was not covered properly, sometimes it was burned for volume reduction, and there was little effort to control storm water runoff and downward migration of water that had come into contact with the waste (Barlaz, 1997). Today landfills have become highly engineered facilities and may be variously regarded as an:

- ultimate option which may require further treatment facilities for generated products
- anaerobic digester for methane generation or
- anaerobic filter for industrial liquid and sludge effluent treatment (Senior,1990)

1.4 Classification of landfills

Landfills differ from one another in terms of size, type and potential threat to the environment. Using the classification system developed by the DWAF, (1998) landfills are grouped according to:

- The type of waste involved
- The size of the waste stream, and
- The potential for significant leachate generation (DWAF, 1998.)

Two classes of waste are general and hazardous wastes. General waste is a generic term of waste that, because of its composition and characteristics does not pose an immediate threat to public health or the environment if properly managed. Examples include domestic, commercial, certain industrial wastes and builders rubble (DWAF, 1998). It may, however, with decomposition, infiltration and percolation, produce leachate with an unacceptable pollution potential (DWAF 1994). General waste is also referred to as municipal solid waste

(MSW). Hazardous waste is waste that can, even in low concentrations, have a significant adverse affect on public health and or the environment. This would be because of its inherent chemical and physical characteristics, such as toxic, ignitable, corrosive, carcinogenic or other properties (DWAF 1998).

1.5 Alternatives to landfills

Alternatives to landfilling as a waste disposal option are numerous. These include thermal treatment, physiochemical treatment, biological treatment or bioremediation, and recycling or resource recovery (Lombard *et al.*, 1992). Most of the options mentioned above are often costly and result in residues or discards, which will require ultimate disposal (Lombard *et al.*, 1992). In addition, they carry problems of their own. For example, recycling is hampered by market instability for certain materials and there are technical barriers for the recycling of mixed materials. Biological treatments like composting can cause environmental pollution, often compounded by the production of pathogenic organisms (Rosenberg, 1993).

1.6 Sanitary landfills

Sanitary landfilling is a method of disposing of waste on land without causing nuisances or hazards to public health or safety. It uses the principles of engineering to confine the waste to the smallest practical area, to reduce it to the smallest practical volume and to cover it with layer of earth at the conclusion of each day's operations or at such less intervals as may be acceptable (DWAF, 1998). Vegetation serves to minimize erosion of the final cover and to promote evapotranspiration.

A sanitary landfill consists of several major components (Barlaz, 1997). The lowest component is the liner system, which includes layers;

(i) to minimize leachate migration to the groundwater and

(ii) to collect leachate for treatment.

A common system used to minimize leachate migration begins with a layer of low permeability soil like clay. A flexible membrane liner (FML) is often placed above the clay layer. Together

the soil and the FML are referred to as the composite liner. A drainage layer designed to promote the collection of leachate is placed above the composite clay liner. This layer consists of sand. Slotted pipe is placed in the sand layer at intervals of 30 to 60m to collect leachate to route it to a treatment system. A protective barrier is then installed above the leachate collection system to protect it from the equipment used to place and compact the waste. Waste may then be placed above the protective layer (Barlaz, 1997). A final cover is then applied after compaction, which includes a layer of low permeability soil, overlaid with a layer that will support vegetative growth.

Methane and carbon dioxide are the major terminal products of anaerobic decomposition in landfills. These and other gases are typically vented through wells placed in the waste to minimize their migration off site. The gas may then be vented, flared to reduce the release of methane or recovered as energy (Barlaz, 1997).

1.7 Gas production in a sanitary landfill

The breakdown of organic material in the absence of oxygen results in the production of methane (CH_4), carbon dioxide (CO_2) and other volatile organic compounds. The resulting gas mixture is saturated with moisture and is referred to as landfill gas.

Moisture content of the waste placed in a landfill is an important factor that affects the landfill gas production. The moisture content of waste in a landfill may change over time depending on the rainfall, control of run-off and the management of the site. In general, the higher the moisture content, the greater the gas production (Lombard *et al.*, 1998).

Table 1.1 Typical composition of landfill gas (Lombard *et al.*, 1998)

Component	% by Volume
CH_4	64
CO_2	34
H_2	2

Methane (CH_4) is a colourless, odourless, flammable, non-toxic gas that is lighter than air with a vapour density of 0.6. CH_4 is explosive between the concentrations of 5% - 15.5 by volume of air. Carbon dioxide (CO_2) is a colourless, odourless, non-flammable toxic gas that is heavier than air with a vapour density of 1.53. At a level of 3% by volume in air, breathing is laboured and with resultant headaches. The accepted safety limit for CO_2 is 1.5% by volume in air and the concentrations above this limit are regarded as hazardous (Lombard *et al.*, 1998).

Landfill gas from an active extraction scheme usually has a CH_4 content of 40-50%. Lombard *et al.*, (1998) have found that in the water surplus areas of Kwa-Zulu Natal, landfill sites that are anaerobic, with a depth greater than 2m and sufficiently moist, will begin to generate sufficient volumes of landfill gas within a period after 6-9 months after deposition.

There are various uses for landfill gas according to Lombard *et al.*, (1998). However, in South Africa the relatively low cost of energy is a serious constraint on the viability of the uses of landfill gas. The primary economic benefit of landfill gas extraction, whether by flaring or recovering it for use lies in extending the life of the landfill sites. This occurs when airspace is conserved due to the accelerated settlement of the landfill as a result of optimising the catabolism of the biodegradable fraction of the waste (Lombard *et al.*, 1998).

1.8 Leachate production in a sanitary landfill

One of the most important aspects that must be taken into consideration during the operation and management of landfills is their capacity to generate leachate and particular attention must be paid to the protection of surface and groundwaters (Senior, 1995).

Under normal circumstances general waste has moisture content of between 10 % and 20% when it reaches the landfill site. Without the addition of excess moisture from an external source, the rain infiltrating the waste should evaporate as a result of the average annual evaporation exceeding the rainfall, thus resulting in a negative water balance. Should further moisture however be added from an external source the water reaches a point at which it has no more absorption capacity, with the field capacity of the waste, the excess moisture is released as leachate. This moisture is usually very dark in colour with a distinctive odour (du Preez and Pieterse, 1998).

A pertinent environmental parameter affecting leachate composition is the age of the landfill. Leachate from young landfills contain high chemical oxygen demand (COD) as a result of the presence of fatty acids. They also tend to be easily degradable and are characterized by high biological oxygen demand to chemical oxygen demand ratios (BOD:COD), higher pH and lower heavy metal concentrations (Harris, 1988). As the proportion of liable organic compounds decreases with age, biological leachate treatment processes tend to become less effective (Henry *et al.*, 1987).

Leachate formed from general waste can be high in iron, chlorides, organic nitrogens, phosphate and sulphates as well as pathogens. If released directly into the environment, it could present a health risk, as both surface and ground water resources being used by the surrounding community could be contaminated (du Preez and Pieterse, 1988). du Preez and Pieterse, 1998 performed a comparison between landfill leachate and raw sewage typical of Southern Africa to highlight the pollution potential of landfill leachate.

Table 2.1 Comparison of landfill leachate and raw sewage typical of Southern Africa

(Lombard *et al.*, 1992)

Constituent	Leachate (mg/*l*)	Raw Sewage (mg/*l*)
pH	4.9 - 8.4	6.8 – 7.5
COD	246.0 – 75 000.0	72.0 – 1 500.0
Chlorine	116.0 – 2 096.0	30.0 – 79.0
Nitrogen	0.2 – 1 106	1.6 – 33.0
Cadmium	0.003 – 17.0	0.004 – 0.016
Total Chrome	0.03 – 0.18	0.05 – 0.13
Copper	0.03 – 0.75	0.03 – 0.11
Total Iron	2.0 – 1000.0	0.35 – 5.6

1.9 Waste composition in a sanitary landfill

The waste deposited in landfill is a heterogeneous mixture including putrescible material (subject to microbial degradation), plastic, glass, ash and metal (Cummings and Stewart, 1994). The putrescible fraction, being the largest portion of the waste is more useful for biodegradation studies (Barlaz, 1997).

Published work indicate that cellulose and hemicellulose are the principal biodegradable components of waste (Barlaz, 1997) these having their source from paper and paper-related products which were discarded. Garden trimmings also contribute to an increase in cellulose and hemicellulose concentrations. The other major organic component of waste, lignin is recalcitrant under anaerobic conditions (Young and Frazer, 1987). In addition, lignin interferes with the decomposition of cellulose and hemicellulose by physically impeding microbial access to these degradable carbohydrates. Other biodegradable organics present in smaller concentrations are proteins and soluble sugars (Barlaz, 1997).

1.10 Biological decomposition of waste

The decomposition of waste to methane is a microbially mediated process that proceeds along pathways similar to those documented for anaerobic sludge digestion (Barlaz and Ham, 1990).

Landfill bacteria are a wide range of organisms capable of utilizing many components of landfill waste for growth. The bacterial populations present in a landfill can be defined in terms of trophic groups, that is, by the substrate, which they utilize for growth (Cummings and Stewart, 1994). These may be divided into hydrolytic bacteria, which hydrolyse polymers such as cellulose, acidogenic bacteria which convert sugars to carboxylic acids, and acetogenic bacteria and methanogens which convert carbon dioxide and hydrogen to acetate and methane respectively (Cummings and Stewart, 1994).

Despite the presence of thousands of landfills worldwide, their microbiology is poorly characterized compared to that of anaerobic sludge digesters (Mah and Sussman, 1967; Chatrain and Ziekus, 1986 cited in Barlaz and Ladapo, 1997), the rumen (Byrant, 1959; Miller *et al.*, 1986 cited in Barlaz and Ladapo, 1997) and rice paddies (Murase and Kimura, 1994 cited in Barlaz and Ladapo, 1997). In contrast to the rumen and sludge digesters, retention times in landfills are essentially infinite. However, the ecosystem changes over time with respect to pH and the nature of available carbon (Ladepo and Barlaz, 1997).

When the waste is deposited, a complex series of biological and chemical reactions are initiated. Initially oxygen entrained in the refuse at burial is depleted, and the system becomes anaerobic. Due to an imbalance among the fermentative, acetogenic and methanogenic activities, carboxylic acids accumulate once a landfill becomes anaerobic, resulting in an acidic pH. Over time, acetogenic and methanogenic activities increase, leading to vigorous methane production. Initially methane production results from consumption of the accumulated soluble fermentation products. Later, methane production is dependent upon hydrolysis of cellulose

and hemicellulose, the principal biodegradable components of refuse. (Barlaz and Ladapo, 1997). In the presence of nitrate or sulphate, electrons will be diverted to these electron acceptors prior to and concurrent with methanogenesis respectively (Barlaz *et al.*, 1989).

1.11 Decomposition of cellulose

Cellulose is a major component of landfill waste, which has been shown to contain approximately 30% paper (ETSU 1993 cited in Westlake *et al.*, 1995). Processed paper not only constitutes the vast majority of cellulosic waste, but also comprises a very large part of environmental waste as a whole. These include newspapers and magazines, telephone books and packaging materials (Rosenberg, 1993). There is substantial evidence that paper, particularly newspaper is recalcitrant to degradation and can persist in landfill for decades (Booth 1965 cited in Cummings and Stewart, 1994). What causes this apparent lack of degradation is unclear. It may be due to physical factors of the landfill environment, or the paper itself may be resistant to microbial attack (Cummings and Stewart, 1994).

