UNIVERSITY OF KWAZULU-NATAL

Analysis of indigenous herbivore faecal matter as a potential source of hydrolytically active microorganisms

By

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Declaration

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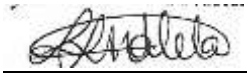

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Abstract

Microbial hydrolases play an important role in a variety of enzymatic processes employed in industry. Among the promising environmental sources explored for the presence of microbial hydrolases is herbivore faecal matter. However, information is lacking on the microbiological status of South African indigenous herbivore faeces. The objective of this study was therefore to analyse faecal matter from zebra, giraffe and impala as a potential source of hydrolytically active microorganisms and the related enzyme that might have potential for industrial applications. Hydrolase groups targeted were amylase, cellulase, esterase and proteases. Zebra giraffe and impala faeces were collected in Bisley Valley Nature Reserve over a period of 2 years during the study. Soil samples around the areas of faecal collection were used as controls. Samples were also compared to local Nguni cow faeces. Hydrolytic activity of the samples was measured through the fluorescein diacetate (FDA) assay and dehydrogenase activity through the triphenyltetrazolium chloride (TTC) assay. Microbial diversity and abundance in samples was estimated using aerobic viable plate counts, with additional use of media targeting specific hydrolase producers. The FDA assay and viable plate counts were used to analyse samples representing different seasons (winter of 2011 and summer of 2012) and to analyse the impact of storage conditions on the abundance of target microorganisms (storage over a period of four weeks at 30°C and 4°C). Randomly selected isolates producing the targeted hydrolases from faeces of zebra, giraffe and impala were characterised using microscopy, biochemical tests, MALDI-TOF MS and 16S rRNA gene sequence analysis. Selected isolates from zebra giraffe and impala were additionally analysed for their ability to use organic polymers (cellulose, skim milk, starch and Tween 80) as the sole carbon source. Faeces from zebra produced the highest hydrolytic and dehydrogenase activity. Overall, faecal matter from indigenous herbivores produced a higher hydrolytic activity than Nguni cow dung and soil controls. The measured microbial hydrolytic activity of samples collected in winter was higher than that of faecal samples collected in summer for all wild herbivores. Storage of samples caused fluctuations in microbial diversity and activity within 2 weeks of storage at both 4°C and 30°C. Selected faecal isolates that produced the targeted hydrolases were assigned to the genera *Bacillus* and *Arthrobacter* and did show specific enzyme activities not exceeding results reported in previous studies. This study demonstrated that faecal matter from zebra, giraffe and impala is a promising source for hydrolytically active microorganisms and the related hydrolases.

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To my Father in heaven, thank You faithful Father for Your grace to finish strong.

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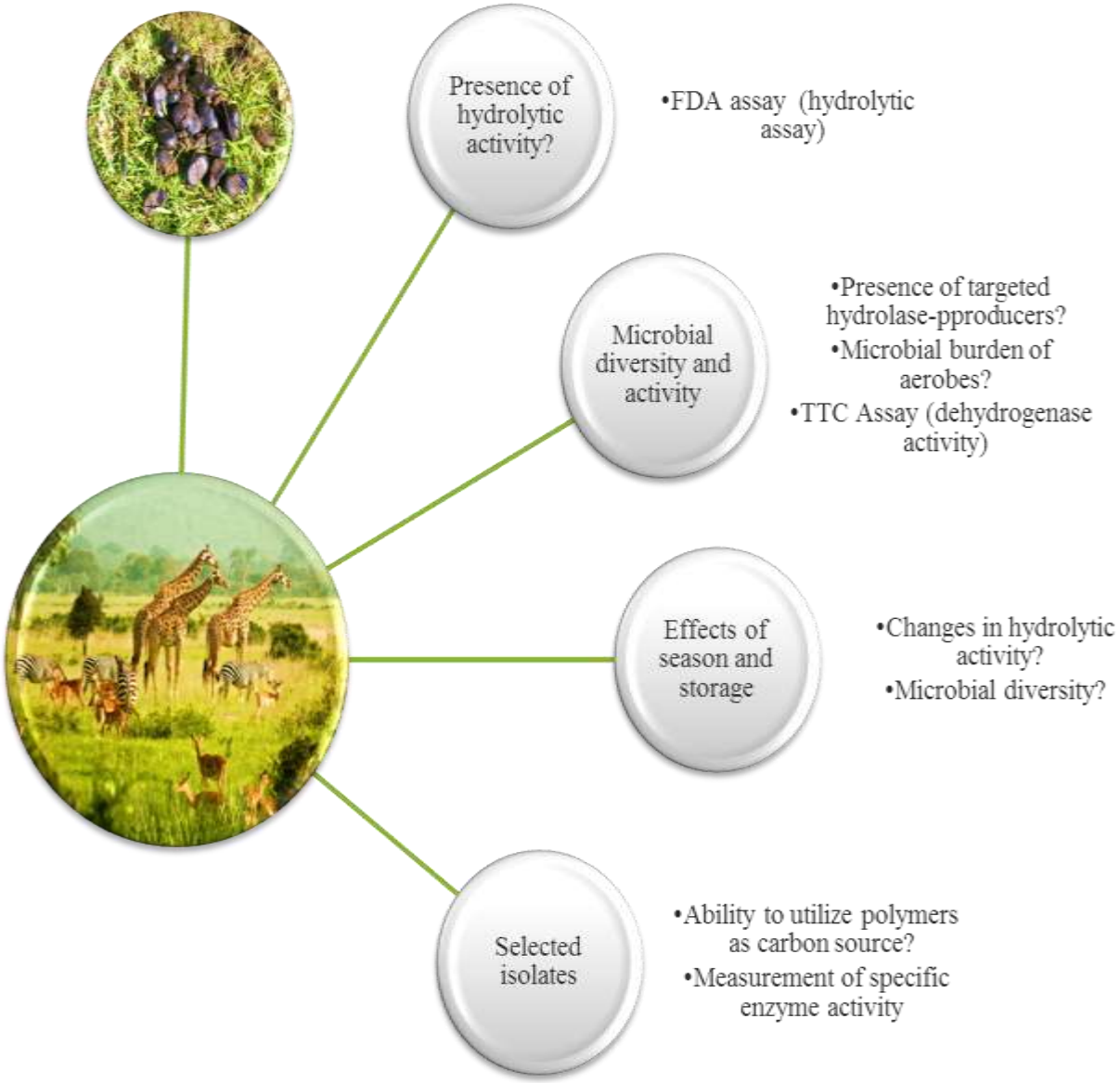
Conceptual Framework



1. Literature Review

1.1 Introduction

African indigenous ungulates have been under extensive review in an attempt to understand the multiple factors that contribute to their nutrition (Moon, 2010, Odadi *et al*., 2011). In countries such as Kenya studies were conducted on the implications of sex, age and size of wild ungulates on diet quality (Ngethe, 1976, Keesing, 1998; Odadi *et al*., 2011), comprising of behavioral and interactional analysis in natural environments (Udén and Van Soest, 1982, de Garine-Wichatitsky *et al*., 2004; Odadi *et al*., 2011) . Diet quality is commonly determined by analysis of faeces or rumen fluid. Fistulation of cows is a common experimental technique to analyse rumen fluid. This gives a clearer understanding of digestion within ruminants and how diet quality (Wrench *et al*., 1996; Wrench *et al*., 1997) affects digestion. Faecal analysis is an alternative, less invasive natural approach that yields a similar overview of dietary intake (Kohn and Wayne, 1997; Masunga *et al*., 2006).

Apart from reflecting the dietary intake of an animal, faecal matter has other characteristics that draw scientific interest; it provides an insight into the microbial community and interactions within the digestive systems of organisms. Faeces have also been used as a substrate for the isolation of coprophilic microorganisms such as fungi, (Abdullah, 1982; Nyberg and Persson, 2002) cellulolytic and predatory bacteria, particularly myxobacteria (Smit and Clark, 1971; Memela and Schmidt, 2013) In addition, faeces have been used to study the diversity and succession of coprophilic fungal classes (Angel and Wicklow, 1975; Nannipieri *et al*., 2003). In the specific case of indigenous South African ungulates, detailed analyses from a microbiological perspective appear to be lacking. Presently available data reports basic characteristics of cattle faecal matter such as chemical oxygen demand, pH (Shehu *et al*., 2012), and viable counts for different target organisms (Gong, 2007). So far, fungal succession (Dix and Webster, 1994), rate of dung decomposition (Omaliko, 1981) and the forage preference based on carbon output in faeces (Sponheimer *et al*., 2005) have been studied from faecal matter of ungulates such as impala.

In response to a need to isolate and develop novel microbial enzymes for industrial and bioremedial purposes (Maki *et al*., 2009), faecal matter of ungulates, moose and smaller mammals has been investigated for its potential to contain industrially applicable microbial enzymes (Angel and Wicklow, 1975; Doi, 2008; Fon and Nsahlai, 2012). Cellulolytic strains

from genera such as *Bacillus*, *Clostridium* and *Ruminococcus* are examples of successfully isolated microorganisms sourced from rumen fluid and faeces of cows (Varel *et al*., 1984). In the search for novel microbial enzymes, hydrolases are prioritised due to their attractive financial revenue outputs (Kirk *et al*., 2002; Goyal *et al*., 2005; Maciel and Ribeiro, 2010).

Currently, only a few microbial hydrolytic strains have been reportedly isolated from indigenous ungulates (Sadhu *et al*., 2011). In South Africa, a study measuring fibrolytic activities from zebra, horses and wildebeest faeces found that these materials displayed considerable fibrolytic activity, which was attributed to exocellulase enzymes (Kohn and Wayne, 1997). This served as an indicator for the presence of hydrolytic microbes within herbivore faeces and drew interest into further microbial analysis and the potential isolation of hydrolytic strains from faecal matter of indigenous South African ungulates.

This review aims to highlight the importance of hydrolases and to assess ungulate (zebra, giraffe and impala) faeces as potential microbial hydrolase sources, based on established facts regarding these animals.

1.2 Environmental and industrial applications of biocatalysts

Microbial enzymes play an important role in catalysis of industrial and environmental processes. Microbial enzymes comprise of six main groups; namely hydrolases, transferases, ligases, oxidoreductases, lyases and isomerases. Hydrolases are among the frequently used enzymes involved in numerous industrial processes. These enzymes function by the cleavage of polymeric bonds with water, thus producing monomers or less complex variants of the specific polymer (Karigar and Rao, 2011). Their application in the environment and industry is a constantly evolving field of study; more unique and efficient enzymes with higher tolerance under harsh conditions are continuously mined based on industrial needs.

Technological advances in industrial processes have produced negative environmental impacts that have resulted in rapid waste and by-product formation. The pace of industrial processes far surpasses the waste disposal procedures, which results in an accumulation of wastes that are not of notable value to nature or industry in their native forms (Eshun *et al*., 2012). In 2010, wood waste in Ghana was 62% of the initial wood input, which is mostly of no value and is inevitably disposed as waste (Eshun *et al*, 2012). Similar occurrences ensued in fishing and agricultural industries, with waste products amounting to more than 50% of the initial raw material input (Rustad, 2003). Microorganisms are pivotal in combating pollution

from the accumulation of environmental wastes (Baker, 2002; Lo *et.al*., 2009), as the microbial hydrolysis of agricultural and industrial wastes produces mostly less toxic and even industrially re-usable by-products (Karigar and Rao, 2011). These wastes are rich microbial nutrient sources, which are typically composed of cellulose and starch, protein, lignin and esters, depending on the waste source. Generally, these wastes are proven sources of diverse microorganisms and their potentially useful enzymes. As a result, there have been many studies conducted on samples such as wastewater sludge and agricultural waste, as a potential source of microorganisms with hydrolytic enzymes. This information has led to increased interest in the isolation and adaptations of microbial enzymes (Bhaskar *et al.*, 2008).