Fermentation of cellulose yields a range of volatile acids, which make up a major part of the leachate organic carbon in the early stages of waste degradation (Pohland, 1975) and will when present, increase the leachate hazard. The acids will also act as substrates for methanogens, thus contributing to the risks associated with the migration of landfill gas. According to Barlaz *et al.* (1989), the degradation of cellulose and hemicellulose components of MSW accounted for 91% of the methane generation of waste samples studied.

Despite the importance of cellulose hydrolysis to energy generation from landfill gas and to the potential for environmental pollution arising as a result of leachate migration, very little is known of the cellulolytic bacteria within landfill or their biochemical properties (Westlake *et al.*, 1995). Westlake and Archer (1990) obtained nine pure cultures of cellulolytic bacteria from various sites. Seven were identified as *Eubacterium*; the remaining two were *Clostridia*. Another study isolated ten cellulolytic bacteria from a solid waste digester, believed to be

broadly based study enumerated the bacteria from three landfills in the USA. This found large numbers of fermentative bacteria, but failed to identify any cellulolytic isolates (Palmisano *et al.*, 1993). The authors provided the following reasons; (i) the numbers of cellulolytic bacteria were low in the landfill sampled (ii) difficulty in extracting the bacteria from refuse (iii) inadequacy of cellulase detection or their inability to grow on solid media provided.

Xingdong and Barlaz (1996) reported the occurrence of cellulolytic bacteria in fresh refuse samples. Publications by Barlaz, Schaefer and Ham (1989 a, b) have identified techniques for enumerating cellulolytic bacteria from landfill and have monitored the population development of key groups of bacteria as well as methane production in refuse on a laboratory scale. These studies showed that polymer hydrolysis was the rate-limiting factor in the conversion of waste to methane. The cellulolytic bacteria play a major role in polymer hydrolysis within landfill and therefore a greater understanding of these bacteria is essential to our understanding of waste degradation (Westlake, 1990). By isolating and characterising cellulolytic bacteria from landfill optimum growth conditions can be identified and this information used in formulating landfill practices for maximum landfill gas production (Westlake, 1990) and prevention of pollution.

1.12 Aims and Objectives

The objective of this study is to provide information on the bacterial species responsible for the anaerobic degradation of cellulose in nearby landfills by;

(i) Implementing published anaerobic techniques to allow for the isolation of cellulose degrading organisms.

(ii) Characterizing each isolate morphologically and biochemical

The aim would be to show which species anaerobic cellulolytic organisms were present in the landfill and in what approximate numbers. Also, to characterize the organisms morphologically and biochemical. This research is preliminary in enhancing the rate and extent of cellulose degradation within the landfill, by obtaining a better understanding of the microorganisms that contribute to cellulose biodegradation, thereby resulting in greater production of methane gas and possibly faster stabilization of the landfill.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Waste Management in South Africa

Until 1997, waste management was not regarded as a national priority issue in South Africa. The waste management that took place focused mainly on waste disposal and was reactive in that it addressed needs as they occurred. Holistic integrated waste management planning was rarely, if ever undertaken. The low priority that was historically accorded to waste management has resulted in waste impacting detrimentally on the South African environment and on human health (Report 970296, 1999).

Today the Integrated Waste Management (IWM) approach maintains that waste management can be planned in advance (DWAF, 1988). Advance planning means an orderly process can ensue involving:

- Waste prevention : the prevention and avoidance of the production of certain hazardous wastes or hazardous substances, sometimes by regulation.

- Waste minimisation: the economic reduction of the volume of waste during production, by means of different processes or clean technology.

- Resource recovery: recycling or the recovery of energy through incineration and biodegradation.

- Waste treatment: the treatment of waste to reduce waste volumes of hazardousness.

- Waste disposal: the environmentally safe disposal of waste (DWAF, 1988).

2.2 Waste disposal on landfills and it's environmental impact

Landfills are the most widely used option for waste disposal throughout South Africa; almost every municipality has a landfill site. There are estimated to be 1 200 landfill sites in South Africa. All these sites, according to current legislation must be licensed by the Department of Water Affairs and Forestry. However, only 300 permits for disposal sites were issued by mid 1998. Thus, at the time many municipal landfills were not yet licensed and not all licensed sites were run according to the licence requirements. Furthermore, there were many other small landfills outside municipal boundaries on farms and plots which were unlicensed and unregulated, hence the promulgation of the Environment Conservation Act, No 73 in 1989.

Uncontrolled landfilling can have an adverse impact on the environment. These can be divided into short term and long term impacts. Short-term impacts include problems such as noise, flies, odour, air pollution, unsightliness and windblown litter. Such nuisances are generally associated with a waste disposal operation and should cease with the closure of the landfill. Long-term impacts include problems such as pollution of the water regime and landfill gas generation. Such problems are generally associated with incorrect landfill site selection, design, preparation or operation and may persist long after the landfill site has closed (DWAF, 1988).

2.3 Landfills as a waste repository

A landfill is a disposal alternative for solid waste as well as certain industrial wastewater treatment sludges and agricultural residues. There is a limit to the types of waste that can be recycled or composted and combustion is typically more expensive than a landfill (Barlaz, 1997), thus disposal in a landfill is a simple and inexpensive way to dispose of solid waste.

In a landfill, both organic and inorganic wastes are deposited in low-lying land that has minimal property value. Each day's waste deposit is covered with a layer of soil to prevent odour problems, the attraction of rodents and insects and to prevent fires from occurring. In this way a sanitary landfill is created. Biological decomposition of waste then takes place.

2.4 Biological refuse decomposition

Biological decomposition of refuse in landfill does not occur immediately. A period ranging from months to years is necessary for the proper growth conditions and the required microbiological system to become established. Thus, most research on refuse decomposition has been conducted by using laboratory simulations in which the rate of decomposition is accelerated. (Barlaz, 1997). A characterisation of refuse decomposition describing chemical and microbiological characteristics has been developed by using the data from laboratory-scale reactors (Barlaz, 1989). Refuse decomposition is described as occurring in an;

- aerobic phase
- anaerobic acid phase
- accelerated methane production phase and
- a decelerated methane production phase

The description in summarized in Figure 2.4.1.

In the aerobic phase (phase 1), both oxygen and nitrate are consumed, with soluble sugars serving as the carbon source for microbial activity. All the trophic groups required for refuse methanogenesis (cellulolytics, acetogens and methanogens) are present in fresh refuse, though there is little change in their populations. In the anaerobic acid phase (phase 2), carboxylic acids accumulate and the pH decreases as result of an imbalance between fermentative activity and acetogenic and methanogenic activity. There is some cellulose and hemicellulose decomposition in phase 2. The methanogen population

begins to increase and methane is detected in landfill gas. In phase 3, the accelerated methane production phase, there is a rapid increase in the rate of methane production to some maximum value. A methane concentration of 50 to 60% is characteristic of this phase, as are a decrease in carboxylic acid concentrations, an increase in pH, little hydrolysis of solids and increases in the population of cellulolytic, acetogenic and methanogenic bacteria. The accumulated carboxylic acids are the principal substrate supporting methane production in this phase. The fourth phase is termed the decelerated methane production phase. The methane concentration, pH and cellulolytic and methanogenic populations remain at values similar to those in phase 3. Concurrently the methane production rate decreases the acetogenic population increases and the carboxylic acids are depleted. In addition, as carboxylic acid concentrations decrease, there is an increase in the rate of cellulose plus hemicellulose hydrolysis. While acid utilisation limits methane production in phase 2 and 3, solids hydrolysis limits methane production in phase 4.

There are limitations to the to the four-phase description of refuse decomposition presented here as it applies to full-scale landfills. First the time required for the onset of each phase may be significantly longer that than the times shown in Figure 1. Second gas and leachate samples from the landfills may reflect a composite of refuse in several different states of decomposition, depending on the manner in which landfills are filled and leachate and gas are collected. Third, in the presence of significant sulphate concentrations, electrons would be diverted from methane production to sulphate reduction. Fourth, the presence of nitrate would stimulate denitrification and nitrogen gas production would inhibit methanogenesis.

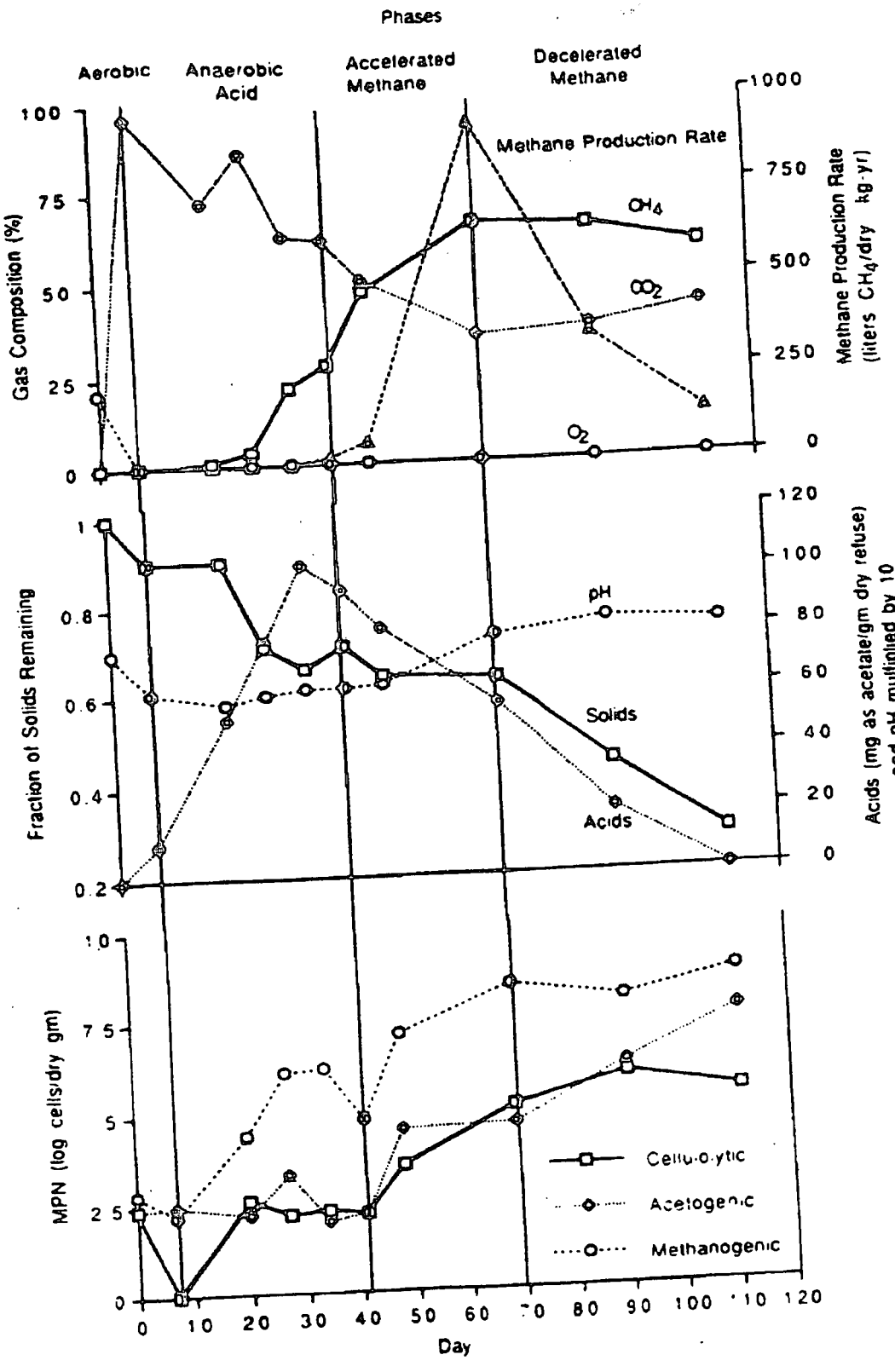

Figure 2.4.1 Summary of observed trends in waste decomposition with leachate recycle (Barlaz et al., 1989 in Barlaz, 1997)

2.5 Factors limiting decomposition in landfills

A number of factors including moisture content and moisture flow, pH, particle size, inoculum addition, nutrient concentrations and temperature have been shown to influence the onset and rate of methane production in a laboratory scale reactor (Barlaz *et al*, 1990). All these variables will affect the environmental conditions present in a landfill and these conditions in turn control microbial growth. The two variables that appear to be most critical in controlling refuse methanogenesis are moisture and pH. Adequate moisture and pH about neutral are required for refuse methanogenesis. In addition, high moisture will promote the dissolution and mixing of soluble substrates and nutrients and will provide a mechanism for microbial transport within a landfill. While mixed refuse contains all the microbes required for refuse decomposition, they are undoubtedly not well distributed among all the degradable components of refuse (Barlaz, 1997).

2.6 Microbiological studies

A major challenge in studying landfill microbiology is the issue of system heterogeneity. To address this issue researchers have developed a number of approaches, including small grab samples that are not representative, multiple replicate samples at full scale landfills and laboratory simulations with shredded refuse. The appropriate system for the study of landfill microbiology as well as the appropriate measures will be unique for each set of research objectives. The types of measurements used to study landfill microbiology are analogous to those used in other ecosystems and include total and viable cell counts, enzyme activity, carbon turnover and the production of terminal end-products (Barlaz, 1997).

2.6.1 Systems for the study of refuse decomposition and landfill microbiology

A number of systems have been used to study refuse decomposition and landfill microbiology, with each system typically optimised for a specific objective.

The ideal landfill simulation is a field-scale landfill containing several thousand kilograms of refuse. While a system of field-scale test cells represents a near-perfect landfill simulation, the system has its drawbacks: it is expensive, and the nature of the simulation makes it difficult to obtain representative samples and to ensure 100% recovery of the gas produced. Furthermore, obtaining the desired data requires several years of monitoring. Even when field-scale test cells are constructed, variability can be a problem. Given the time and expense associated with field-scale cells the majority of work on parameter assessment has been conducted by using laboratory scale simulations.

As an alternative to the construction of field-scale test cells, refuse samples can be collected from full-scale landfills for certain study objectives. Such samples may be used for the isolation or enumeration of bacteria, enzyme assays, measurement of methane production rate of an excavated sample and measurement of chemical parameters such as pH, solids (cellulose, hemicellulose, lignin), and soluble organics.

Where laboratory simulation of a landfill is desired, reactors ranging from 2000 to 3.000 litres have been used. The largest reactors were about were about 4.2m long and 1m in diameter and held approximately 400kg of refuse (Pohland and Gould, 1986). These reactors were large enough to obtain representative samples of refuse. However, their size made them difficult to control and manipulate and expensive to replicate. In addition it would not be possible to obtain representative sub-samples over time.

Work has also been conducted in 2-to-4 litre reactors containing 1 to 2 kg of shredded refuse (Barlaz *et al*, 1989; Rhew and Barlaz, 1995). In the initial use of these reactors, multiple replicate reactors were set up and were destructively sampled over time to monitor microbial population development during decomposition (Barlaz *et al,* 1989). Recently this concept has been expanded to add a ^{14}C-radiolabeled material to the reactor to evaluate its conversion to $^{14}CH_4$ and $^{14}CO_2$ under the simulated landfill conditions (Bonner *et al,* 1995). The use of shredded refuse makes it possible to work

with smaller quantities of refuse and still have a representative sample. However, shredding refuse provides a degree of mixing not typically found in landfills. This mixing, coupled with the decreased particle size, likely enhances biodegradation.

Irrespective of the quantity of refuse used, the time to the onset of methane production varies. In the absence of some enhancement, 10 to 20 years may be required to complete the refuse decomposition cycle. This incubation period is not practical for many experiments and strategies to enhance decomposition in laboratory scale systems have been developed. These include:

- incubation at about 40°C
- the use of shredded refuse and
- the use of leachate recycle and neutralization.

It has been reported that 40°C is the optimum temperature for mesophilic refuse decomposition (Jones and Grainger, 1983).

Leachate recycle and neutralization involve addition of sufficient moisture to a refuse sample during the reactor filling operation to allow for the free drainage of liquid into a liquid collection system. This liquid can then be externally neutralised and recycled through the top of the rector. If needed or as part of the test method, nutrients could also be added prior to recycling. This process will move refuse from the anaerobic acid phase to the accelerated methane production phase in 1 to 2 months.

Another technique that has been successfully used to accelerate the onset of methane production in laboratory-scale systems is the addition of a seed. A seed of well-decomposed refuse has been shown to repeatedly initiate decomposition of fresh refuse or individual components thereof (Barlaz, 1992). It will eliminate the anaerobic acid phase of decomposition, and methane production will begin almost immediately. Because carboxylic acids will not accumulate during the anaerobic acid phase, they will not be

present in large concentrations, and the increase in the methane production will not be as sharp as it would be in the absence of the seed.

In the absence of such enhancement tests to assess the impact of variables would take years to complete. Work has been conducted to show that there are no significant differences in the total anaerobic population or sub-populations of cellulolytic, hemicellulolytic, acetogenic or methanogenic bacteria in refuse incubated with or without leachate recycle (Barlaz *et al*, 1992).

The biochemical methane potential (BMP) technique has been modified for use in studies to assess the ultimate biodegradability of refuse samples. The BMP test was originally developed to measure the anaerobic biodegradability of soluble organic chemical in a small batch reactor such as a 125m/ serum bottle (Shelton and Tiedje, 1984). It has since been adapted in various ways for measurement of the biodegradability of solid samples (Owens and Chynoweth, 1993; Wang *et al*, 1994).

Anaerobic biodegradation is mediated by the activity of bacterial consortia with a wide range of activities and syntrophic interactions. Because of the syntrophic interactions, the study of individual members of anaerobic consortia has always been difficult. A multi-stage continuos culture technique has been applied to the study of landfill microbiology (Coutss et al, 1987; James and Watson, 1993; Parks and Senior, 1988). In this system, reactor vessels of increasing size are connected in series and the vessels are operated as a plug flow chemostat. As the reactor size increases, the imposed dilution rate decreases. This selects for faster growing bacteria in the top vessel and the slower growing bacteria in subsequent vessels. This technique is reported to separate habitat domains while still permitting the overlap of activity domains required for syntrophic activity. The operating requirements of this system are fairly complex.

2.7 Sample collection and processing

Refuse samples required for filling reactors to begin decomposition studies can be obtained from refuse collection vehicles en route or upon delivery at a landfill or combustion facility. The major precaution is to verify that the refuse originated in residential areas only and does not contain unusual materials (Barlaz, 1997). Refuse may also be excavated from a landfill either for use as a seed or to obtain samples for microbiological and chemical analyses.

Representative samples are more important when refuse is to be excavated from a landfill for microbiological and chemical analyses. Given the heterogeneous nature of landfills, the best approach is to obtain multiple samples over a preselected grid pattern. Samples may be obtained either with a bucket-auger that has the capability to obtain deeper samples (Sulfita *et al.*, 1992). The process of bringing refuse to the surface with an auger will likely reduce the particle size sufficiently that shredding is not required. Refuse excavated with construction equipment will probably require shredding. In each case, large samples (approximately 1000 kg) should be excavated and then reduced by using quartering techniques to obtain as representative a sub-sample as possible.

When excavated samples are to be used for microbiological analyses, there is often concern regarding the effect of sample exposure to air. The microorganisms responsible for the terminal reactions in refuse decomposition-acetogens and methanogens-are obligate anaerobes. Those responsible for the hydrolytic and fermentative processes may be facultative or obligate anaerobes. It has been shown that exposure of refuse samples in an active state of methane production to air for 2 to 4 h does not decrease the time required for these samples to resume methane production once replaced in an anaerobic system (Bogner, 1990;Bogner, 1992). While practical steps to minimise air exposure are warranted, extraordinary steps are not.

2.8 Processing samples for microbiological analyses

The first step in many microbiological measurements is the formation of a liquid extract from the excavated solids. The basic technique of this study was to blend refuse in anaerobic phosphate buffer (23mM, pH 7.2) for about 60s in a 4*l* blender. The buffer was boiled under nitrogen but a reducing agent, cysteine hydrochloride was not added because some oxidation occurs during the inoculum formation procedure and the oxidized form of cysteine exerts toxicity. The blended material was then squeezed by hand (covered with gloves) and the squeezings were used as the inoculum (Barlaz, 1997).

Treatments evaluated to increase the efficiency of cell extraction included pre-chilling the refuse at 4°C, multiple blendings and hand squeezings, and the use of blended refuse prior to hand squeezings. The additional treatments did not increase the most probable number (MPN) of cellulolytic bacteria above the population measured by blending followed by hand squeezings. The inoculum formation technique was validated by the addition of a spike of rumen fluid to refuse followed by the recovery of the spiked cellulolytic bacteria, using the inoculum formation technique and MPN enumerations. The results showed no evidence that the refuse was exerting a toxic effect on the rumen cellulolytic bacteria or that the refuse bacteria were irreversibly attaching to the refuse. The use of a blender was appropriate because all work was conducted with shredded refuse.

The efficiency of a stomacher for extraction of microbial cells from refuse was evaluated (Maule *et al.*, 1994). It was reported that 93.4% of the cells that were extractable were extracted in two cycles of the extraction procedure.

2.9 Microbiological measurements

Techniques that have been used for the enumeration of landfill bacteria include acridine orange direct counts (AODC) (Palmisano *et al,* 1993), MPNs (Barlaz et al, 1989; Qian and Barlaz, 1996), agar plate counts (Maule *et al,* 1994) and roll tubes (Palmisano et al, 1993;

Westlake and Archer, 1990). Irrespective of the enumeration technique the first step must be the formation of a liquid inoculum.

Adaptation of the AODC procedure to refuse has been presented by Palmisano *et al* (1993). Briefly, between 0.5 and 1g of refuse was fixed in formaldehyde. Samples were the diluted to a known volume in sodium pyrophosphate and incubated for 5 to 10 min. Next samples were cooled to 0°C and alternately sonicated and cooled. Samples were then stained, filtered and counted. The medium for enumeration of the total anaerobic population contained ten soluble carbon sources (cellobiose, glucose, maltose, xylose, galactose, arabinose, mannose, starch, glycerol and galacturonic acid), each at a concentration of 2.5mM. Carbon sources were representative of refuse hydrolysis products. Microbial growth on cellulose was detected by visible disappearance of ball-milled Whatman no. 1 filter paper. Filter paper strips would be more representative of the particle size of the refuse.

Acetogenic bacteria were enumerated on the basis of conversion of butyrate (40mM) to acetate and hydrogen and subsequent conversion of the hydrogen to methane by a pure culture of Methanobacterium *formicicum* (Mackie and Byrant, 1981). The methane concentration in tubes containing butyrate was compared with those in tubes without butyrate at each dilution. Tubes in which the methane concentration was significantly greater than that in the controls could be considered positive. Methanogen MPN tests were performed with either 80mM acetate or 202.6kPa of H2/CO2. Tubes were considered positive if they contained greater than 0.5% (vol/vol) methane. MPN tubes were incubated for 30 days except for the acetogen tubes, which were incubated for 30 days. Roll tubes have been used for the enumeration and isolation of cellulolytic bacteria from refuse (Palmisano *et al*, 1993; Westlake and Archer, 1990). In this procedure, the tubes are inspected visually or with the assistance of a microscope for a zone of clearing indicative of the consumption of insoluble cellulose. Researchers typically used ball-milled cellulose as the cellulose source.