For example, microbial enzymes are increasingly being applied in the conversion of raw material in biofuel production. The increased depletion of fossil fuels (e.g. coal) as well as the detrimental environmental effects due to their usage has resulted in a global energy crisis. Simple sugar crops such as sugarcane were previously employed as raw material for biofuel production; however, this has threatened food security; especially in developing countries (Subhadra and George, 2011). Seaweed (Wargacki *et al*., 2012), *Miscanthus* and post-harvest plant waste material (Rackemann and Doherty, 2011) are examples of cited substrates that could be exploited to generate bioenergy (Gressel, 2008; Rackemann and Doherty, 2011; Wargacki *et al*., 2012). These plant material substrates are high in lignin and cellulosic fiber, making their hydrolysis specific to cellulolytic or lignolytic microorganisms. Cellulolytic microorganisms are typically found in soils, bovine guts and faeces of organisms that feed on forage (Varel *et al*., 1984; Doi, 2008).

Industrially, hydrolase enzymes grossed $ 1 billion annually in 1998 (Rao *et al*., 1998) and have been at the hub of food, beverage and textile production (Banat, 1995; Rao *et al*., 1998; Rustad, 2003; Ray, 2011). Developments in production over the years have led to prioritisation of cost-efficiency with high product yield in fermentation processes (Kirk *et al*., 2002). More than 95% of global industry fermentations utilise hydrolase biocatalysts, namely lipases, proteases and carbohydrases (Kirk *et al*., 2002). The hydrolases currently utilised in industry have been mutated over time to increase product yields, which takes time. As a result, more efficient hydrolases are being mined through exploring the potential of currently unexploited environmental sources for increased enzyme competency.(Sivaramakrishnan *et al*., 2006). With the use of microbial enzymes, industrial processing has interlinked with

bioremediation and the use of agricultural waste as a substrate for fermentation (Rustad, 2003; Karigar, 2011)

Fermentation forms the basis of general industrial product processing. There are two general types of fermentation employed in industrial production; submerged fermentation (SMF) and solid-state fermentation (SSF). Both of these fermentation methods have some advantages and drawbacks. SSF is frequently used in industry due to its cost-effectiveness; however it is not as effective in physico-chemical parameter control during fermentation as SMF (Latifian *et al*., 2007). On the other hand, submerged fermentation has been less utilised over the years due to the higher costs and lower product yields, regardless of affording efficient parameter control. This shift in prioritising saving has put a lot of pressure on developing enzyme competence of hydrolases that are able to remain stable in solid-state fermentation, in addition to further stability enhancement of currently used commercial enzymes (Kirk *et al*., 2002; Sivaramakrishnan *et al*., 2006; Latifian *et al*., 2007). The enzymes of interest in this review are carbohydrate, protein and ester hydrolases.

1.3 Isolation and characteristics of microbial hydrolases

1.3.1 Cellulolytic microorganisms

Cellulolytic microorganisms can be found in various environmental habitats and are usually not solely dependent on cellulose; this means that these microorganisms are usually able to utilise other carbon sources. A majority of isolated efficient cellulose degraders are anaerobes; mostly isolated from sewage and sludge waters. A high number can also be found in bovine, equine and other animal faeces (Varel *et al.,* 1983). Herbivore faeces generally contain cellulose degraders, as they feed on highly fibrous material which is broken down by microbes in the animals gut (Mullings and Parish, 1984). Some thermophilic cellulose degraders have been isolated from hot springs e.g. *Clostridium thermocellum* (Zverlov, 2008), displaying active cellulase enzymes. Amongst aerobes, strains from genera such as *Bacillus*, *Streptomyces* and *Micrococcus* have been isolated as sources for cellulase enzymes (Hoskisson and Hobbs, 2005). A *Micrococcus* sp. strain was found recently, exhibiting cellulose hydrolytic activity of 0.73 U/mg protein (Sadhu *et al*., 2011), which is higher than the cellulase activity produced by microbial isolates from invertebrates such as caterpillars and termites, which did not exceed 0.40 U/mg in cellulose endoglucanase activity (Gupta *et al*., 2012)

Screening of cellulolytic microorganisms generally occurs by staining of cellulose containing media after colonies have formed and checking for zones of hydrolysis (Fig 1). Gram's iodine, Congo red and 1% hexadecyltrimethyl ammonium bromide (HAB) have been used as stains for screening cellulose degraders on carboxymethylcellulose (CMC) agar. Gram's iodine was found to be the most easily visible indicator among the three stains (Kasana *et al.*, 2008).

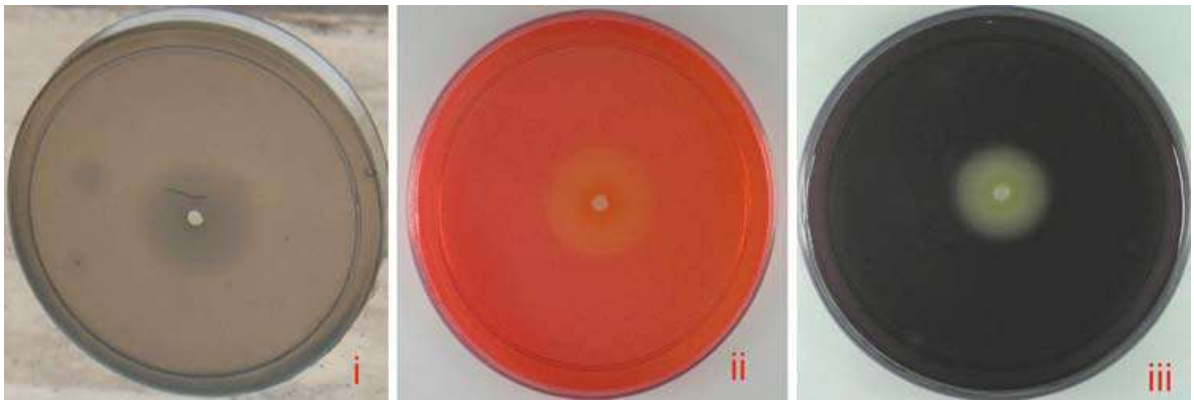


Figure 1: Comparison of HAB (i), Congo red (ii) and Gram's Iodine stain (iii) for the demonstration of cellulase positive colonies (Kasana *et al*., 2008).

The term "cellulase enzymes" generally refers to enzymes involved in hydrolysis of cellulose and other β-glycosides to simpler forms, with examples such as hemicellulases, lignocellulase and β-glycosidase. These enzymes are classified as either endo- or exoglucanases, based on whether these enzymes cleave molecules internally or externally. Cellulose can be broken down through oxidative degradation or hydrolytic attack (Varel *et al*., 1984, Maki *et al*., 2009). Oxidative degradation can be caused by the reaction of cellulose with hydrogen peroxide. Hydrolysis on the other hand, is caused by enzymes that initially cause the rupturing of the hydrogen bonds in the crystalline structure of cellulose, which causes the molecules to become linear and leads to the production of cellobiose units via hydrolysis (Fig 2). Further hydrolysis would result in the transformation of cellobiose to glucose (Wilson, 2008) consequently, bacteria can break cellulose down via fermentation of glucose to form volatile fatty acids such as propionic and butyric acid (Gascoigne and Gascoigne, 1960).

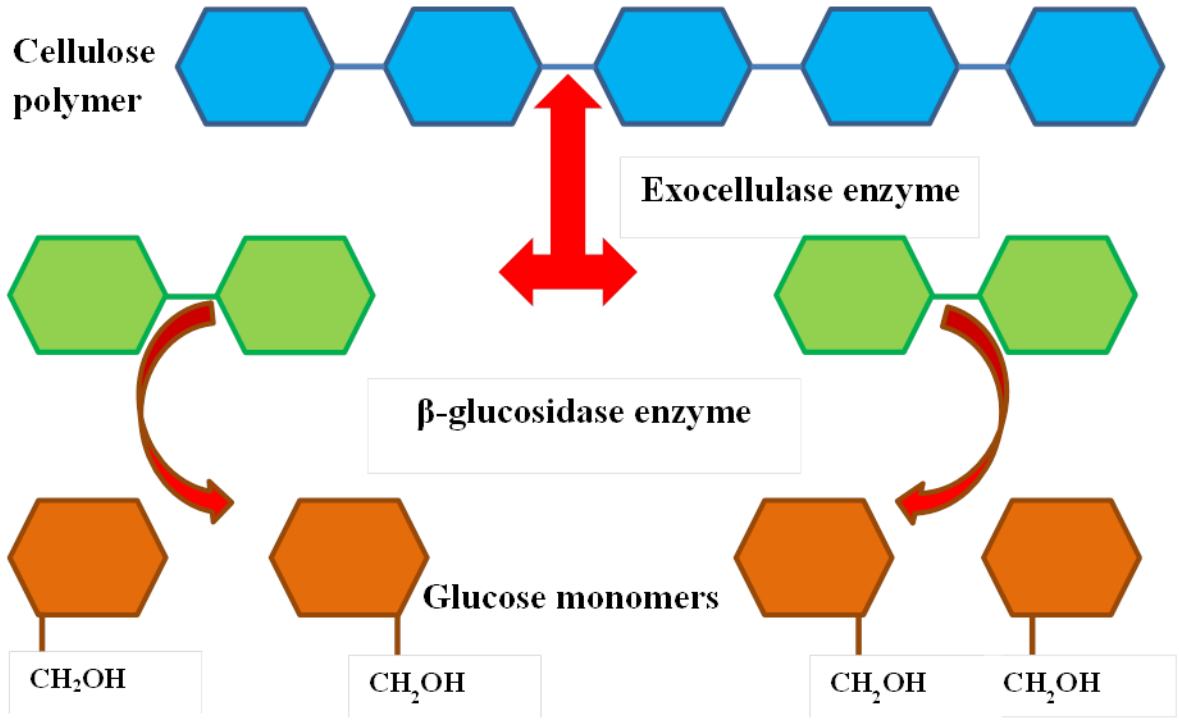


Figure 2: Cellulose polymer hydrolysis into simpler units (adapted from Wilson, 2008).

Cellulose hydrolysing enzymes typically exhibit optimum performance at pH ranges that are between 5.8 and 7.0 and are unstable at temperatures beyond 55°C (Ørskov and Ryle, 1990). This affects the enzyme's ability to affiliate itself with the substrate as well as the speed at which it catalyses the degradation of the cellulose substrate. Most mesophilic bacterial cellulases have an optimal temperature range of 36-40°C, whilst thermophilic cellulose degraders have optima of up to 80°C (Ørskov and Ryle, 1990).

1.3.2 Proteolytic microorganisms

Bacteria and fungi are known to produce proteases with varying properties. Fungi generally have a wide variety of proteases that range from being acidophilic to alkaliphilic. However, these have a lower reactivity rate and a lower temperature tolerance as compared to bacterial proteases (Rao *et al*., 1998). Most of the reported proteolytic microbes are from the genus *Bacillus* and generally have similar characteristics, which is neutral pH optima and a higher temperature tolerance as opposed to fungi. Alkaline tolerant proteases that are produced by bacteria have a wider substrate spectrum, temperature tolerance of as high as 60°C and a pH tolerance of up to pH 10 as opposed to neutral proteases (Rao *et al*., 1998). The majority of

proteases in industry are sourced from *Bacillus* spp. isolates, which are also present in faecal matter (Table 1).