2.10 Habitat simulation and incubation conditions

The cultivation media and growth conditions that have been used with anaerobes are as diverse as those that have been used with non-anaerobic microorganisms. When attempting to isolate a new anaerobic microorganism, different sets of factors have to be evaluated to decide on the type of growth conditions and media to be used. If the organism to be isolated is part of the indigenous microbial population, then its nutritional and physiological makeup will reflect the nutrient status and physiochemical properties of its natural habitat, namely the inoculum source. Thus the use of an isolation medium that simulates the properties of the natural habitat will enhance the probability of isolating the desired microorganism. In many instances, information on the nutrient mineral makeup of the natural habitat is not known, and a chemically defined, habitat-simulating medium cannot be readily formulated. In these cases an alternate approach can be the incorporation of an extract made from the habitat into the isolation medium. For example, in studies of the rumen microflora, clarified rumen fluid is typically added to the medium. In landfill microbiology, adjustments can be made to the medium pH and incubation temperature. (Barlaz, 1997).

Another important aspect in formulating growth media for anaerobic microorganisms is determining the type of reducing agent to use. The exclusion of oxygen from all types of media and buffers is needed to avoid oxygen toxicity, but the addition of reducing agents is necessary to obtain low levels oxidation-reduction potentials. Cysteine hydrochloride and sodium sulphide are the preferred compounds and are used either alone or in combination with one another. These agents should be prepared in solutions and added to growth media that have been made basically oxygen-free. Reducing agents should not be used as a major means of removing oxygen, because the interaction of these agents, especially cysteine hydrochloride, with oxygen will produce excessive hydrogen peroxide or oxidized sulphur compounds, which will be toxic to the microorganisms.

Finally, since most anaerobes produce short-chain fatty acids as fermentation products, a lowering of the pH in the medium during growth will occur. Thus most media need to contain some type of buffer. The most simple and commonly buffer system is carbon dioxide-bicarbonate. Besides using buffers in growth media, one can help prevent low-pH effects by limiting the amount of fermentable substrate to about 0.3% (wt/vol) or less, by periodically adjusting the culture medium by alkaline addition; or by the inclusion of larger amounts of peptide materials, from which ammonia can be made by microbial degradation.

2.11 Molecular techniques for the study of landfill microbiology

The limitations of traditional laboratory culture techniques for the study of microorganisms in the environment are well known and are unique to landfill microbiology. These limitations have stimulated the development of a number of molecular techniques.

Antigenic fingerprinting has been used to characterize methanogens isolated from refuse samples. The relatedness of seven methanogenic isolates to a reference methanogen culture collection was reported (Effland, 1977). However this technique can be applied only to previously isolated species and does not aid in the detection of uncultured species. Maule et al (1994) were the first to apply a DNA-based technique to landfill samples. They prepared 13 species of methanogens and used them to check for each species in landfill leachate. Five species were shown to be present. The authors concluded that "if suitable genetic markers for the detection and quantification of other microbiological groups such as acetogens, cellulose degraders and sulphate reducers can be found, DNA-based systems may supersede culture based methods for monitoring microbiological changes in landfill." Raskin *et al* (1994) monitored the start-up phase of an anaerobic digestor by using oliginucleotide probes complementary to conserved tracts of the 16s rRNSs of phylogenetically defined groups of methanogens. Silvey and Blackall (1995) used denaturing gradient electrophoresis to monitor population shifts in leachate from a

laboratory-scale landfill simulation. While the use of molecular techniques holds much promise for advancing our understanding of landfill microbial ecology, results to date are preliminary (Barlaz, 1997).

2.12. Cellulose degradation in anaerobic environments

2.12.1 Cellulose as a substrate

Cellulose in the most abundantly produced bioploymer in terrestrial environments. Cellulose is a homopolymer consisting of glucose units joined by B-1.4 bonds. The disaccharide cellobiose is regarded as the repeating unit in cellulose in as much as each glucose unit is rotated by 180° relative to it's neighbour (Leschine, 1995).

2.12.2 Cellulolytic enzyme systems of anaerobes

One of the most important features of cellulose as a substrate for microorganisms is its insolubility. Bacterial and fungal degradation of cellulose and other insoluble polymers occurs exocellularly, either in association with the outer cell envelope layer or extracellularly. To function these enzyme systems must be stable in the exocellular environment e.g. they must be reasonably resistant to proteolytic attack. Also the products of cellulose hydrolysis may be available as carbon and energy sources for other microbes that inhabit environments in which cellulose is biodegraded, thereby forming the basis of many interactions between microorganisms in these environments.

The mechanism by which cellulases from anaerobic bacteria catalyse the depolymerization of crystalline cellulose is poorly defined, despite numerous investigations. However this mechanism is fundamentally different from that of the cellulase systems of most aerobic fungi and bacteria. In early studies of bacterial cellulases the fungal system was employed as a model. The cellulase system of fungi (eg. *Trichoderma reesei*) comprise three main activities; (a) endoglucanases, which randomly hydrolyse 1.4-B bonds within cellulose molecules, thereby producing reducing

and non-reducing ends; (b) exoglucanases, which cleave cellobiose units from the non-reducing ends of cellulose polymers; and (c) b-glucosidases, which hydrolyse cellobiose and low molecular weight cellodextrins, thereby yielding glucose. These enzymatic components act synergistically in the hydrolysis of crystalline cellulose. Synergism has been explained by the proposal that the endoglucanses attack amorphous regions of cellulose fibres, forming sites for exoglucanases which can then hydrolyse cellobiose units from more crystalline region of the fibres. Finally B-glucosidases, by hydrolysing cellobiose prevents the accumulation of this disaccharide, which is an inhibitor of exoglucanase activity.

In contrast to the cellulase systems of aerobic fungi, the cellulases of most anaerobic microorganisnms are organized into large, multiprotein complexes (Bayer *et al.*, 1994; Beguin and Aubert, 1994; Beguin *et al.*, 1992). In general, these complexes efficiently catalyse the hydrolysis of cellulose as long as they can retain their integrity, but even partial dissociation of the complexes as occurs under relatively mild conditions causes loss of most activity against crystalline cellulose. Some of the proteins that result from the disassociation of the large complexes have endoglucanase, cellobiohyrolase, or xylanase activity: others do not appear to have enzymatic activity and may have a structural function (e.g. as a scaffolding protein) and /or may be involved in attachment of the complex to the substrate (Cavedon *et al.*, 1990;Hon-nami *et al.*, 1986; Kobayashi, Romaniec, 1990). None of the individual proteins in the complexes have been found to have significant activity against crystalline cellulose. The most thoroughly investigated cellulase complex, that of *Clostridium thermocellum,* was termed the cellulosome (Lamed and Bayer, 1988; Lamed and Bayer, 1991; Lamed *et al.,* 1983). On the cell surface, these multiprotein mutifunctional enzymes appear as polycellulosomal aggregates and promote adherence of the bacterium to the cellulose (Bayer and Lamed, 1986; Lamed *et al,* 1991). Possibly the cellulosome catalyses multiple, nearly simultaneous cuttings of the glucan chain (Felix and Ljungdahl, 1993).

2.13 Microbial decomposition of cellulose

A community of physiologically diverse microorganisms is responsible for the anaerobic degradation of cellulose. The metabolic versatility of anaerobes arises largely because they can perform various fermentations and respirations employing diverse electron acceptors (e.g. carbon dioxide, inorganic sulphur compounds inorganic nitrogen compounds) in place in oxygen (Lungdahl and Eriksson, 1985).

In the absence of oxygen and certain other exogenous inorganic electron acceptors [e.g. nitrate, Mn(IV), Fe (III), sulphate (Lovley and Goodwin, 1988], cellulose is decomposed by the anaerobic community into CH_4, CO_2 and H_20. Through a complex microbial food chain (Miller, 1991) shown diagrammatically in Figure 2.13.1 .The processes are similar in most anaerobic soils and sediments (Breznak and Kane, 1990) and in anaerobic digestors. Celluloytic microbes produce enzymes that depolymerize cellulose, thereby producing cellobiose, cellodextrins and some glucose. These sugars are fermented by cellulolytic and other saccharolytic microorganisms. By keeping cellobiose concentrations low, thus preventing the inhibition of the cellulase system by this product of cellulose hydrolysis. These fermentations yield CO_2, H_2, organic acids and alcohols. Very little H_2 escapes into the atmosphere because it is immediately consumed by methanogens or homoacetogens. Methanogens use H_2 to reduce CO_2 to CH_4 and homoacetogens use H_2 to reduce CO_2 to acetate. Syntrophic bacteria play a key role in the conversion of cellulose to CH_4 and CO_2. These organisms ferment fatty acids or alcohols and produce acetate, CO_2 and H_2. They grow only in the presence of H_2-consuming organisms through interspecies H_2 transfer. Syntrophic bacteria grow very slowly and thus the fermentation of fatty acids is usually the rate-limiting step in anaerobic decomposition of cellulose (Miller, 1991).

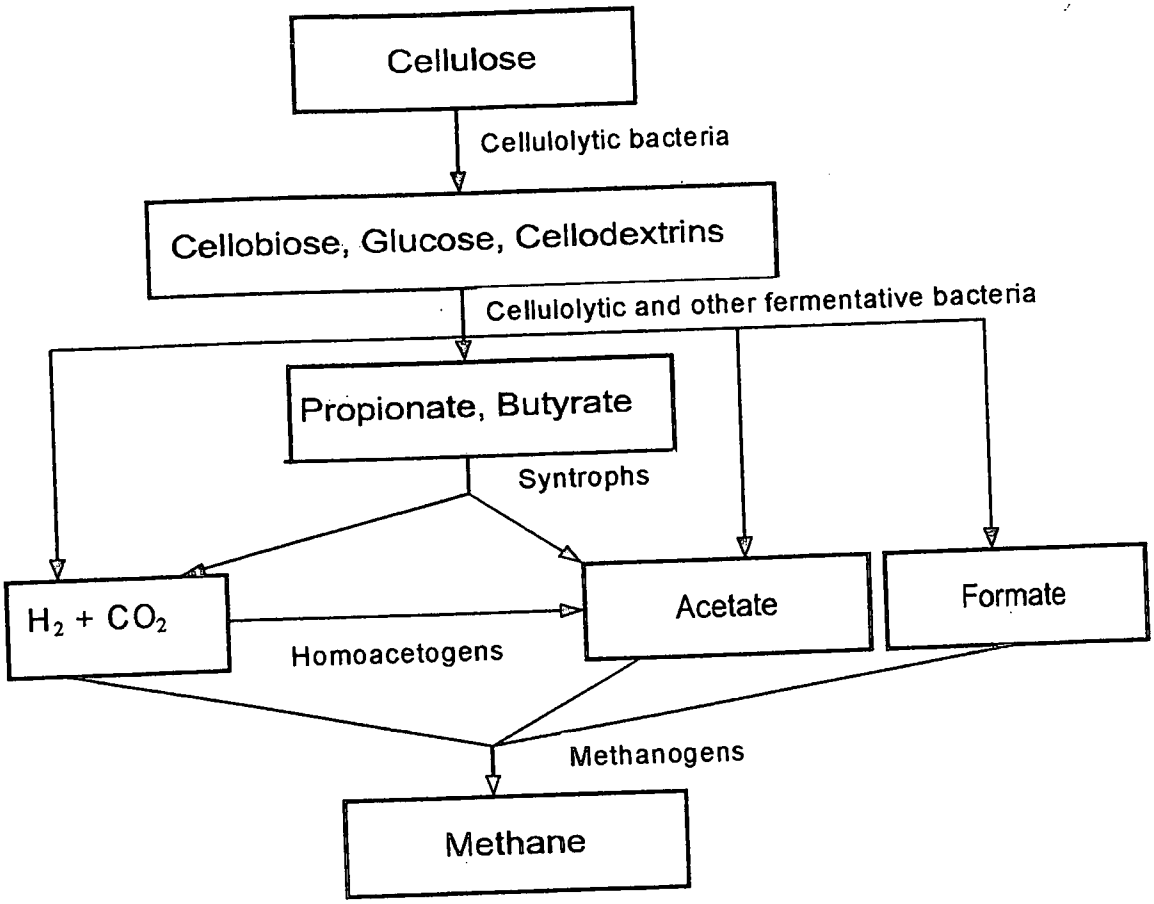

Figure 2.13.1 Diagrammatic representation of anaerobic cellulose degradation by microbial communities in soils and freshwater sediments. (Leschine, 1995)

2.14. Diversity of cellulose degrading microorganisms

Several mesophilic cellulolytic anaerobes have been isolated from soils, sediments and composts from geographically widely separated locations. *Clostridium papyrosolvens* NCIB 11394 was isolated from estuarine sediments (Madden *et al.*, 1982) and another strain of this species, strain C7, was isolated from the sediment of a freshwater swamp (Leschine and Canale–Parole, 1983; Pohlschroeder *et al.*, 1994). *Clostridium lentocellum* was isolated from samples from samples of the same estuarine sediments from which *C. papyrosolvens* NCIB 11394 was derived (Murray *et al.*, 1986). *C. lentocellum* differs from other mesophilic, cellulolytic bacteria in several ways, but perhaps the most significant is the relatively slow rate of cellulose degradation by cultures of this bacterium. Clostridium cellulofermentans inhabits dairy farm soil. Cellulolytic anaerobes isolated from wetwood

of trees are similar in many respects to isolates from soil. And these trees are probably infected with soil bacteria. Although Clostridium cellobioparum was originally isolated from bovine rumen contents (Hungate, 1944), this clostridium is reportedly also found in soil (Skinner, 1960).

All of the mesophilic strains that have been examined are motile, rod shaped organisms with similar cell envelope ultrastructure. Also they have similar G+C contents. Cells of almost all strains stain gram-negative, and most, but not all form spores. All ferment cellulose, cellobiose, and glucose, but also components of the hemicellulosic portion of biomass (eg.xylan, pentoses). Of those strains examined, all form acetate, ethanol, CO_2 and H_2 as primary products of fermentation. Other free-living cellulose fermenting mesophiles have been isolated from composts e.g. *C. cellulolyticum* is just one example (Petitdemange *et al.*, 1984).

2.15. Cellulose degradation in landfills

Cellulose is the principal biodegradable constituent of refuse under the anaerobic conditions typical of landfills (Barlaz *et al.*, 1990). Processed paper waste constitutes the vast majority of cellulosic waste. These include newspapers and magazines, telephone books and packaging (Rosenberg, 1993). Within a landfill newsprint has been legible many years after initial compaction (Booth, 1965) and illustrates how slow cellulose degradation within this environment can be. Moisture content is probably the single most important variable affecting the rate of degradation (Boyd, 1981) and will affect rates of diffusion of substrate and products as well as affecting hydrolytic reactions such a cellulose degradation. However, was it not for cellulolytic bacteria within the landfill, the breakdown of cellulosic material and subsequent methane production would be very limited.

Publications by Barlaz, Schaefer and Ham 91989a, b) identified techniques for enumerating cellulolytic bacteria from landfill and have monitored the population development of key groups of bacteria as well as methane production in refuse, on a laboratory scale. These studies showed that polymer hydrolysis was the rate-limiting factor in the conversion of refuse to methane. The cellulolytic bacteria play a major role in determining the rate of polymer hydrolysis within landfill and hence a greater understanding of these bacteria is essential to our understanding of refuse degradation (Westlake, 1990). While much information concerning anaerobic degradation of cellulose is available (Aubert et al., 1966) and the processes in landfill gas production have been described (Senior and Balba, 1987), very little is known of the bacteria responsible for cellulose degradation and subsequent methane production within the landfill environment. However a relatively large number of bacteria have been isolated from environments such as the rumen and whose cellulolytic properties are well characterized (Westlake, 1990). In order for a bacterium to be considered truly cellulolytic, it must be able to hydrolyze crystalline cellulose and must therefore possess a range on enzymes including carboxymethylcellulase (CMCase), cellobiohydrolase and B-glucosidase.

2.16 Methods for the isolation of cellulolytic microorganisms

The use of thin agar layers containing finely divided cellulose for direct counts has been suggested to seriously underestimate the number of cellulolytic organisms present (Byrant and Burkey, 1953), although careful control of the nature of this substrate may overcome this problem (van Gylswyk, 1970). The alternative method of using a non-selective medium to isolate as wide a range of organisms as possible followed by the testing of ech isolate for cellulolytic activity, severely limits the nature and number of studies that can be undertaken because of the time and labour required. In both cases a degree of ambiguity arises because of the wide range in time requires for different species to produce obvious

degradation of the substrate (visible degradation of intact filter paper, for example may require as little as two days or longer than two months).

Methods have been developed to overcome some of these problems, using either dye-labelled insoluble substrates, which releases a dye upon hydrolysis (Smith, 1977) or a soluble substrate (carboxymethylcellulose) which is precipitated with detergent to visualize a zone of hydrolysis on an agar plate (Hankin and Anadnostakis, 1977). However these techniques were still limited in their lack of sensitivity and by the restricted range of substrates which can be tested (Teather and Wood, 1982).

Teather and Wood (1982) found that the use of Congo red as an indicator for B-glucan degradation in agar medium provided the basis for a rapid and sensitive screening test for cellulolytic bacteria. Incubation times were reduced from weeks or months to less than one day and results were generally unambiguous. The method is readily adaptable to either direct counts of viable cellulolytic organisms, using roll tubes, or to the screening or characterization (or both) of large numbers of isolates, using replica plating techniques.

Cummings and Stewart (1994) studied the degradation of newspaper by landfill bacteria by using an accurate model of the environment. They found that the optimal conditions for cellulolysis of newspaper and subsequent production of methane by the isolated were 37°C and a pH close to neutrality. Temperatures and pH outside the optimal range significantly inhibit both cellulolysis and the volumes of methane produced. The refractory nature of newspaper towards landfill cellulolytic bacteria was probably due to the presence of high concentrations of lignin, which protected the cellulose from bacterial degradation. Increasing the surface area of paper by milling had a negligible effect on both cellulolysis and methanogenesis. Ink decreased both the solubilization of the paper and methane production, apparently by acting as a barrier to the bacterial attachment required for degradation. Kauri and Kushner (1985) found that it was not essential that bacteria make contact with the substrate in order to degrade it. They found that when a number of bacterial species were separated from cellulose on plates by membrane filters

or by layers of agar they could still degrade the cellulose, they could still degrade the cellulose.

In order to understand the processes of waste degradation in the landfill and thereafter optimize these processes, either for the generation of energy or minimization of landfill pollution, it will be necessary to gain a better understanding of the diversity of bacterial species within the waste and their biochemical activity,

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Sampling

3.1.1 Landfills

Sampling was performed six times throughout this research, each time at a different location on the landfill. Construction equipment available at the landfill site was used to excavate to a maximum depth of 4m. The landfill expanded over 44 hectares, was approximately 17 years old and had a depth of 30m. The compaction rate was Approximately 1300kg/m^3 and a moisture content of 40%. Only municipal solid waste was disposed of at the time. No co-disposal was being practised

For the first sampling, 20kg refuse was collected. Leachate was collected in a bucket once it had drained through the soil layers at the excavation point. It was then transferred to an aspirator allowing minimum headspace. The refuse was placed in a plastic bag and transported to the laboratory where it was sorted. Decomposing plant and paper material together with the soil were retained for microbiological analyses. Plastic, glass and metal materials were discarded. The bulk refuse and leachate samples were stored at 2°C to decrease the rate of chemical and microbiological reactions within the refuse. For subsequent samplings 5 kg samples were collected, since 20-30g of sample were being used for the analyses. In addition, containers used for sample collection were flushed with oxygen-free nitrogen prior to sampling and the refuse was not sorted, to eliminate exposure to oxygen.

3.1.2 Other potential sources of cellulolytic bacteria

Six different samples were collected. Anaerobic sludge was obtained from an active bioreactor, decomposing bagasse from the lowest portion underneath the bagasse heap. Fresh cow manure was obtained several hours after deposition and pond

sediment from a depth of 30cm on pond banks. Rumen contents were obtained from a freshly slaughtered animal. A compost sample was obtained from underneath a compost heap. Sampling containers were flushed with oxygen-free gas prior to collection of samples. Samples were processed within 4 hours of collection

3.2 Processing samples for microbiological analyses

The technique for inoculum formation constituted a combination of two methods. Firstly, the refuse (20g) was blended in anaerobic phosphate buffer (180m*l*) (23.7mM, pH 7.2) (Appendix A) for approximately 60s in a 1L blender (Barlaz *et al.*, 1989). The homogenized solids were transferred into a stomacher bag and homogenized in a stomacher for a further 60s to extract any unremoved microbial cells from the refuse. (Maule *et al.*, 1994). The extraction procedure using the stomacher was repeated twice. The solids were filtered through wire gauze and hand squeezed to obtain the liquid extract which was to be used as the inoculum. All the above manipulations were performed in aerobic conditions, as the anaerobic chamber could not accommodate a blender and a stomacher because of space limitations. The inoculum was transferred to the anaerobic chamber soon after preparation.

Samples from the other sources were treated in the same manner. To induce spore formation, the rumen sample was heat shocked at 70°C for 15min.

3.3 Growth Conditions

All media were pre-reduced and cultures were incubated in an anaerobic chamber (Labotec) in an atmosphere of 10 % CO_2, 10% H_2 and 80% N_2 at temperatures ranging from 30°C to 37°C. A glove bag was used for the incubation of cultures at higher temperatures (>37°C). The bag was filled with oxygen-free high purity nitrogen, sealed and placed into an incubator in an aerobic environment.

To obtain oxygen free high purity nitrogen, an Oxisorb Filter [Messer Fedgas (Pty) Ltd.] was fitted onto the gas line. Residual traces of oxygen were removed down to concentration smaller than 10 ppb. From the filter, for flushing purposes, the gas was led through rubber tubing which ended in a 10cm, 18 gauge stainless steel syringe needle bent into a hook so that it could be hung over the rim of the culture tube.

Resazurin (Appendix B) was the indicator used to show the Redox State of the medium. Resazurin has an E^{o} (redox potential) of –51mV. It goes from blue to pink to completely colourless when the redox potential is lowered to approximately 110mV or lower. When reoxidized resazurin becomes pink and does not turn blue. A medium containing resazurin with no pink colour is a very good assurance of anaerobicity, although this does not indicate the low redox potential of –330mV needed for growth of some anaerobes.

Reducing agents cysteine hydrochloride and sodium sulphide were used at concentrations of 0.5% and 1.5 respectively (Appendix C).

3.4 Carbon sources for cellulolytic bacteria

3.4.1 Liquid Media

Whatman No. 1 filter paper strips (5cmX1cm) were used in liquid medium. Cellobiose, a soluble cellulose source was used to initiate growth of the cellulolytic bacteria.