Table 1: Examples of microbial proteases being utilised in industry (Gupta *et al.*, 2002)

Supplier	Trade name	Source	Application
Norvo Nordisk, Denmark	Alcalase	*Bacillus licheniformis*	Textiles
	Savinase	*Bacillus* sp.	Detergents, textiles
	Esperase	*Bacillus lentus*	Detergents, food, silk degumming
Genencor International, USA	Purafact	*Bacillus lentus*	Detergent
	Primatan	Bacterial source	Leather
Solvay Enzymes, Germany	Opticlean	*Bacillus alcalophilus*	Detergent
	Optimase	*Bacillus licheniformis*	Detergent
Amano Pharmaceuticals, Japan	Proleather	*Bacillus* sp.	Food
	Collagenase	*Clostridium* sp.	Technical

Proteolytic microbes are ubiquitous and can be found in environmental conditions that are favourable to their nutritional needs, ranging from rumen fluid to milk samples (Kopecny and Wallace, 1982; Nielsen, 2002), garden soil and exotic locations such as hot springs (Kumar and Bhalla, 2005). The first isolation of proteases from ungulates was reported in 1960 from sheep (Blackburn and Hobson, 1962). Since then more and more proteases have been isolated from microbes for various industrial processes.

In the hydrolysis of waste material from fish processing, *Bacillus licheniformis* was found to possess the most efficient proteases to produce fish hydrolysate. These enzymes were reported to be active at neutral and alkaline conditions. The isolation of these microorganisms can be achieved by regular screening using a medium containing proteins such as casein or azocasein (Blackburn and Hobson, 1962; Kopecny and Wallace, 1982)

There are various classes of proteases in microbial and higher taxa organisms (Rao *et al.*, 1998). In microorganisms, the following classes have been found; acidic, neutral or alkaline proteases, although acidic proteases are seldom found in bacteria. These classes are further divided into four subclasses which are defined by the catalytic residue at their active site.

These are metallo, serine, carboxyl and thiol proteases. In microbial cells, these proteases are either produced extracellularly or within the cell. Extracellular proteases are the most commercially utilised e.g. subtilisin. Much has been reported on the isolation of novel serine alkaline proteases (Rao *et al*., 1998; Gupta *et al*., 2002; Kumar and Bhalla, 2005). This is due to their ubiquity, temperature tolerance and ability to withstand higher alkalinity as compared to neutrophile proteases. As a result, alkaline proteases are commonly applied in industrial processes. However, some of the microbes possessing these proteases can grow under neutral conditions as well (Kumar and Bhalla, 2005).

1.3.3 Amylolytic microorganisms

Amylolytic microorganisms are also ubiquitous and there is plenty information on *Bacillus* and *Pseudomonas* spp. as industrially utilised amylase-possessing microorganisms (Ray, 2011). Most isolates that have been discovered are mesophilic *Bacillus* spp. strains and a lot of research has gone into isolating halotolerant bacteria. Apart from the ability to withstand higher salt concentrations, most of the enzymes in halotolerant microorganisms are thermotolerant. Examples of discovered halotolerant thermophiles are from genera such as *Halobacter*, *Bacillus* and *Halomonas*. Microbial amylases are dominant in industry as opposed to plant or animal amylases; this is because of their stability and the ease with which microorganisms can be mutated to produce amylases with the desired enzyme characteristics (de Souza and Oliveira ê Magalhaes, 2010).

Primary screening of amylolytic isolates is typically done on starch agar with positive colonies displaying a clear halo after staining with Grams iodine whilst negative isolates remain purple, as starch gives a bluish colour when reacted with iodine (Kasana *et al*., 2008; Maki *et al*., 2009). The explanation for the colour development is the amylose-iodine complex formed when intact starch is present, though the exact chemistry of the reaction is unknown (Rendleman, 2003).

There are a number of amylases that are applied in industrial processes. The main amylases used are α-amylase, β-amylase, isoamylase, glucoamylases and pullulanases (Maitin *et al*., 2001). The α-amylases comprise the largest family of the amylase enzymes and their mode of action is basically the hydrolysis of the internal α(1,4)-glycosidic bond in polysaccharides (Sivaramakrishnan *et al*., 2006). The β-amylases hydrolyse starch to maltose whilst pullulanases and isoamylase cleave the branch points in the starch molecule which are the α

(1-6)-linkages in the molecule, leaving long linear polysaccharides after hydrolysis. These enzymes are stable under various conditions whilst isoamylases have a lower thermo stability compared to the other amylase enzymes (Ray, 2011).The α-amylases from mesophiles such as *Saccharomyces* spp. showed stability between 25 and 37°C whilst those from most industrially used microorganisms such as *Bacillus* showed stability of up to 42°C (Ray, 2011). Limited literature is available on the de-branching type amylases as compared to α and β-amylases (Ray, 2011). Although these enzymes have varied uses and characteristics, a usage of combined enzymes has been advised in the hydrolysis of starch industrially (Maitin *et al*., 2001).

1.3.4 Esterolytic microorganisms

Lipases or esterases are produced by a large number of bacteria and fungi in nature. Esterases are a sub-class of lipases, which differ from lipases in a few ways; for example, esterases are more regioselective and secondly have a lesser substrate specificity compared to true lipases. Esterases are also less hydrophobic than lipases and also have a preference for shorter triglyceride chains than lipases (Bornscheuer, 2002).

Most microorganisms are known to produce membrane-bound esterases although Gram negative isolates are capable of producing extracellular esterases (Gunasekaran and Das, 2005). The high activity of esterases in microorganisms as well as their easy detection has led to them being used in the identification of *Rhizobium* species in a previous study (Murphy and Masterson, 1970) and their high stereo-specificity is advantageous in producing pure compounds (Bornscheuer, 2002). Although mostly fungal lipases have been industrially applied, microbial esterases are also being isolated for industrial processes (Bornscheuer, 2002; Gunasekaran and Das, 2005; Vakhlu and Kour, 2006). They are of particular interest in industry due to their stability over wide temperature ranges and catabolism of carbohydrate intermediates. Esterases and lipases from *Pseudomonas* have been increasingly employed in synthetic organic chemistry and in the production of pure compounds(Jaeger *et al*., 1996). Esterases are not thoroughly understood, particularly in microbes due to the varying nature of esters across different microbial genera (Jaeger *et al*., 1996; Arpigny and Jaeger, 1999; Gunasekaran and Das, 2005; Topakas *et al*., 2007).

A sub-class of carboxyl esterases termed feruloyl esterases was discovered in 1991. These esterases are of interest due to their relatively recent discovery and applicability in the biofuel

industry. Feruloyl esterases have been applied in hydrolysis of hemicellulose to produce ferulic acid, which is hydrolysed further for use in food flavouring (Topakas *et al*., 2007). Enzymologically, esterases act by cleavage of small ester linkage containing molecules (Arpigny and Jaegar, 1999) or short triglyceride chains, in turn releasing fatty acids and glycerol (Gunasekaran and Das, 2005). Esterases have been isolated from bacterial genera such as *Pseudomonas, Bacillus* and *Staphylococcus* (Arpigny and Jaegar, 1999). These are some of the few bacterial genera in which esterases have been investigated in more detail. Properties of esterases vary and many have been found to function over wide temperature and pH ranges (Aurilia *et al*., 2008).

More research is required, particularly in the enzymology and classification of feruloyl esterases. Microorganisms containing esterases can be screened using *p*-nitrophenyl substrates and generally show Michaelis-Menten enzyme kinetics (Bornscheuer, 2002). Tween 20 and 80 agar with calcium chloride has also been employed as a screening medium, upon which positive isolates produce a calcium oleate or calcium laurate precipitate, which is indicative of ester hydrolysis (Castro *et al*., 1992; Bornscheuer, 2002; Aurilia *et al*., 2008). Screening has also been conducted using rhodamine B agar, which fluoresces under UV light if ester hydrolysis has occurred (Kouker and Jaeger, 1986). In the case of feruloyl esterases, ferulic acid enzyme assays have been developed, that measure enzymatic activity by using high performance liquid chromatography (HPLC) and gas chromatography (GC) (Topakas *et al*., 2007).

1.4 Faecal matter as a source of microbial biocatalyst isolation

Various industrially utilised microorganisms have been isolated from soil, wastewater and extreme environments (Wallace and Brammal, 1985; Vasileva-Tonkova and Galabova, 2003; Lo *et al.*, 2009)

Faecal matter is among the potential sources of useful microbial isolates that are applicable in industry or in bioremediation as it is rich in microbial load and diversity (Gong, 2007). In addition, it is excreted in large quantities in agricultural farms and can thus serve as an abundant substrate (Table 2) (Dix and Webster, 1994; Hansen, 2006). The abundance is proportional to the farming activities and increase in the number of livestock reared. When measured, faecal material in most agricultural farm animals was found to have a COD ranging from 30-6000 mg/l (Maranon *et al*., 2006), making it a potentially rich substrate for

microbial colonisation before and after defecation as the COD is within ranges measured in raw sewage, which is considered rich in microbial load and diversity (Hur *et al*., 2010).

Table 2: Estimates of annual manure production in some agricultural farm animals (Hansen, 2006)

Animal	Animals per AU (1000lbs)	Annual manure production per AU (tons)
Beef cattle	1	11.5
Dairy cattle	0.74	15.24
Swine (breeders)	2.67	6.11
Swine (other)	9.09	14.69
Hens (laying)	250	11.45
Pullets (<3 months)	250	8.32
Pullets (>3 months)	455	8.32
Broilers	455	14.97
Turkey (slaughter)	67	8.18

*Key AU= animal unit (5 acres of land) 1000lbs=450kg

Faecal properties and rumen contents of ungulates have been analysed in detail for agricultural and dietary purposes. Faeces of domesticated ungulates such as cows have been analysed for general microbial counts and basic characteristics such as pH, with hydrolytically active strains of bacteria being isolated (Anthony and Smith, 1974) . However, a detailed culture based microbiological analysis of other ungulate faeces such as giraffe and impala has not been done previously. This is an interesting option to search for new of microbial hydrolases since these animals also feed on an herbivorous diet similar to domestic ungulates. The similarity in diet indicates that similar hydrolytically active microorganisms may be present in wild ungulate faeces. This is true especially for wild indigenous ungulates who feed on a wild fibrous diet. There is a lack of data on hydrolytic microbes from zebra, giraffe and impala faeces apart from established cellulose-degraders. Cellulolytic bacteria have been isolated not only from cattle and sheep but as well as from zebra faeces. A cellulolytic *Microbacterium* sp. strain was isolated from zebra (*Equus zebra*) faeces in Kolkata (India). This strain exhibited cellulase activity of 0.73 U/mg with carboxymethyl cellulose as a substrate (Sadhu *et al*., 2011) indicating the presence of hydrolytically active strains within such faeces.

Herbivore and omnivore faecal properties have been analysed more from an animal science perspective, however, the microbial communities and hydrolytic activity in these faecal

samples were not studied for application purposes discussed in this review. Microorganisms able to hydrolyse multiple complex polymers have been sourced from faecal matter; both amylolytic, lipolytic, cellulolytic and proteolytic bacteria were found in cow dung samples (Gong, 2007). There have also been reports of various anaerobic microorganisms e.g. *Clostridia* spp. that have useful enzymes which can be applied in bioremediation being sourced from pigs (Varel *et al*., 1984), indicating that there are useful and stable enzymes that can be isolated from faecal microorganisms.

The Nguni cow is primarily a beef breed cow, indigenous to Southern Africa. Farming methods for this breed are well established as this knowledge has been passed on from generations of farmers. Nguni cows are particularly well known for surviving on poor forage and are less susceptible to ticks, which makes tick control easier. Nguni cattle, which are described as a "hardy" breed differ from breeds such as the Jersey cow, which is primarily a milk breed cow (Tada *et al*., 2013). Not only is the Nguni breed adaptable and easily farmed, but it plays a major role in agricultural advancement in South African underdeveloped areas. These cows feed on both natural veld and permanent pastures; they are also used in commercial farming (Nowers and Welgemoed, 2010).

1.5 The ungulates of interest: Zebra, giraffe, impala

1.5.1 Foraging in ungulates

Ungulate foraging habits have an effect on faecal output. Factors such as sex, diet, age, digestive system, body size and mass, chewing mechanism and seasonal changes are amongst the most studied influences on nutrition and faecal composition of ungulates (Ngethe, 1976; Ngethe and Box, 1976; Odadi *et al*., 2011). Generally, herbivorous ungulates feed on highly fibrous material, which varies in composition based on the feeding habits of the ungulate. Ungulate feeding habits are categorised as either grazing or browsing. Grazers generally feed on grasses, whilst browsers feed on leaf material. Some ungulates such as impala feed on either grasses or leaves, depending on the season and food availability. The specific grasses and leaf material that ungulates forage on in nature cannot be certain but the general properties can be estimated. A previous study analyzing the properties of non-wood fibres found that certain grasses contained about 33-38% of cellulose (Han, 1998). Another study found that grasses generally contained 25-40% of cellulose. Other major components were

hemicellulose and lignin (Sun and Cheng, 2002). Leaf material contained about 20% of cellulose in comparison (Sun and Cheng, 2002).

1.5.2 Zebra

Zebra (*Equus burchelli*) is an equine hindgut fermenting ungulate. It is indigenous to Africa and is a grazer in feeding habits. Zebras have been described as one of the most successful non-ruminant ungulates that share the plains with ruminants (Estes, 1991, Neuhaus and Ruckstuhl, 2002). These ungulates weigh between 220-250 kg and males are slightly heavier than females. Zebras are group feeders that segregate according to sex during feeding. No significant differences between the feeding habits of males or females are on record to date. These ungulates feed on grasses and were found to have a preference for *Themeda triandra* and *Cynodon dactylon* grass species when foraging in Kenyan savannas (Stewart and Stewart, 1970). Being grazers, zebras have a similar diet to cows and other bovids. Based on a study conducted in Kenya, no competition for forage was observed between bovids and equids and relations appeared to be mutualistic amongst these grazers (Odadi *et al*., 2011).

Zebras spend a large part of their day foraging and resting. Due to their digestive system, their food is digested faster than in ruminants and digestion of cellulose is not as efficient (Odadi *et al*., 2011). As a result, more time is spent on foraging to obtain adequate nutrition as digestion is relatively fast (Neuhaus and Ruckstuhl, 2002). Zebra and wildebeest faeces produced high fibrolytic activity and viable counts (Fon and Nsahlai, 2012), probably due to primary digestion occurring in one chamber unlike in ruminants, where the hindgut is for secondary digestion. This results in excretion of active microorganisms, which can be aerobically cultured (McSweeney *et al*., 1999).

1.5.3 Giraffe

Giraffe (*Giraffa camelopardalis*) is a ruminant browser indigenous to Africa. Like zebra, giraffes are segregated according to sex during foraging and are group feeders. A giraffe weighs 1800-2200 kg, with males being significantly larger than females. This is evident in their feeding habits, as males can usually survive on a less nutritious diet and spend less time foraging. Females are more selective and spend longer amounts of time foraging (Ginnett and Demment, 1997). Giraffe ungulates calve throughout the year, despite their narrow forage niche. This indicates an ability to obtain sufficient nutrition throughout the year, regardless of season (Leuthold, 1978). The steady nutrition of these ruminants may be due to their height

advantage, which reduces competition for food sources when feeding on very high trees. Giraffe diet is mainly *Acacia* plants during the green season and may change to evergreen species over the dry season. These ungulates spend more time foraging during the dry season (Leuthold, 1978; Pellew, 1984).

Giraffe are entirely dependent on leaves and shrubbery throughout the year and spend a large amount of time foraging and ruminating (Ginnett and Demment, 1997). As browsers, their diet quality is more nutritious compared to grazers and some of the herbage they feed on is higher in cellulosic content than grasses. Their diet has been studied extensively through faecal analysis but the microbiology is not well researched.

1.5.4 Impala

Impala (*Aepyceros melampus*) is indigenous to Africa and is a ruminant mixed browser. Impala generally feed on browse or grasses, depending on the availability of forage. It has been known to browse and graze in different seasons. The choice of diet depends largely on the location of the impala and the herbage available at that time. In South Africa impala were found to feed on 63% of grasses based on faecal analysis. It is supposed however, that this is not always constant and can vary (Sponheimer *et al*., 2003). Impala is a small ruminant, generally weighing approximately 50 kg and because of the smaller size, feeding time is not as lengthy as that of zebra and giraffe. Impala has advantages in being a mixed feeder such as being able to feed on a larger variety of herbage and not spending large amounts of time selecting forage in dry or rainy seasons (Sponheimer *et al*., 2003).

The effect of impala, springbok and kudu diets on their meat quality has also been analysed, but no microbial analysis was conducted on these ungulates (Hoffman, 2007). Stable carbon isotopes have been used to study the diet quality of impala in comparison to kudu competitors. Impala was found to take smaller bites of the herbage compared to kudu, which took greater bites and spent more time foraging (Sponheimer *et al*., 2005). Faecal analysis of impala forage consisted of incompletely digested cellulosic material and the diversity of plant material consumed could be obtained (Stewart, 1971). There is only limited literature available on the microbiological analysis of impala faecal output or rumen contents, emphasising the potential avenue of study.

1.6 Intestinal micro flora and findings from other animals

The intestinal micro flora from herbivorous ungulates such as cows (Artan *et al*., 1996)has been analysed as well as the micro flora from other organisms such as caterpillars and termites (Gupta *et al*., 2012) or fish (Austin, 2006). In general, microbial numbers are considered high when they are in the range of 10^6 to 10^8 cfu per gram in fish intestines (Austin, 2006). Termites and soil invertebrates exhibited intestinal microbe numbers of up to 10^{11} per ml, with *Bacillus* being detected as part of the microbial diversity (König, 2006). A study of the viable microbial population in faecal samples of herbivores such as rabbits that exhibit coprophagous behaviour found protease-producing microorganisms to comprise 50% of the microbial life in the faeces. Cellulolytic microorganisms from guinea pigs, rabbits and swine faeces were found to exceed 10^5 cells/g (Yoshida *et al*., 1968; McBee, 1971). In cows, cellulolytic microorganisms were found in the range of 10^9 cells per gram dry weight (Gong, 2007). However, literature assessing microbial numbers and diversity in zebra, giraffe and impala faecal matter is limited.

Potential of indigenous ungulate faecal matter as a source for hydrolytic enzymes

The prospects of using cellulolytic enzymes in industry have been reviewed and many advantages are established. Firstly, enzyme stability is usually greater if isolates have been sourced from environments containing complex polymer substrates e.g. cellulose. Secondly, the microorganisms capable of utilising cellulose are generally able to tackle other substrates as well (Maki *et al*., 2009) making cellulolytic microorganisms versatile for industrial applications.

Factors that make faecal matter an attractive source for microbial hydrolases are mainly the diet of ungulates, which comprises of cellulose rich material thus requiring hydrolytic activity to enable microorganisms to utilise such organic polymers as carbon and energy sources. At the same time, the lack of research in the area of faecal microbiology of indigenous South African ungulates shows the potential merit of such investigations. Due to the limited data, currently reference would have to be made based on available data for cattle faecal matter as an example for a herbivorous ungulate (Odadi *et al*., 2011).

Based on available data and feeding habits, it could be hypothesised that these indigenous animals are potentially promising objects of microbiological research. Recent publications (Köhn and Wayne, 1997; Fon and Nsahlai, 2012) discussed the presence of fibrolytic activity

in faeces and found zebra faeces to be among the samples exhibiting higher activity compared to wildebeest, horses and other mixed faeces. Sadhu *et al*. (2011) are one of the few examples reporting a cellulolytic bacterial isolate from zebra faeces. Their isolate exhibited a high and inducible cellulase activity based on analysis via the DNS (dinitrosalicylic acid) assay and was able to utilise more than one substrate efficiently. This confirms that there is a potential for the isolation of other microorganisms with even more efficient hydrolytic enzymes potentially applicable in industry. The combination of ruminant and hindgut ungulates in the study will provide insight into general microbial properties of indigenous ungulates, as well as providing an interesting comparison to what is currently known for domesticated ungulates. An investigation into microbiological properties of the faeces of three different ungulates will provide an overview covering different animals and at the same time offer a preliminary assessment of such faeces as a source for microbial hydrolases.

1.6 References

Abdullah, S.K. 1982. Coprophilous mycoflora on different dung types in southern desert of Iraq. *Sydowia***,** XXV**,** 1-5.

Angel, K. & Wicklow, D.T. 1975. Relationships between coprophilous fungi and faecal substrates in a Colourado grassland. *Mycologia*, 67**,** 63-74.

Anthony, R. G. & Smith, N. S. 1974. Comparison of rumen and faecal analysis to describe deer diets. *Journal of Wildlife Management,* 38**,** 535-540.

Arpigny, J. L. & Jaeger, K. E. 1999. Bacterial lipolytic enzymes: classification and properties. *Biochemistry Journal,* 343**,** 177-183.

Artan, R., Cook, D. F., Dadour, I. R. & Ali, D. N. 1996. Effect of diet on the excretion profile of ivermectin in cattle faeces. *International Journal for Parasitology,* 3**,** 291-295.

Aurilia, V., Parracino, A. & D' Auria, S. 2008. Microbial carbohydrate esterases in cold adapted environments. *Gene*, 410**,** 234-240.

Austin, B. 2006. The bacterial microflora of fish, revised. *The Scientific World Journal*, 6**,** 931-945.

Baker, A. 2002. Fluorescence properties of some farm wastes: implications for water quality monitoring. *Water Research*, 36**,** 189-195.

Banat, I. M. 1995. Biosurfactants produvtion and possible uses in microbial enhanced oil recovery and oil pollution remediation: a review. *Bioresource Technology*, 51**,** 1-12.

Blackburn, T. H. & Hobson, P. N. 1962. Further studies on the isolation of proteolytic bacteria from the sheep rumen. *Journal of General Microbiology,* 29, 69-81.

Bornscheuer, U. T. 2002. Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS Microbiology Reviews,* 26**,** 73-81.

Castro, G. R., Stettler, A. O., Ferrero, A. M. & Sineriz, F. 1992. Selection of an extracellular esterase-producing micro-organism. *Journal of Industrial Microbiology,* 10**,** 165-168.

De Garine-Wichatitsky, M., Fritz, H., Gordon, I. J. & Illius, A.W. 2004. Bush selection along foraging pathways by sympatric impala and greater kudu. *Oecologia,* 141**,** 66-75.

De Souza, P. M. & Oliveira e Magalhaes, P. 2010. Application of microbial alpha-amylase in industry- a review. *Brazilian Journal of Microbiology,* 41**,** 850-861.

Dix, N. J. & Webster, J. 1994. *Fungal ecology,* London, Chapman & Hall Ltd.

Doi, R. H. 2008. Cellulases of mesophilic microorganisms: cellulosome and noncellulosome producers. *Annals of the New York Academy of Science,* 1125**,** 267-279.

Eshun, E., Potting, J. & Leemans, R. 2012. Wood waste minimization in the timber sector of Ghana: a systems approach to reduce environmental impact. *Journal of Cleaner Production,* 26**,** 67-78.

Estes, R. D. 1991. *The behavior guide to African mammals: including hoofed mammals, carnivores, primates,* University of California Press.

Fon, F. N. & Nsahlai, I. V. 2012. The *in vitro* fermentation of maize stover as affected by faecal bacteria obtained from ungulates. *South African Journal of Animal Science,* 42**,** 478-482.

Gascoigne, J. A. & Gascoigne, M. M. 1960. *Biological degradation of cellulose,* Carlifonia, Butterworth.

Ginnett, T. F. & Demment, M. W. 1997. Sex differences in giraffe foraging behaviour at two spatial scales. *Oecologia,* 110**,** 291-300.

Gong, C. 2007. Microbial safety control of compost material with cow dung by heat treatment. *Journal of Environmental Sciences,* 19**,** 1014-1019.

Goyal, N., Gupta, J. K. & Soni, S. K. 2005. A novel raw starch digesting thermostable α-amylase from *Bacillus* sp. I-3 and its use in the direct hydrolysis of raw potato starch. *Enzyme and Microbial Technology,* 37**,** 723-734.