3.4.2. Solid Media

Initially unmilled Whatman CF 11 cellulose powder was used in solid media. At a later stage in the research, the powder was subjected to ball-milling for 72 h. The milling was intended to increase the susceptibility of the cellulose particles to enzyme

attack (Greeves, 1971). Whatman No.1 filter paper was also subjected to ball-milling and was used as a fine powder. Carboxymethyl cellulose (CMC), medium viscosity, a water-soluble cellulose derivative was used for the detection of cellulase production because it is degraded quickly by microorganisms (Mandels *et al.*, 1976). Cellulose powders manufactured by Sigma and Aldrich were also used. These were not subjected to ball-milling. Cellobiose was also used in solid media.

3.5 Preparation of pre-reduced anaerobic media

The successful cultivation of an anaerobic microorganism requires techniques to effectively remove oxygen (air) from the both the medium and the gas phase in contact with the medium (Ljungdahl and Wiegel, 1986)

3.5.1 **Modified Schott Bottle** (Rogers, 1990)

Initially, media was prepared in bulk by the use of the modified Schott bottle implemented by Rogers (1990). The lid of the bottle was modified to possess the following:

(a) an inlet for flushing with oxygen-free gas

(b) an exhaust line

(c) a sampling line and

(d) an injection port for the inclusion of reducing agents.

Using this bottle, media was autoclaved with the inclusion of the indicator, resazurin. The media was then allowed to cool whereafter it was transferred to the anaerobic chamber. Here sterile reducing agents were added and the media dispensed into desired aliquots into previously autoclaved universal tubes.

3.5.2 Other methods

Solid media: The medium was autoclaved without the reducing agent and when cool a reducing agent is added and the plates were poured aerobically. As soon as the plates were set, they were stored under anaerobic conditions for 24 hours (Reid, S. 1998, personal communication). Any tubes, which appeared turbid or were pink (oxidized) discarded.

Broth medium: The broth medium containing the reducing agent was steamed in the autoclave for 20 min to reduce it. The pressure valve on the autoclave was kept opened during steaming. Thereafter the medium was dispensed into universal tubes and flushed with oxygen free high purity nitrogen prior to autoclaving (Reid, 1998 personal communication).

3.6 Culture Vessels

3.6.1 Universal tubes and serum bottles

Liquid media was prepared in bulk with the inclusion of the indicator and dispensed into desired quantities prior to autoclaving. The universal tubes were closed with lids containing rubber seals in the inside of the lid. Serum bottles were closed with rubber stoppers and sealed with aluminium crimps. Once cooled, they were transferred to the anaerobic chamber were sterile reducing agents were added.

3.6.2 Roll-tubes and Lee tubes

These techniques were used when using solid media to identify zones of clearing caused by anaerobic cellulose-degrading microorganisms. Agar content was raised to 2 % to allow for a firmer layer of agar.

For the roll-tube technique, implemented by Hungate (1944) sterile tubes, which were 12cm in length, with a diameter of 2cm, were used. 10ml of inoculated molten media

(25°C) were aseptically dispensed into each tube and the tube was rolled on a flat surface until the medium was set in a thin layer on the inside wall of the tube. Tubes were incubated for the specified period.

Lee tubes are similar to roll tubes except that they possess an inner wall that allows for easier formation of the thin agar layer. Inoculation was also performed before the agar was allowed to set.

3.7 Media preparations

Table 3.7.1 shows the media preparations that were used to isolate the anaerobic, cellulose degrading bacteria. All the preparations were pre-reduced.

Table 3.7.1 Media preparations for the growth of anaerobic, cellulolytic bacteria. (Appendix D to Appendix L, consecutively)

Medium	Cellulose source	Temp, pH	Phase	Reference
1.MS	Ball-milled Whatman No.1 fp	35°C, 7.2	Solid/liquid	Westlake *et al.*, (1995)
2. Enrichment – Cellulolytic Clostridia	Whatman No.1 fp strips	35°C, 7.0	Liquid/Solid	Skinner, F. (1971)
3. Cellulolytic Thermophilc	Ball-milled CF11	65°C, 7.2	Solid/liquid	Atlas, R and Parks, L. (1993)
4.Anaerobic celluloytic	Cellobiose	35°C, 6.5	Solid/liquid	Atlas, R and Parks, L. (1993)
5.Cellulose congo red agar	Carboxymethyl cellulose,cellobiose	35°C, 7.0	Semi-solid	Hendricks *et al.*, (1995)
6.Cellulolytic Clostridia	Cellulose powder (Sigma)	35°C, 7.1	Liquid	Atlas, R and Parks, L. (1993)
7.*Clostridium* cellulolytic	Cellulose powder (Aldrich)	35°C, 7.5	Solid	Atlas, R and Parks, L. (1993)
8.Clostridium cellulose	Ball-milled CF11	35°C, 7.0	Solid/	Atlas, R and Parks, L. (1993)
9.Basal cellulolytic	Cellobise	35°C,7.0	Solid/liquid	Hobson, P and Mann, S. (1971)

Initially, Reinforced Clostridial media, Robertsons Cooked Meat Broth and Enrichment media were used. These were used to enrich for all microorganisms and thereafter; subcultures would be performed on selective media containing cellulose

or a cellulose derivative as the sole carbon source. Later, in the research, pre-reduced Brain Heart Infusion (BHI) medium was used as the enrichment medium as this medium is known to be very rich. The BHI was pre-reduced.

3.8. Isolation of cellulolytic microorganisms

Serial dilutions (10^{-1}–10^{-4}) of the enriched cultures were prepared using the anaerobic phosphate buffer as the diluent. From these, 1 ml was used to inoculate media shown in the Table 3.7.1. For samples taken from the landfill, landfill leachate was added to the medium as enrichment Clarified rumen fluid (Appendix M) was used for isolation from rumen contents. Rumen fluid provides growth factors such as branched-chain volatile fatty acids. For samples taken from other sources the Basal medium was used which was supplemented with cellobiose.

Incubation was performed in the anaerobic chamber at a temperature of 25°C for a minimum period of 7 days. When the cultures were visually turbid and the filter paper strips showed signs of degradation, this was used as an indication of microbial activity. Once growth was evident, pre-reduced solid media with the same composition as that of the liquid media was inoculated and incubated at the same temperature. After incubation colonies surrounded by zones of clearings were regarded as being capable of degrading cellulose anaerobically.

3.9 Methods for screening of cellulose degrading bacteria

3.9.1 Overlay technique (Teather and Wood, 1982)

The ability to degrade soluble B-D glucans was tested by overlaying colonies grown on petri dishes in an anaerobic chamber with 4ml of the same medium without the normal cellulose source and containing 0.8% agar and 0.05 to 1 % of the test substrate. The test substrate used was carboxymethylcellulose, medium viscosity, obtained from Sigma.

After an appropriate incubation period at 25°C, the agar medium was flooded with an aqueous solution of Congo red (1mg/ml) for 15min. The Congo red solution (Appendix N) was then poured off, and plates were further treated by flooding with 1M NaCl for 15min. The visualised zones of hydrolysis could be stabilized for two weeks by flooding the agar with 1M HCl which changes the dye colour to blue and inhibits further enzyme activity.

3.9.2 Use of aniline blue-black (Kauri and Kushner, 1988)

A 0.005% solution of aniline blue-black dye was prepared and added to the medium before autoclaving. Observations for zones of clearings were performed after incubation as the hydrolyzed cellulose would not bind the dye (Kauri and Kushner, 1988).

3.9.3 Additional treatments encourage cellulose biodegradation

- This was done by ball-milling Whatman No. 1 cellulose powder and and Whatman No1 filter paper separately for 72h to decrease the particle size of the cellulose.
- To increase contact between the substrate and the bacteria, after the agar had cooled; it was gently turned over with a sterile spatula so that the sedimented cellulose would be on the top and in direct contact with the cellulolytic microbe.
- Cellulose powder was soaked in distilled water for 24 hours.
- Clarified rumen fluid was added to the medium as a supplement

3.10 Rehydration of anaerobic cellulolytic reference organisms

Two reference organisms were used in this research. *Clostridium stercorarium* (ATCC 21399) and *Clostrium cellulovorans* (ATCC 43279). The former grows at an optimum temperature of 65°C and the latter at 30°C.

3.11 Morphological and biochemical tests of isolates

Gram reaction, spore reaction and the catalase reaction were determined by the methods of Cappucino and Sherman. (1992). Fermentation tests were performed using the 20A API identification kit for anaerobes.

CHAPTER 4

RESULTS AND DISCUSSION

Figures 4.1 to 4.7 shows photographs taken at different stages of progress in this research.

Figure 4.1 Sampling points

Figure 4.1 shows the approximate positions at which excavations for sampling were performed. The landscape of the landfill changed continually because of daily deposition and covering of the waste. The above positions were selected to obtain waste samples that varied in age. Position 4.1.2 was the present site for waste deposition at which the waste was approximately two weeks old. The waste at position 4.1.4 was eight years old and was the oldest position sampled. The remaining positions contained waste that was greater than one year in age.

Figure 4.2 Excavation for sampling

Figure 4.2 shows the construction equipment used at the landfill site in order to obtain samples from underground, approximately 4m. Barlaz et al (1995) used a drilling rig equipped with a flight auger and sampled from depths of 5m, 6m and 18m. The use of drilling equipment would be more advantageous for sampling, since there would be less exposure to the atmosphere on removal of the sample and samples from greater depths could be obtained.

A methane concentration of 50 to 60% was indicative of the latter phase (Barlaz, 1997). Cellulose degrading organisms are in the log phase of growth at this phase of decomposition. Methane concentrations were not measured in this research thus it was uncertain at precisely which stage the decomposition was, at the time of sampling.

Figure 4.3 Decomposing waste

Figure 4.3 shows the decomposing refuse shortly after sampling. The refuse was brown to black in colour with a sharp acidic odour. Leachate (not shown here) was brown in colour and also accompanied by the acidic odour. The odour indicated that fermentation was occurring.

Figure 4.4 Legible newspaper from a landfill sample

The sample in Figure 4.4 was collected in 1998 and a newspaper buried in 1991 was found to be legible after 7 years. This emphasizes that the rates of decomposition in landfills are slow. Rosenberg (1993) mentioned that newspapers could remain legible for up to 30 years in a landfill. Cummings and Stewart (1994) suggested that the major resistance of newsprint to degradation by bacteria seemed to be the high proportions of lignin present. It was found that newspaper manufactured from wood pulp contained more lignin than newspaper manufactured from recycled paper and presumably for this reason, the latter was subject to slightly more solubilization by bacteria.

In addition, temperature and pH of the waste were also crucial for optimal newspaper degradation. Ink could also prove to be a problem on heavily printed paper, forming a barrier to the attachment of bacteria. Paper pretreatments had no significant impact on cellulolysis (Cummings and Stewart, 1994).

Figure 4.5 Modified Rogers bottle for the preparation of pre-reduced media

The use of the Rogers modified Schott bottle as shown in Figure 4.5 was successful in that pre-reduced media was achievable. However, this method was not practical in this research. One example was that of space constraints in the anaerobic chamber which made tasks such as dispensing of media from this bottle awkward and subsequently the chances of media becoming contaminated increased. The method suggested by Reid (1998) was less complex in terms of preparation and ensured that reduced media was obtained and this method was used in subsequent media preparations. Figure 4.5.1 shows the media containing the redox indicator, resazurin, hence the blue-purple colour. At this stage, the media was oxidized and was unsuitable for the cultivation of anaerobic, cellulolytic bacteria. Figure 4.5.2 shows the media in the reduced state.