Gressel, J. 2008. Transgenics are imperative for biofuel crops. *Plant Science,* 174**,** 246-263.

Gunasekaran, V. & Das, D. 2005. Lipase fermentation: progress and prospects. *Indian Journal of Biotechnology,* 4**,** 437-445.

Gupta, P., Samant, K. & Sahu, A. 2012. Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. *International Journal of Microbiology,* 2012**,** 1-5.

Gupta, R., Beg, Q. K. & Lorenz, P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology,* 59**,** 15-32.

Han, J.S. 1998. Properties of non-wood fibres. *Proceedings of the Korean Society of Wood Science and Technology Annual Meeting*

Hansen, D. J. 2006 Manure as a nutrient source. In *The Mid-Atlantic Nutrient Management Handbook,* Mid-Atlantic Regional Water Program, 208-224.

Hoffman, L. C., Kroukamp, M., Manley, M. 2007. Meat quality characteristics of Springbok (*Antidorcas marsupialis)*. 4: Sensory meat evaluation as influenced by age, gender and production region. *Meat Science, ,* 774-778.

Hoskisson, P. A. & Hobbs, G. 2005. Continuous culture – making a comeback? *Microbiology,* 151**,** 3153-3159.

Hur, J., Lee, B.-M., Lee, T.-H. & Park, D.-H. 2010. Estimation of biological oxygen demand and chemical oxygen demand for combined sewer systems using synchronous fluorescence spectra. *Sensors,* 10**,** 2460-2471.

Jaeger, K. E., Liebeton, K., Zonta, A., Schimossek, K. & Reetz, M. T. 1996. Biotechnological application of *Pseudomonas aeruginosa* lipase: efficient kinetic resolution of amines and alcohol. *Applied Microbiology and Biotechnology,* 46**,** 99-105.

Karigar, C. S. & Rao, S. S. 2011. Role of microbial enzymes in the bioremediation of pollutants: a review. *Enzyme Research,* 2011.

Kasana, R. C., Salwan, R., Dhar, H., Dutt, S. & Gulati, A. 2008. A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Current Microbiology,* 57**,** 503-507.

Keesing, F. 1998. Impact of ungulates on the demography and diversity of small mammals in central Kenya. *Oecologia,* 116**,** 381-389.

Kirk, O., Borchert, T. V. & Fuglsang, C. C. 2002. Industrial enzyme applications. *Current Opinion in Biotechnology,* 13**,** 345-351.

Kohn, M. H. & Wayne, R. K. 1997. Facts from faeces revisited. *Trends in Ecology & Evolution,* 12**,** 223-227.

König, H. 2006. *Bacillus* species in the intestine of termites and other soil invertebrates. *Journal of Applied Microbiology,* 101**,** 3 620-627.

Kopecny, J. & Wallace, J. R. 1982. Cellular location and some properties of proteolytic enzymes of rumen bacteria. *Applied and Environmental Microbiology,* 43**,** 1026-1033.

Kouker, G. & Jaeger, K. 1987. Specific and sensitive plate assay for bacterial lipases. *Applied and Environmental Microbiology,*53**,** 211-213

Kumar, D. & Bhalla, T. C. 2005. Microbial proteases in peptide synthesis: approaches and applications. *Applied Microbiological Biotechnology,* 68**,** 726-736.

Latifian, M., Hamidi-Esfahani, Z. & Barzegar, M. 2007. Evaluation of culture conditions for cellulase production by two s mutants under solid-state fermentation conditions. *Bioresource Technology,* 98**,** 3634-3637.

Leuthold, W. 1978. Ecological Separation among Browsing Ungulates in Tsavo East National Park, Kenya. *Oecologia***,** 35**,** 241-252.

Lo, Y., Saratale, G. D., Chen, W., Bai, M., & Chang, J. 2009. Isolation of cellulose-hydrolytic bacteria and applications of the cellulolytic enzymes for cellulosic biohydrogen production. *Enzyme and Microbial Technology***,** 44**,** 417-725.

Maciel, M. J. M. & Ribeiro, H. C. T. 2010. Industrial and biotechnological applications of ligninolytic enzymes of the *Basidiomycota*: A review. *Electronic Journal of Biotechnology,* 13**,** 14-15.

Maitin, V., Kavitha, R. & Umesh-Kumar, S. 2001. Properties of an extracellular amylase purified from a *Bacillus* species. *World Journal of Microbiology and Biotechnology,* 17**,** 823-826.

Maki, M., Leung, K. T. & Qin, W. 2009. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *International Journal of Biological Sciences,* 5**,** 500-516.

Maranon, E., Castrillon, L., Fernandez, J. J. & Fernandez, Y. 2006. Anaerobic mesophilic treatment of cattle manure in an upflow anaerobic sludge blanket reactor with prior pasteurization. *Journal of the Air & Waste Management Association,* 56**,** 137-143.

Masunga, G. S., Andresen, O., Taylor, J. E. & Dhillion, S. S. 2006. Elephant dung decomposition and coprophilous in two habitats of semi-arid Botswana. *Mycological Research,* 110**,** 1214-1226.

Mays, P. A. & Edwards, G. S. 2001. Comparison of heavy metal accumulation in a natural wetland and constructed wetlands receiving acid mine drainage. *Ecological Engineering,* 16**,** 487-500.

Mcbee, R. H. 1971. Significance of intestinal microflora in herbivory. *Annual Review of Ecology and Systematics,* 2**,** 165-176.

Mcsweeney, C. S., Dalrymple, B. P., Gobius, K. S., Kennedy, P. M., Krause, D. O., Mackie, R. I. & Xue, G. P. 1999. The application of rumen biotechnology to improve the nutritive value of fibrous feedstuffs: pre- and post-ingestion. *Livestock Production Science,* 59**,** 265-283.

Memela, N. S. & Schmidt, S. 2013. Fishing with bait for bacterial predators in Zebra droppings – isolation and characterisation of a bacteriolytic *Myxococcus* strain. *In:* Mendez-Vilas, A. (ed.) *Microbial pathogens and strategies for combating them: science, technology and education, Vol 2 pp 1104-1111* Spain: Formatex Research Centre.

Moon, Y. H., Ok, J., Lee, S. J., Ha, J. K., Lee, S. S. 2010. A comparative study on the rumen microbial populations, hydrolytic enzyme activities and dry matter degradability between different species of ruminant. *Animal Science Journal,* 81**,** 642-647.

Mullings, R. & Parish, J. H. 1984. Mesophillic aerobic Gram-negative cellulose degrading bacteria from aquatic habitats and soils. *Journal of Applied Bacteriology,* 57**,** 455-468.

Murphy, P. M. & Masterson, C. L. 1970. Determination of multiple forms of esterases in *Rhizobium* with paper electrophoresis. *Journal of General Microbiology,* 6**,** 121-129.

Nannipieri, P., Ascher, J., Ceccherini, M., Landi, L., Pietramellara, G. & Renella, G. 2003. Microbial diversity and soil functions. *European Journal of Soil Science,* 54**,** 655-670.

Neuhaus, P. & Ruckstuhl, K. E. 2002. The link between sexual dimorphism, activity budgets, and group cohesion: the case of the plains zebra (*Equus burchelli*). *Canadian Journal of Zoology,* 80**,** 1437-1441.

Ngethe, C. 1976. Preference and daily intake of five East African grasses by zebra. *Journal of Range Management,* 29**,** 510.

Ngethe, C. & Box, T. W. 1976. Botanical composition of eland and goat diets on an acacia-grassland community in Kenya. *Journal of Range Management,* 29**,** 290-293.

Nielsen, S. S. 2002. Plasmin system and microbial proteases in milk: characteristics, roles and relationship. *Journal of Agricultural and Food Chemistry,* 50**,** 6628-6634.

Nowers, C. B. & Welgemoed, J. 2010. The economic viability of finishing Nguni weaners on natural veld and permanent pastures. *South African Journal of Animal Science,* 40**,** 422-423.

Nyberg, A. & Persson, I. 2002. Habitat differences of coprophilous fungi on moose dung. *Mycology Research,* 106**,** 1360-1366.

Odadi, O. W., Jain, M., Van Wieren, S.E., Prins, H.H.T. & Rubenstein, D.I. 2011. Facilitation between bovids and equids on an African savanna. *Evolutionary Ecology Research,* 13**,** 237-252.

Omaliko, C. P. E. 1981. Dung deposition, breakdown and grazing behaviour of beef cattle at two seasons in a tropical grassland ecosystem. *Journal of Range Management,* 34**,** 360-362.

Ørskov, E. R. & Ryle, M. 1990. *Energy nutrition in ruminants,* Elsevier Science Publishers, Cambridge.

Pellew, R. A. 1984. Food consumption and energy budgets of the giraffe. *Journal of Applied Ecology,* 21**,** 141-159.

Rackemann, D. W. & Doherty, W. O. 2011. The conversion of lignocellulosics to levulinic acid. *Biofuels, Bioproducts and Biorefining,* 5**,** 198-214.

Rao, M. B., Tanksale, A. M., Ghatge, M. S. & Deshpande, V. V. 1998. Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews,* 62**,** 597-635.

Ray, R. R. 2011. Microbial isoamylases: an overview. *American Journal of Food Technology,* 6**,** 1-18.

Rendleman, J. A. 2003. The reaction of starch with iodine vapor. Determination of iodide-ion content of starch–iodine complexes. *Carbohydrate Polymers,* 51**,** 191-202.

Rustad, T. 2003. Use of marine by-products. *Electronic Journal of Environmental, Agricultural and Food Chemistry* [Online], 2. [Accessed 1 October 2012].

Sadhu, S., Saha, P., Mayilraj, S. & Maiti, T. K. 2011. Lactose-enhanced cellulase production by *Microbacterium* sp. isolated from faecal matter of Zebra (*Equus zebra*). *Current Microbiology,* 62**,** 1050-1055.

Shehu, B., Abubakar, U. I. & Ismail, N. 2012. Anaerobic digestion of cow dung for biogas production *ARPN Journal of Engineering and Applied Sciences,* 7**,** 169-172.

Sivaramakrishnan, S., Gangadharan, D., Nampoothiri, K. M., Soccol, C.R. & Pandey, A. 2006. Alpha-amylases from microbial sources – an overview on recent developments. *Food Technology and Biotechnology,* 44**,** 173-184.

Smit, M. & Clark, A. G. 1971. The observation of myxobacterial fruiting bodies. *Journal of Applied Microbiology,* 34**,** 399-401.

Sponheimer, M., Grant, C. C., De Ruiter, D. J., Lee-Thorp, J. A., Codron, D. M. & Codron, J. C. 2003. Diets of impala from Kruger National Park: evidence from stable carbon isotopes. *Koedoe,* 46**,** 101-106.

Sponheimer, M., Grant, C. C., De Ruiter, D. J., Lee-Thorp, J. A., Codron, D. M. & Codron, J. C. 2005. Assessing diet in savanna herbivores using stable carbo isotope ratios of faeces. *Koedoe,* 48**,** 115-124.

Stewart, D. R. M. & Stewart, J. 1970. Food preference data by faecal analysis for African Plains ungulates. *Zoologica Africana,* 5**,** 115-129.

Stewart, D. R. M. 1971. Food preferences of an impala herd *Journal of Wildlife Management,* 35**,** 86-93.

Subhadra, B. & George, G. 2011. Algal biorefinery-based industry: an approach to adress fuel and food insecurity for a carbon-smart world. *Journal of Science, Food and Agriculture,* 91**,** 2-13.[Accessed 23 July 2012].

Sun, Y. & Cheng, J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology,* 83**,** 1-11.

Tada, O., Muchenje, V. & Dzama, K. 2013. Preferential traits for breeding Nguni cattle in low-input in-situ conservation production systems. *SpringerPlus,* 2**,** 195. [Accessed 23 May 2014].

Topakas, E., Vafiadi, C. & Christakopoulos, P. 2007. Microbial production, characterisation and applications of feruloyl esters. *Process Biochemistry,* 42**,** 497-509.

Udén, P. & Van Soest, P. J. 1982. Comparative digestion of timothy (*Phleum pratense*) fibre by ruminants, equines and rabbits. *British Journal of Nutrition,* 47**,** 267-272.

Vakhlu, J. & Kour, A. 2006. Yeast lipases: enzyme purification, biochemical properties and gene cloning. *Electronic Journal of Biotechnology,* 9**,** 69-85.

Varel, V. H., Fryda, S. J. & Robinson, I. M. 1984. Cellulolytic bacteria from pig large intestine. *Applied and Environmental Microbiology,* 47**,** 219-221.

Vasileva-Tonkova, E. & Galabova, D. 2003. Hydrolytic enzymes and surfactants of bacterial isolates from lubricant-contaminated wastewater. *Zeitschrift für Naturforschung* 58**,** 87-92.

Wallace, R. J. & Brammal, M. L. 1985. The role of different species in the hydrolysis of protein in the rumen. *Journal of General Microbiology,* 131**,** 821-832.

Wargacki, A. J., Leonard, E., Win, M. N., Regitsky, D. D., Santos, C. N. S., Kim, P. B., Cooper, S. R., Raisner, R. M., Herman, A., Sivitz, A. B., Lakshmanaswamy, A.,

Kashiyama, Y., Baker, D. & Yoshikuni, Y. 2012. An engineered microbial platform for direct biofuel production from brown macroalgae. *Science,* 335**,** 308-315.

Wilson, D. B. 2008. Three microbial strategies for plant cell wall degradation. *Annals of the New York Academy of Science,* 1125**,** 289-297.

Wrench, J. M., Meissner, H. H. & Grant, C. C. 1996. Environmental factors that affect the concentration P in N faecal samples collected for the determination of nutritional status. *Koedoe,* 39**,** 1-6.

Wrench, J. M., Meissner, H. H. & Grant, C. C. 1997. Assessing diet quality of African ungulates from faecal analyses: the effect of forage quality, intake and herbivore species. *Koedoe,* 40**,** 125-136.

Yoshida, T., Pleasants, J. R., Reddy, B. S. & Wostmann, B. S. 1968. Efficiency of digestion in germ-free and conventional rabbits. *British Journal of Nutrition,* 22**,** 723-737.

Zverlov, V. V., Schwarz, W. H. 2008. Bacterial cellulose hydrolysis in anaerobic environmental subsystems - *Clostridium thermocellum* and *Clostridium stercorarium*,thermophilic plant-fiber degraders. *Annals of the New York Academy of Science,* 1125**,** 298-307.

2. Microbial analysis of indigenous faecal matter properties as a potential source of useful bacterial hydrolases

2.1 Introduction

Hydrolytic enzymes or hydrolases are biocatalysts that break substrate bonds by a reaction with water. Naturally, enzymes are contained in cell structures and serve as biocatalysts in biochemical processes within organisms. Enzymes form an important part of industrial and environmental processes and have been applied for centuries in food processing and alcoholic beverage industries, e.g. the processing of beer, wine and cheese. Advances in biotechnological industries have led to an increase in development of competent enzymes, particularly hydrolases (Kirk *et al.*, 2002). Developments in biotechnology resulted in a shift from artificially mutated enzymes towards more naturally competent enzymes. The ideal sources of natural microbial enzymes for industrial applications would be easily available, cheaper and still functionally similar to mutated and higher taxa enzymes traditionally used in industry. This prompted the investigation of microbes as enzyme sources. From the literature, it is evident that most proteolytic microorganisms that were sourced for proteases inhabited soil, water, hot springs and bovine guts (Blackburn and Hobson, 1962; Varel *et al*., 1984; Vasileva-Tonkova and Galabova, 2003, Doi, 2008). There are limited numbers of prokaryotic microorganisms whose enzymes are commercially used in industrial processes; particularly from the genera *Bacillus*, *Pseudomonas*, and *Halomonas* (Gupta *et al*., 2002; Kirk *et al*., 2002; Sanchez-Porro *et al*., 2003). Common industrial processes are carried out under specified conditions to produce desired products, which require stable enzymes. It is therefore more feasible to find enzymes able to perform optimally under defined physical and chemical parameters than to adjust parameters (Sanchez-Porro *et al*., 2003). This is because some specific industrial processes can only occur under high temperatures or alkalinity to produce the desired products (Cherry and Fidantsef, 2003), which means isolation of industrially suitable hydrolase enzymes is required.

Microbial hydrolases are being used for bio remedial processes in oil polluted soils, plant waste sites and degradation of wastes in the fishing industry for production of less complex polymers. For example, cellulosic material is broken down to produce glucose, which is subsequently used for anaerobic production of biofuels (Bhaskar *et al*., 2008).

Environmental challenges such as fossil fuel depletion, pollution of natural habitats (e.g. rivers and soils) as well as biotechnological advances call for the isolation of competent

hydrolase-producing microbes. The purpose of this is to discover novel hydrolases that can produce better yields in industrial processes or function in the breakdown of pollutants to less harmful or environmentally beneficial by-products (Kirk *et al*., 2002).

Faecal matter, particularly of indigenous herbivores, has not yet been extensively studied as a source for the isolation of hydrolytic microorganisms. A study investigating environmental sources for cellulolytic microorganisms found more than 20 cellulolytic microbe species of which at least two were from bovine faeces (Doi, 2008). Such findings have highlighted the potential of various faecal substrates as sources of industrially valuable microbial enzymes. Presently there are only a few publications on hydrolytic isolates from zebra faeces (Sadhu *et al*., 2011; Laho *et al*., 2012). At the same time, data are lacking on the faeces of animals indigenous to southern and eastern Africa like giraffe, zebra and impala in terms of their potential as sources of industrially applicable microorganisms and microbial enzymes. Based on their diet of grasses, evergreen leaves and other shrubbery which is high in fibre (Pellew, 1984; Keesing, 1998; de Garine-Wichatitsky *et al*., 2004) it is highly likely that these herbivores possess cellulolytic microbes in their faeces.

Cellulolytic microorganism have been put under the spotlight due to a variety of reasons, one being the renewable energy crisis (Maki *et al*., 2009). Cellulases are used in biofuel generation by hydrolysing cellulosic plant waste material which is an essential part of the first hydrolysis step of the so-called anaerobic food chain. Apart from cellulases, there are three other major groups of hydrolases significant to industry: esterases, amylases and proteases which are widely used in industrial processes (Kirk *et al*., 2002; Langeroodi and Semnani, 2009; Ray, 2011). The study objectives were to: (i) analyse the faecal matter of indigenous zebra (*Equus burchelli),* giraffe (*Giraffa camelopardalis*) and impala (*Aepyceros melampus*) for microbial hydrolytic activity (ii) assess microbial diversity regarding protease-, amylase-, esterase- and cellulase-producing microorganisms (iii) determine the potential of faecal matter as a source for hydrolase-producing microorganisms.

2.2 Materials and methods

2.2.1 Sample collections

Fresh faeces from zebra, giraffe and impala were collected at the Bisley Valley Nature Reserve in Pietermaritzburg (29°39'44"S 30°23'25"E) from January 2011 to January 2012. Soil samples around faecal collection points were collected as respective controls. Soils were

accordingly coded as GSC (giraffe soil control), ISC (impala soil control) and ZSC (zebra soil control). All soil and faecal samples were collected in clean plastic bags and kept on ice during transport and then stored at 4°C. Fresh faeces from pasture-fed Nguni cows (Ukulinga Research Farm, UKZN Pietermaritzburg (29°24′E, 30°24′S)) were used for reference and comparison. They were collected and stored in a similar manner as for the other samples. Unless otherwise specified, all experimental work on samples was performed in the laboratory on the same day within 3-4 hours after collection.

2.2.2 Moisture content estimation

Moisture content measurements of samples were established through drying 1 g of samples in a drying oven at 100°C for 48 hours. Samples were then cooled to room temperature and reweighed. Based on the dry weight obtained, moisture content was estimated as percentages.

2.2.3 Chemical oxygen demand

The soluble organic fraction in fresh faeces and soil samples was quantified as soluble chemical oxygen demand (sCOD). 1 g of fresh sample was added to 99 ml of distilled water, homogenised by shaking at 150 rpm for 20 minutes and centrifuged for 5 minutes at 10000x g. 3 ml of these 100-fold dilutions were added to Merck 14541 COD kit tubes (25-1000 mg/l). Tubes were heated at 148°C for 2 hours after which they were removed, shaken and left to cool for 30 minutes. Measurements were performed using a Merck Spectroquant Nova 60 system. Distilled water was used as a control. Values were expressed as sCOD per gram dry weight.

2.2.4 Measurement of pH

The pH of samples was measured using the amended ISO 10390 (2005) method, in which 50 ml of 0.1M Calcium chloride solution was added to 10 g of air-dried (48 hours in the dark), homogenised sample material. Measurements were taken using a calibrated pH electrode (Crison, MicropH 2001, USA) after 5.5 hours incubation at 25°C.

2.2.5 Hydrolytic activity

Overall hydrolytic activity in the samples was quantified using a modified fluorescein diacetate (FDA) assay essentially following the procedure of Green *et al*. (2006) while the biomass related dehydrogenase activity was determined with the triphenyltetrazolium chloride (TTC) assay (Stevenson, 1959).

The FDA assay was carried out in duplicate Erlenmeyer flasks containing 50 ml 60 mM sodium phosphate buffer pH 7.6, to which 500 µl of 4.8 mM fluorescein diacetate in acetone and 1 g of fresh environmental sample material was added. Flasks were incubated in a water bath at 30°C for 1.5 hours either at 150 rpm or statically in the dark for 1.5 hours. The reaction was terminated with addition of 2 ml acetone. The supernatant was clarified by centrifugation at 20000 x g for 5 minutes. Absorbance of samples was measured at 490 nm using a Shimadzu 1240 spectrophotometer. Fluorescein concentrations were extrapolated from calibration curves based on concentrations of 0, 1.75, 2.5, 3.75 and 5.0 µg per ml Hydrolytic activity was reported in µg of fluorescein formed per gram dry weight per hour of incubation. Flasks containing environmental sample and buffer without FDA were prepared similarly and used to correct for background absorbencies, whilst phosphate buffer with FDA served as a control for abiotic cleavage. The activity for zebra faecal matter which had been autoclaved at 121°C for 15 minutes was measured for comparison.

For the TTC assay 6 grams of fresh sample was added to 7.5 ml deionised water and 3ml 3% TTC in water in duplicate flasks with 0.1 g glucose added as substrate and flasks without glucose. Triphenylformazan (TPF) was extracted from samples using methanol after 1 week incubation in the dark at room temperature. The TPF concentration was obtained by extrapolation from a TPF standard curve (0, 5, 10, 15, 20, 25 and 30 µg/ml) with spectrophotometric measurement at 485 nm. The activity was reported as µg of TPF formed per gram dry weight.