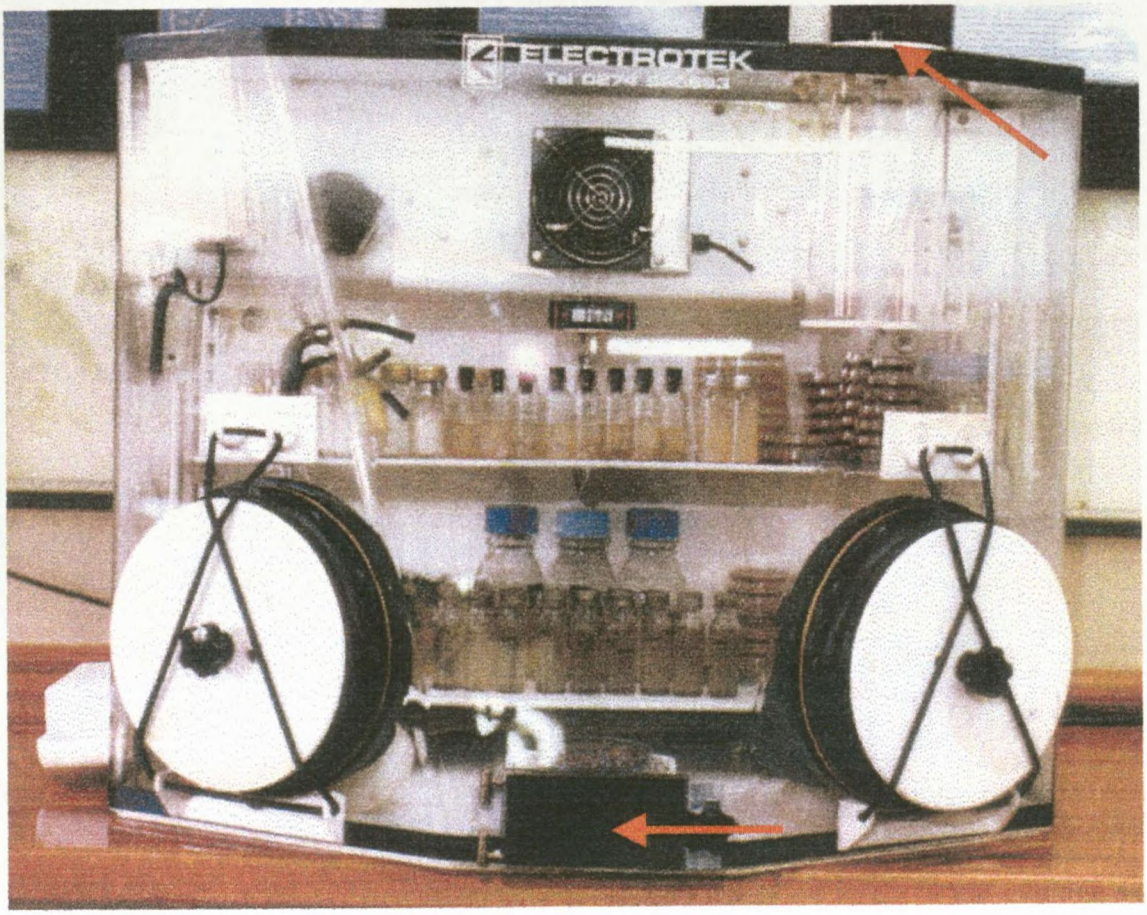

Figure 4.6 Anaerobic chamber

Figure 4.6 shows the anaerobic chamber used in the research. Manipulations and incubations were performed in the same space. Media were introduced through a port at the top of the chamber or through a port at the front. The chamber automatically flushed with oxygen free gas, however oxygen entered when the ports were opened. Media left in the incubator overnight would change from the reduced state to the oxidised state once the chamber was opened. The platinum catalyst was recharged twice and the silica gel changed daily or as required. This helped in reducing condensation in the chamber but did not aid in maintaining reduced media.

Figure 4.7 Degradation of filter paper strips in universal bottles

Figure 4.7 shows the universal tubes most widely used in this research. Figure 4.7.1 shows the filter paper that has not been degraded. Figure 4.7.2 shows partial degradation of the filter paper and Figure 4.7.3 shows complete degradation after 62d. Media levels were low in Figures 4.7.2 and 4.7.3 due to sampling.

Serum bottles with rubber seals and aluminium crimps were used in the initial enrichment process. An attempt at utilizing roll-tubes and Lee tubes were made, but was not successful, as the film of media did not form evenly on the inner surfaces. Tubes were rolled manually.

Table 4.1 Growth and cellulose degradation in various media preparations

Medium	Growth(+) / No Growth(-)	Visible cellulose degradation
1. MS	+	-
2.Enrichment – cellulolytic Clostridia	+	+
3. Cellulolytic thermophilic	+	-
4. Anaerobic cellulolytic	+	+
5. Cellulose congo red agar	+	-
6. Cellulolytic Clostridia	+	-
7. *Clostridium* cellulolytic	+	-
8. Clostridium cellulose	+	-
9. Basal cellulolytic	+	-

Of the nine media preparations shown in Table 4.1, enrichment medium for cellulolytic Clostridia and anaerobic cellulolytic medium, showed hydrolysis of the Whatman No 1 filter paper strips in liquid media. Filter paper degradation was visible after a 30d-incubation period in enrichment media. According to Skinner (1971), digestion of the filter paper usually began after 7d. The long incubation time may have been due to the medium not being sufficiently reduced and the available oxygen present in each tube had to be used by the oxygen utilizing organisms in the inoculum. Furthermore, the preparation used by Skinner (1971) did not include an indicator to check for anaerobiosis. Reducing agents were also omitted in Skinner's method, thus the onset of anaerobic conditions could have been delayed, hence the long incubation period.

Table 4.2 Degradation of filter paper (fp) in liquid medium 2 inoculated with processed samples of decomposing paper and plant material

Sample	10^{-1}	10^{-2}	10^{-3}
Paper	Gas production Degradation of fp	Gas production, Yellow pigment Degradation of fp	Gas production Yellow pigment Degradation of fp
Plant		Gas production Degradation of fp	Gas production Degradation of fp

When enrichment medium was inoculated with decomposing paper and plant samples at different dilutions, the results showed degradation of the filter paper (Table 4.2). The paper appeared as a mass of fluff (Figure 4.7). Gas production was also evident observed as bubbles near or at the surface of the medium. The gas produced was not analysed. In addition, a yellow pigment was seen in the 10^{-2} and 10^{-3} dilutions inoculated with decomposing paper. This is in agreement with the findings of Weimer and Zeikus (1977), that the above characteristics were initial evidence of cellulose fermentation by *Clostridium thermocellum*. Although this organism is a thermophile, the above characteristics were obtained at mesophilic temperatures. The long incubation time (28 days) required to obtain similar results as those obtained at thermophilic temperatures (14 days) could be attributed to the mesophilic temperatures used. On the other hand, Leschine and Canale-Parola (1983) found that thermophilic bacteria do not necessarily degrade cellulose at a faster rate than mesophilic bacteria and that factors other than temperature limit the rate of cellulose fermentation.

When solid media (pH 7) containing ball-milled Whatman No. 1 filter paper was inoculated no zones of clearing were visible. The enrichment culture medium after degradation was approximately pH 5. This was in accordance with Leschine and Canale-Parola (1983) who found that after cellulose degradation, the pH decreased

from 7.1 to 5.3. This was expected, as there would be a production of acids during fermentation. It is possible then that the sudden pH change from 5 to 7 caused the cellulolytic organisms to cease metabolism and consequently not degrade the cellulose in solid media as the enzyme systems would be affected. Jones and Grainger (1983) found that the cellulases were the only active at pH 5.0 and activity decreased in both dry and saturated samples. In addition, low cellulase activity was recorded throughout the research of Jones and Grainger (1983). They contributed this to several factors such as the combination of cellulose with lignin, cellulases are susceptible to inactivation by refuse-derived proteolytic enzymes. The high concentration of ammonium in landfill leachate may also reflect either poor utilization of landfill cellulose or the temporal separation of proteolysis and cellulolysis.

In addition, the cellulose in the solid media settled to the base of the petri dish, so enzymes may not have diffused to the carbon source. Kauiri and Kushner (1988), stated that it was not essential that bacteria make contact with the substrate in order to degrade it. The long milling process of the filter paper (72h) might have caused contamination of the cellulose powder with heavy metal(s), thereby also affecting enzyme systems. Cellulose powders from Sigma and Aldrich was also used, but a positive result was not obtained.

Table 4.3 Growth and cellulose degradation of samples other than those from the landfill

Sample	Growth (+)/ No growth (-) on cellobiose agar	Visible cellulose degradation
Anaerobic sludge	+	+
Bagasse	+	-
Compost	+	+
Fresh Cow Manure	+	-
Rumen Contents	+	+
Pond Sediment	+	-

Samples were collected from other sources because of difficulty experienced in isolating the desired microorganisms from the landfill.

The results show that the basal medium containing cellobiose supported microbial growth from all the sources sampled other than the landfill. Although the medium was a basal medium for cellulolytic bacteria, it supported growth and cellulose degradation from the rumen contents sample. This was expected since rumen fluid was a constituent of the medium. In addition, heat shock treatment could have resulted in the germination of spores. Cellulose degradation occurred after 16d .

The anaerobic sludge sample also showed cellulose degradation after 14 days. No degradation was achieved with from the other samples. It is thought that the cellulolytic organisms were not extracted or that these samples were exposed to higher oxygen concentrations in the soils from which they were collected whereas the rumen and sludge samples were collected from definite anaerobic environments.

The anaerobic cellulolytic medium is recommended for the cultivation of *Clostridium cellulovorans* (Atlas and Parks). This organism is cellulolytic and was used as a reference strain (ATCC 35296) in this research. However, this organism did not grow on rehydration. According to Sleat *et al.,* (1984) this bacterium lyses cellulose in roll tubes within 24 to 48h and is an obligate anaerobe. The latter property makes it extremely oxygen intolerant and would require oxygen concentrations of less than 5ppm for survival (Hespell, 1990). The anaerobic chamber had automatic flushing to maintain a constant anaerobic atmosphere, however it is uncertain as to what levels the oxygen increased as other manipulations were performed in the chamber.

Cellulolytic thermophilic media was used to rehydrate a second reference strain, *Clostridium stercorarium* (ATCC 21399). The thermophilic organism was placed in an anaerobic bag filled with oxygen-free nitrogen gas and incubated aerobically at 65°C. Again, no growth was achieved. According to Madden (1983), after 7 days, colonies were surrounded by clear zones. In addition, this organism was also a strict anaerobe and the gas was found to be escaping from the bag. Only one sample was available, therefore it was not possible to repeat the experiment.

The overlay technique and the aniline blue method did not yield positive results. It is thought that even though the organisms degrade filter paper in liquid medium they may not necessarily show degradation on solid media. The reason for this in uncertain.

Treatments such as ball milling or turning the agar had little or no effect in terms of producing zones of clearing indicative of cellulose degradation. Wemer and Zeikus (1990) found that cellulose digestion by mixed ruminal microflora was not enhanced by treating the cell with phosphoric acid at a concentration which has been shown to increase pore volumes fourfold.

Morphological tests showed that the colonies obtained from a culture that degraded filter paper in liquid medium were 1 to 2mm in diameter and were cream in colour on solid media. They were predominantly gram positive, produced spores and were catalase negative. The latter property is characteristic of anaerobes as they do not possess the enzymes catalase and superoxide dismutase. The isolates were found to be of the genus Clostridium and cellobiose was fermented. However, fermentation of cellobiose does not necessarily mean that the organism is cellulolytic thus the failure to produce zones of hydrolysis on solid media was of concern.