2.2.6 Bacterial enumeration

Viable counts of heterotrophic aerobes were determined with spread plating onto PC agar. Decimal dilutions of environmental samples (ranging from 10^{-3} to 10^{-8}) were established by initially adding 10 g of each sample material to 90 ml (10^{-1}) peptone water (8.5 g NaCl, 0.5 g peptone per litre, pH 7.2) followed by homogenisation at 150 rpm for 15 minutes and subsequent decimal dilutions up to 10^{-8}. Samples (100µl) from each decimal dilution were then spread-plated in triplicate onto plate count agar (PC agar) (5 g tryptone, 2.5 g yeast extract, 1 g glucose, 14 g agar per litre, pH 7.2); Tween 80 agar (5 g peptone, 3 g meat extract, 10 ml Tween 80, 100 mg $CaCl_2x2H_2O$, 15 g agar per litre, pH 7.2); Skim milk agar (10 g skim milk powder, 3 g meat extract, 5 g NaCl, 2 g Na_2HPO_4, 15 g agar, 0.05 g bromothymol blue per litre, pH 7.2); Carboxymethylcellulose agar (CMC agar) (2 g $NaNO_3$, 1 g K_2HPO_4, 0.6 g $MgSO_4$, 0.6 g KCl, 2 g carboxymethylcellulose sodium salt, 0.2 g

peptone,17 g agar per litre, pH 7.2) and Starch agar (3 g beef extract, 10 g soluble starch, 12 g agar per litre, pH 7.2). The colony counts were established after 24-48 hour incubation at 30°C. CMC and starch agar plates were flooded with Gram's iodine to test for cellulase and amylase positive colonies respectively which displayed a clear halo; a positive protease reaction on skim milk agar was displayed by a change of colour from green to midnight blue due to casein hydrolysis; esterase positive colonies on Tween 80 agar produced calcium oleate which precipitates around the colonies. For weighted means calculations only plates with colony numbers of 10-300 per plate were considered.

2.2.7 Isolation and analysis of unknown isolates

Ten individual bacterial colonies that displayed cellulase activity on CMC plates were selected using a sterile inoculation loop from zebra, giraffe and impala faeces spread plates and all together were then inoculated into one Erlenmeyer flask containing 30 ml of sterile nutrient broth (Merck). This mixed culture was incubated at 25°C at 150 rpm for 24 hours. Cells were harvested by centrifugation of 1 ml culture at 20000 x g for 10 minutes, washed twice with 0.9% saline solution and resuspended in 1 ml 0.9% saline solution. The cell concentration was determined using a Helber (Thoma Ruling, Marienfeld, Germany) bacterial counting chamber at 400x magnification with a Motic (BA6310) phase contrast microscope. 1 ml of cell suspension (10^8 per ml) in 0.9% saline was tested for hydrolytic activity using the FDA assay.

2.3 Results

2.3.1 Qualitative analysis of faeces

Samples were initially tested for pH and moisture content (Table 3) with soil samples used as controls to compare with findings from the faeces. The pH range of faecal samples was found to be around neutral to alkaline with impala and zebra having a similar alkaline pH while giraffe faeces were close to neutral pH. In contrast, all soil samples were slightly acidic with zebra soil having the lowest pH (5.81) of all representatives. The sCOD of the samples varied, with all the faecal samples having a much higher sCOD (between 57 and 80 mg/g) than the matching soil controls (between 7 and 9 mg/g). This is indicative of the difference in soluble organic matter content between the collected soil and faeces. The cow faecal material had a significantly higher sCOD of 339 mg/g than the wild ungulate faeces and the highest moisture content (54%) whilst the pH was near neutral and similar to that of giraffe. With 49% zebra faeces had the highest moisture content of the wild ungulates, while giraffe and

impala were almost identical with 27% and 30% respectively. Generally, the soil samples had the lowest moisture content with a more acidic pH and a much lower sCOD in comparison to the faecal samples, highlighting the differences between the composition of the faeces and the soil samples.

Table 3: Initial analysis of freshly collected zebra, giraffe and impala faeces and respective soil controls over different sampling occasions (2011-2012)

Source	pH	Percentage moisture	sCOD (mg/g)
Zebra	8.18	49%	80
Zebra soil control	5.81	4%	7
Impala	8.32	30%	57
Impala soil control	6.34	7%	9
Giraffe	7.34	27%	73
Giraffe soil control	5.95	4%	8
Cow	**7.39**	**54%**	**339**

*Bold: reference data measured from Nguni cow faeces. All data shown are the means of triplicate measurements on different occasions

2.3.2 Analysis of metabolic activity through FDA and TTC assay

Hydrolytic activity was measured using the fluorescein diacetate (FDA) assay and dehydrogenase activity through the triphenyltetrazoliumchloride (TTC) assay. In the FDA assay, flasks subjected to shaking during incubation showed higher hydrolytic activities than flasks kept under static conditions (Table 4) which is in line with previous reports in the literature. Overall, faecal samples exhibited higher hydrolytic activity than soil samples which is not unexpected on microbiological grounds. Under shaking conditions, zebra faeces showed the highest fluorescein formation rate at 1228 µg x g^{-1} x h^{-1}, followed by giraffe (1095 µg x g^{-1} x h^{-1}) and impala (905 µg x g^{-1} x h^{-1}) while the impala soil control had the highest activity (121 µg x g^{-1} x h^{-1}) of all control soil samples. The large differences between the hydrolytic activities of faeces and their respective soil controls suggest a different microbial burden and diversity. The Nguni cow faeces yielded a lower hydrolytic activity in comparison to other ungulates but were higher than soil control samples, again indicating the uniqueness of microbial communities in each sample.

Samples were also analysed for dehydrogenase activity using the TTC assay. Results (Table 4) indicate that faecal samples had a much higher metabolic activity in comparison to the soil control samples; which is similar to the trend observed in the FDA assay. Addition of glucose to samples resulted in greater TPF yield i.e. dehydrogenase activity than in samples incubated

without glucose. The highest activity was again observed in zebra faeces. A comparison between the different soil controls indicated that the giraffe soil control had higher dehydrogenase activity than the other soils. The dehydrogenase activity in Nguni cow faeces was higher than that in soils but was in a lower range than the activity found in the faeces of the wild ungulates.

Table 4: Hydrolytic and dehydrogenase activities of fresh faecal and soil matter

Sample flask	Fluorescein released per hour (µg x g^{-1})	Sample flask	TPF formation (µg x g^{-1})
Zebra shaker	1228	Zebra	1475
Zebra static	467	Zebra + glucose	2947
Zebra soil control shaker	120	Zebra soil control	14
Zebra soil control static	99	Zebra soil control + glucose	78
Giraffe shaker	1095	Giraffe	1072
Giraffe static	181	Giraffe + glucose	2081
Giraffe soil control shaker	69	Giraffe soil control	73
Giraffe soil control static	1	Giraffe soil control + glucose	270
Impala shaker	905	Impala	1255
Impala static	530	Impala + glucose	3207
Impala soil control shaker	121	Impala soil control	10
Impala soil control static	26	Impala soil control + glucose	240
Cow shaker	**525**	**Cow**	**715**
Cow static	**7**	**Cow + glucose**	**949**

*Bold: reference data measured from Nguni cow faeces; Fluorescein activity values refer to µg of fluorescein released per hour per gram dry weight of faecal matter or soil; TPF formation values refer to µg of TPF formed per gram dry weight of faecal matter or soil All data shown are the means of triplicate measurements on different occasions

To verify that microorganisms present in faeces could hydrolyse FDA, a mixed culture (final assay concentration of 10^8 cells/ml) created from 10 randomly selected colonies from zebra, giraffe and impala faeces was examined for activity via the FDA assay. This mixed culture yielded 92 µg fluorescein per 10^8cells per hour under shaker conditions and only 2.1 µg per 10^8 cells per hour under static conditions respectively. In comparison, autoclaved zebra faeces released fluorescein at concentrations that were beneath the limit of detection, which was <1 µg fluorescein per gram per hour.

2.3.3 Quantitative analysis of aerobic microbial diversity

Once hydrolytic activity was determined, total and specific (hydrolase-producing) viable counts were obtained. The aerobic colony plate counts (Table 5) were recorded in $\log_{10}$ cfu per gram dry weight of faeces or soil. The PC agar used, as a general purpose medium, provides an estimate of the total microbial burden whilst the selective media used (Tween 80,

starch, CMC and casein) provide estimates of microorganisms producing the targeted hydrolytic enzymes. Faecal and soil samples contained all of the targeted hydrolase-producers, in varying proportions. The total viable plate counts (PC agar) of all faecal samples were found to be in a similar range of log 8.13- 8.53 (Table 5), with giraffe having the highest microbial load and Nguni the lowest, while the PC count of all soil samples were about 2 log lower. Regarding the different hydrolase-producing bacterial counts, again the faecal samples showed the highest levels and the soil samples the lowest. However, in most cases the results for hydrolase producers from Nguni faeces were lower than those from the wild ungulate faeces.

Table 5: Log_{10} aerobic plate counts (cfu/g) of fresh samples on Plate count and differential screening agar

Sample	PC agar	Starch agar	Casein agar	Tween 80 agar	CMC agar
Giraffe	8.53	8.51	8.50	7.78	8.40
Giraffe soil control	6.50	6.49	6.30	6.20	6.16
Impala	8.29	8.25	8.25	6.53	8.01
Impala soil control	6.27	6.10	6.08	<4	6.16
Zebra	8.30	8.13	7.55	8.16	7.63
Zebra soil control	6.68	5.92	5.09	<4	6.29
Cow	**8.13**	**7.79**	**6.76**	**7.59**	**7.47**

*****CMC: carboxy-methyl cellulose agar; PC: plate count agar; cfu: colony forming units; bold: Nguni cow reference data <5: lower than detection limit of 10000 cfu/g dry weight

Relative abundances of specific hydrolase-producing microbes were obtained by assuming the colony forming units on PC agar as the total number (100%) of culturable microorganisms (Fig 3). Only the giraffe soil control contained all the targeted enzyme producers while for the other two soils the counts for esterase-producing microorganisms were below the detection limit. Faecal samples contained all the targeted hydrolase-producers, with varying proportions of each hydrolase producer present in faeces of each individual animal. Giraffe and impala had similar proportions of hydrolase-producers overall; with both of them having higher proportions of amylase and protease-producers, with esterase-producers being the smallest percentage. Faeces of zebra and Nguni cow showed similar proportion patterns with esterase-producers as the highest and protease producers as the lowest. Comparing Figure 3 to the FDA and TTC assay findings (Table 4), it appeared that metabolic activity was linked to overall microbial burden and diversity and could not be

attributed to one set of specific hydrolase-producers that contribute to more activity when present in higher proportions.

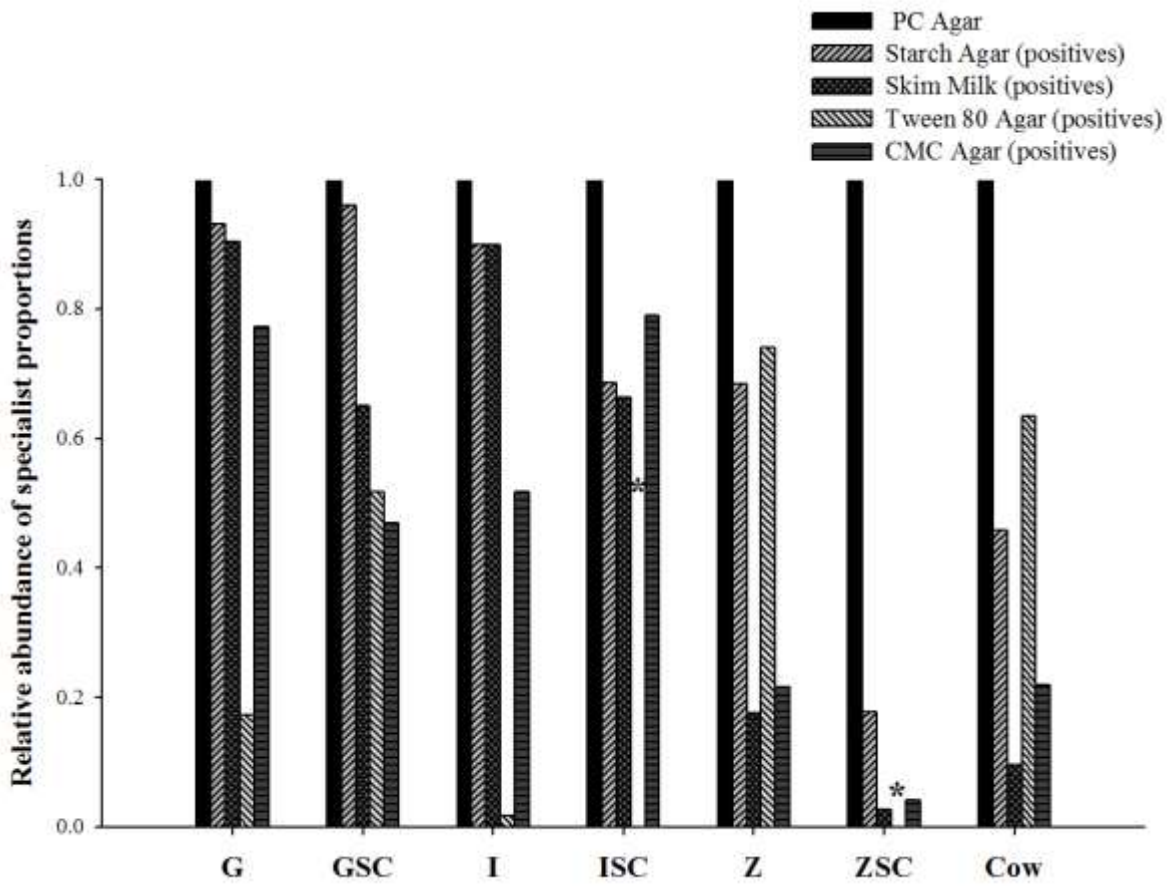


Figure 3: Relative abundance of polymer-hydrolysing microorganisms in fresh faecal and control soil samples