Palmisano et al. (1993) were unable to isolate cellulose degrading bacteria from the landfill. The following were possible reasons:

- low abundance of cellulolytic bacteria in the landfill
- difficulty in extracting bacteria from the refuse
- inadequacy of cellulase detection
- the inability to grow on solid media provided.

The results from this research show that cellulose degradation was achieved in liquid media but not on the solid media. Thus, cellulolytic bacteria do exist in the landfill and

the successful isolation and maintenance of these organisms seems to depend strongly on the nature of the medium on or in which they are cultivated in the laboratory. The solid media provided in this research was not suitable to show cellulose degradation. In addition, the anaerobic conditions would be the other important factor to consider when isolating these microorganisms.

CHAPTER 5

5.0 CONCLUSIONS and RECOMMENDATIONS

5.1 Conclusions

1. All samples taken from anoxic (or presumably anaerobic sites) contained organisms capable of growing under anaerobic conditions at mesophilic temperatures with cellobiose as the sole carbon source.

2. Degradation of the filter paper by samples from the landfill in liquid media is evidence of cellulolytic activity. Although no zones of clearing were visible on solid media. Cellulolytic bacteria might be more active in a mixed culture rather than as pure isolates.

3. Microorganisms were cultivated on cellulose media as naturally existing mixed populations but on solid media colonies of individual organisms were developed. The lack of demonstration of cellulolytic activity on solid media could be attributed to this fact.

4. Since similar results were achieved from samples from the landfill and other sources, the anaerobic techniques employed and equipment might not have been sufficient to maintain an anaerobic environment throughout the isolation procedures.

5. It is important that media contains a buffer in the correct concentrations to maintain the pH at neutral as this is more suitable for the growth of cellulolytic microorganisms.

5.2.1 Recommendations

1. It would be beneficial to measure the methane concentration at the point were sampling is intended as an indication of the phase of decomposition will be obtained and subsequently the predominant microorganisms of that phase determined.

2. Several landfill sites should be sampled.

3. Excavated refuse should be shredded.

4. A laboratory scale model of waste decomposition with leachate recycle could be constructed, conditions maintained constant and enriched for the desired microorganisms. Subsequently the microorganisms could be isolated and studied and parameters such as pH, temperature and moisture content can be changed and the effects on the desired population or species studied.

5. The reference organisms chosen should be more oxygen tolerant, considering the equipment available to create anaerobic conditions.

6. Exposure of media to oxygen should be kept to a minimum.

7. Because of the difficulty in starting anaerobic research of this nature and the long times required for growth, the development of specific molecular probes would facilitate the identification of the desired bacteria from landfill.

8. The preparation and maintenance for pre-reduced media is an important consideration when performing anaerobic isolations.

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APPENDICES

Appendix A

Anaerobic phosphate buffer (23.7mM, pH 7.2)

Calculation : $\frac{23.7mM \times 10^{-3}}{2}$

= 0.01185moles

mass Na_2HPO_4 = number of moles X Molar mass

= 0.01185moles X 141.96g/mol

= 1.6822g

mass NaH_2PO_4 = number of moles X Molar mass

= 0.01185moles X 119.98g/mol

= 1.4218g

Procedure

1. Weigh the two solids into a beaker
2. Add 500m/ distilled water
3. Adjust pH with 0.1N NaOH or 0.1N H_2SO_4
4. Add 1m/ resazurin
5. Place in volumetric flask and make up to 1/
6. Dispense into a 1/ Schott bottle and autoclave at 121°C for 15min
7. Before use add 20m/ reducing agent

Appendix B

Resazurin (redox indicator)

Dissolve 1g resazurin indicator powder (Sigma) in *1000*m/ deionized water to give a final concentration of 0.1% $^{w}/_{v}$.

Appendix C

Reducing agents (van Gylswyk, Uppsala, Sweden, personal communication. letter to author)

The reducing agent is prepared as a combined alkaline solution as follows:

1. Boil 100ml 0.2N NaOH in a narrow mouthed Erlenmeyer Flask for 1 min
2. Cover the mouth with aluminium foil and gas with O_2-free N_2
3. Add 1.25g each of cysteine hydrochloride and sodium sulphide
4. Allow these to dissolve and mix (while gassing)
4. Remove the solution with a syringe fitted with a long cannula (through the foil)
5. Replace the cannula with a needle and inject into a rubber-stoppered bottle, previously purged with O_2-free N_2
6. These can be stored for up to 1 month
7. Use 20ml of reducing agent solution per litre of medium

Appendix D

MS media (modified) (*1l*) (Westlake *et al.*, 1995)

NaOH	4.0g
Yeast extract	2.0g
Trypticase peptones	2.0g
NH_4Cl	1.0g
$MgCl_2.6H_20$	1.0g
2-mercaptoethane-sulfonic acid	0.2g
$KH_2PO_4.3H_20$	0.4g
$CaCl_2.2H_20$	0.4g
$Na_2S.9H_20$	250mg
Disodium EDTA.$2H_20$	5.0mg
$CoCl_2.6H_20$	1.5mg
Resazurin	1.0mg

$MnCl_2.4H_20$	1.0mg
$FeSO_4.7H_20$	1.0mg
$ZnCl_2$	1.0mg
$AlCl_3.6H_20$	0.4mg
$Na_2W0_4.2H_20$	0.3mg
$CuCl_2.2H_20$	0.2mg
$NiSo_4.6H_20$	0.2mg
H_2SeO_3	0.1mg
H_3BO_3	0.1mg
$NaMoO_4.2H_20$	0.1mg
Ball-milled Whatman No. 1 filter paper	0.5% $^{w}/_{v}$

1. Bubble medium with O2 free nitrogen for 1 h before autoclaving
2. Add 2-mercaptoethane-sulfonic acid and sodium sulphide after autoclaving .

Appendix E

Enrichment media for cellulolytic Clostridia (1*l*) (Skinner, F.A, 1971)

$(NH_4)SO_4$	1.0g
K_2HPO_4	1.0g
Mg $SO_4.7H_20$	0.5g
$CaCO_3$	2.0g
NaC*l*	trace

1. Add 2g of chopped filter paper (0.5cmX0.5cm) into universal bottles
2. Dispense media to nearly fill the bottles

Appendix F

Media for Celluloytic Thermophiles (1*l*) (Atlas, R and Parks, L, 1993)

K_2HPO_4	1.65g

NH_4SO_4	1.6g
Yeast extract	1.0g
NaCl	0.96g
Cysteine . HCL.H_20	0.5g
$CaCl_2$	0.096g
$MgSO_4$	0.096g
Cellulose suspension	200ml
Resazurin	1.0ml

Cellulose suspension (200ml)

Cellulose powder , Whatman CF11, ball-milled 8g

1. Add cellulose powder to 200ml of distilled water and mix thoroughly.
2. Add 30g of agar powder to prepare solid media
3. Adjust pH to 7.2 with 5M NaOH

Appendix G

Anaerobic cellulolytic medium (1l) (Atlas, R and Parks, L, 1993)

NH_4Cl_2	1.0g
Cellobiose	1.0g
Yeast extract	1.0g
Mg SO_4	0.5g
KCl	0.5g
L-cysteine.HCl.H_2O	0.5g
K_2HPHO_4	0.4g
Resazurin	1.0mg
Wolfe's mineral solution	20ml
Na_2CO_3 solution	10ml

$Na_2S.9H_2O$ solution (100ml)

Na_2S9H_2O	1.5g

1. Add $Na_2S.9H_2O$ to distilled water and bring volume to 100ml
2. Mix thoroughly
3. Filter sterilize

Appendix H

Cellulose Congo-red medium

K2HPO4	0.5g
MgSO4	0.25g
Cellulose powder (Sigma)	1.88g
Congo red dye	0.20g
Soil extract	100ml
tap water	900ml

1. Autoclave for 20 min at 121°C.
2. Adjust to pH 7 with 1N NaOH.

Appendix I

Cellulolytic Clostridia media(1*l*) (Atlas, R and Parks, L, 1993)

$CaCO_3$	2.0g
$KHPO_4$	1.0g
$(NH_4)_2SO_4$	1.0g
$MgSO_4.7H_2O$	0.5g
NaCl	0.5g
Resazurin	1.0mg
Cellulose powder (Sigma)	20g

Appendix J

***Clostridium* cellulose medium (1*l*)** (Atlas, R and Parks, L, 1993)

$CaCO_3$	5.0g
Polypeptone™	5.0g
$Na_2CO_3.10H_20$	4.0g
K_2HPO_4	2.2g
Yeast extract	2.0g
KH_2PO_4	1.5g
$(NH_4)SO_4$	1.3g
$MgCl_2.6H_2O$	1.0g
Cysteine.HC*l*.H_2O	0.5g
$CaCl_2$	0.15g
$FeSO_4.7H_2O$	6.0mg
Cellulose powder , Whatman CF11, ball-milled	10g
Agar	20g

Appendix L

Basal medium for cellulolytic bacteria (100m*l*)

(Hodson, P.N. and Mann S.O. 1971)

Casitone (Difco)	0.25g
Yeast Extract (Difco)	0.06g
Centrifuged rumen fluid	10ml
Mineral solution A	15ml
Mineral solution B	15ml
Resazurin (0.1%w/v aqueous solution)	0.1ml
Cellobiose	0.25g
Cysteine hydrochloride	0.05g
Sodium bicarbonate	0.2g

Distilled water	60ml
Agar	1g

Mineral Solution A

KH_2PO_4	3g
$(NH_4)_2SO_4$	6g
$NaCl$	6g
$MgSO_4$	0.6g
$CaCl_2$	0.6g

Dissolve all reagents in 1l distilled or deionized water

Mineral Solution B

K_2HPO_4	3g

Dissolve in 1l distilled or deionized water.

Cellobiose, cysteine HCl and sodium bicarbonate are prepared as concentrated solutions, sterilized separately from the bulk medium. Sterilization conditions: 121°C, 15min

Appendix L

Clarified rumen fluid

1. Stomach contents of a freshly slaughtered cow was obtained from the Cato Ridge Abbatoir.
2. Filter contents thorough wire gauze to remove grass and other solid particles.
3. Centrifuge the resulting liquid at 4 500rpm (10 000-20 000rpm is preferable).
4. Autoclave at 121°C for 15min and recentrifuge, until a clear dark brown liquid remains.

$Na_2S.9H_2O$ solution	10ml

Wolfe's mineral solution (1*l*)

Mg $SO_4.7H_2O$	3.0g
Nitrilotriacetic acid	1.5g
NaC*l*	1.0g
$MnSO_4.H_2O$	0.5g
$FeSO_4.7H_2O$	0.1g
$CoCl_2.6H_2O$	0.1g
$CaCl_2$	0.1g
$ZnSO_4.7H_2O$	0.1g
$CuSO_4.5H_2O$	0.01g
$AlK(SO_4)_2.\ 12H_2O$	0.01g
H_3BO_3	0.01g
$Na_2MnO_4.2H_2O$	0.01g

1. Add nitrilotriacetic acid to approximately 500ml of distilled water and adjust pH to 6.5 with KOH to dissolve.
2. Bring volume to 1l with distilled water
3. Add remaining compounds one at a time and mix thoroughly.

Na_2CO_3 solution (300ml)

Na_2CO_3	10g

1. Add Na_2CO_3 to distilled water and bring volume to 100ml
2. Mix thoroughly
3. Filter sterilize

Appendix M

Ball-milling of Whatman No 1 filter paper.

Whatman No 1 filter paper was milled for 72 h and diluted to 3 g (dry weight) per 100ml of distilled water before addition to the medium. Final concentration in the medium was 0.6%.