Key: G: giraffe; GSC: giraffe soil control; I: impala; ISC: impala soil control; Z: zebra; ZSC: zebra soil control, Cow: Nguni cow * indicates numbers below detection limit

2.4 Discussion

The faecal samples in this study were compared with soil collected from the same area to verify whether the surrounding soil differs from faecal matter regarding the microbial burden and hydrolytic abilities. This served to validate that microbial characteristics of faecal samples and soil differ from each other. In addition, Nguni cow dung provided a comparison of the wild ungulates to a domestic ungulate fed on a fibrous diet as well.

2.4.1 Qualitative analysis of faeces

Faecal samples were analysed in terms of sCOD, moisture content, pH, hydrolytic activity and microbial burden, to determine their potential as a source for microbial hydrolases which might be of use for industry. The neutral to slightly alkaline pH of the giraffe and Nguni cow faecal samples upon collection (Table 3) was within the expected pH range of ruminants which is typically between 6-7 (Moran, 2005; Maranon *et al*., 2006). Zebra and impala faeces differed somewhat with a slightly more alkaline pH of above 8. Interestingly, the higher pH could be an indicator that alkalotolerant microorganisms are present within the faeces. Alkalotolerance has also been associated with thermotolerance, which in view of industrial enzyme applications is advantageous because it enables enzymes to remain stable under extreme conditions (Maitin *et al*., 2001) and this may be the case for microorganisms present in alkaline zebra or impala faeces.

Soluble chemical oxygen demand (sCOD) values indicate the presence of organic matter in a sample. A high sCOD value is therefore indicative of potential substrate availability, possibly explaining the abundance of heterotrophic microorganisms in such samples. The soil samples had a more acidic pH and much lower soluble COD content than the faecal samples. The properties of the soil samples (low sCOD, moisture content and below neutral pH) match those reported for infertile soils (Hartemink, 2006a; Hartemink 2006b). This is because lower percentage moisture is related to a lower sCOD as dry acidic soils have a low water binding capacity. The highest soluble sCOD of 339 mg/g was recorded in Nguni cow faeces, while the sCOD values of the other faeces ranged between 57 and 80 mg/g. These values are within the sCOD range of 30-6000 mg/l reported in other studies (Maranon *et al*., 2006; Shehu *et al*., 2012). Additionally, the measured pH of 7.39 is within the range of 7.1-7.4 reported for pasture-fed cows (Artan, 1996; Maranon *et al*., 2006).

No clear correlation was found between moisture content and microbial burden in the faecal and soil samples analysed. However, a previous study showed that faecal microbial diversities differ at high and low moisture contents (Wardle and Parkinson, 1990). At the same time, a high moisture content in faecal samples indicates that the nature of the diet is mostly fibrous material, which is typically taken up when feeding on grasses and leaves, which is water binding in nature (Ziemer *et al*., 2012). As a result, for all the animals analysed in this study, faecal matter had higher moisture percentage values than soils samples. Thus, based on the moisture content, pH and sCOD of the samples, clear differences between faecal material and the surrounding soil were evident.

2.4.2 Analysis of metabolic activity

Microbial activity of samples was measured through colorimetric assays that yield coloured reaction products once hydrolysis (FDA) or reduction (TTC) had occurred. The fluorescein diacetate assay operates on the principle of hydrolytic release of two acetate groups from ester bond cleavage in the colourless esterase substrate FDA to yield yellow-green coloured fluorescein. Cleavage is caused by free and membrane-bound hydrolytic enzymes and the fluorescein produced can be measured at 490 nm (Adam and Duncan, 2001). FDA is a versatile substrate and has been used for the detection of esterases within water bodies and individual cells (Battin, 1997) and in soils (Green *et al*., 2006). However, other hydrolytic enzymes such as amylases can cleave its ester bonds as well (Lundgren, 1981, Green *et al*., 2006). The FDA hydrolysis assay has also been utilised and recommended as an efficient method to estimate active cells within environmental samples (Swisher and Carroll, 1980). The protocol employed in this study has been optimised from Schnurer and Roswall (1982). This takes into consideration the best conditions of most critical parameters such as incubation time and temperature, buffer pH, sample size and reaction-terminating solvents to optimise the accuracy of the fluorescein formation measurement (Green *et al.* 2006). Acetone was used to terminate the reaction in this study. As the use of acetone as a reaction terminator was found to decrease the absorbance of fluorescein produced in samples by up to 37%, (Adam and Duncan, 2001; Green *et al*., 2006), acetone volumes used in this study proto terminate the reactions were reduced to 4% (v/v) to suffice in slowing down the reaction (Green *et al.,* 2006) and readings were taken within 30 minutes to an hour of termination to get stable results.

The FDA assay has been reported to be problematic in case of abiotic cleavage by certain microbiological media components (Clarke *et al*., 2001; Wanandy *et al*., 2005) but this was accounted for through appropriate assay controls in the assay conducted for this study. The fresh samples were used directly and absorbance was corrected for any abiotic cleavage.

The assay yielded two results (Table 4): firstly, that the faecal samples from indigenous herbivores were higher in hydrolytic activity than the soil controls and cow faeces, and secondly, that samples incubated under shaker conditions yielded a higher activity than those under static conditions. This is contrary to the findings of Green *et al* (2006) who reported that shaking was found to decrease the amount of fluorescein release in soil samples. However, the results (Table 4) confirm recommendations made by earlier publications (Swisher and Carroll, 1980; Schnurer and Rosswall, 1982), according to which samples should be shaken during incubation to increase hydrolytic activity which can be attributed to the mixing and homogenisation effects. In addition, aerobic microbial activity has been proven to be optimal under shaker conditions safeguarding the presence of oxygen (Bozic *et al*., 2011). Additionally, shaking enables the distribution of substrate within the medium, which results in higher microbial numbers compared to static conditions (Juergensmeyer *et al.*, 2007) given that fast growing organisms are present. Hydrolytic activity measurements of a mixed culture consisting of 10 isolates from faeces confirmed that activity measured in samples was due to microbial hydrolysis, since selected faecal isolates demonstrated hydrolytic activity independently of the faeces. The obtained activity was much lower than that of the actual faeces but this can be expected as there is a much lower microbial diversity as well as the fact that the isolates may have been poor esterase producers. The fact that a control with autoclaved faeces yielded no measurable activity for FDA hydrolysis confirms that it was the presence of hydrolytically active microorganisms in faeces that hydrolysed FDA to produce fluorescein. Furthermore, the presence of higher hydrolytic activity in the faecal samples indicated that there was a higher number of hydrolase producing microorganisms in faeces than in soil as was confirmed by the plate counts in table 5. An increased degree of FDA hydrolysis relies on the presence of metabolically active microorganisms (Chrzanowski *et al*., 1984) thus making it a useful measure of viable cells (Schnurer and Rosswall, 1982; Clarke *et al*., 2001). The FDA assay method is even used for microbial number determinations in samples as it is more sensitive and cost-effective than other cell quantification assays (Adam and Duncan, 2001).

The TTC assay, which targets a different metabolic activity, was also utilised (Table 4). The TTC assay operates on the principle of reduction of colourless 2, 3, 5-triphenyltetrazolium chloride by active dehydrogenases to produce the corresponding triphenyl formazan (TPF). The water insoluble TPF can be extracted from samples using solvents such as methanol, butanol and ethanol, and is optimally measured at 485nm (Stevenson, 1959). Although the assay has been found to correlate with cell viability in samples (Tergendy *et al*., 1967), it has been reliably used mostly for qualitative analysis of soils and other samples rather than for quantitative studies. It has also been used for determining cell viability within a sample (Mikula *et al*., 2006), as dehydrogenase activity is related to membrane bound microbial respiration and electron transfer. Like the FDA assay, the TTC assay is based on the principle of measuring cell activity through enzymatic activity.

Disadvantages of the TTC assay are a lower sensitivity than for the FDA assay, thus requiring longer sample incubation periods of at least 24 hours (Ishikawa *et al*., 1995). The assay is therefore more suitable for larger samples or aggregates (Mikula *et al*., 2006). In addition, the TTC assay works better under anaerobic conditions as it is inhibited by O_2 as a competing electron acceptor and thus dehydrogenase activity is potentially underestimated (Von Mersi and Schinner, 1991). Therefore, samples analysed by the TTC assay were incubated statically in this study. In addition, build-up of TTC in samples has been found to be toxic to fungal cells when exceeding 30g/l in young cells (4 days old) and 20g/l in older cells (12 days old) (Ghaly and Mahmoud, 2007). As a result, alternative substrates such INT (2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyl-tetrazolium chloride or MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide) have been suggested as being more sensitive and less toxic under both aerobic and anaerobic conditions (Von Mersi and Schinner, 1991; Mosher *et al*., 2003). TTC is nevertheless an accepted and frequently used qualitative measure of dehydrogenase activity in samples and therefore a useful addition to the FDA assay by targeting a different enzyme activity.

The TTC results are in overall agreement with the FDA findings (Table 4). The range in metabolic activity measured without glucose is similar to that of FDA. Addition of glucose confirmed the presence of glucose utilising microorganisms by leading to higher TPF formation due to increased microbial biomass, verifying that the dehydrogenase activity was produced by glucose utilising microorganisms. This is expected, since cellulose is broken down by gut microorganisms in the ungulates to simpler forms, i.e. glucose. Zebra faeces

showed the highest activity in the FDA and TTC assays in comparison to the giraffe and impala faeces. The cow faeces and soil control samples had a lower activity in both assays. The data obtained in this study appear to be slightly lower than reported measurements of 160 µg x g^{-1} fluorescein released in 3 hours from soil (Green *et al*., 2006), with the average soil hydrolytic activity in this study being 126 µg x g^{-1} fluorescein released in 3 hours under static conditions. Comparing these to the faecal hydrolytic activity, the hydrolytic activity measured from the ungulates is considerably higher than that of the soils reported in literature.

These findings indicate that zebra faecal matter has the highest hydrolytic activity, clearly exceeding that observed for soil and other faecal samples analysed in this study. Alkalinity and high dehydrogenase and hydrolytic activity of zebra faeces might indicate that microorganisms contained within zebra faeces are able to function at higher than neutral pH, which is valuable in industrial applications (Horikoshi, 1999).

Comparing the pH (Table 3) and TPF formation measurements (Table 4), seems to indicate that there is a trend of increased TPF formation in samples that have a neutral to slightly alkaline pH, which confirms previous studies showing that the activity of electron transport system (ETS) is enhanced within a pH range of 7.4 to 8 (Trevors, 1984), which includes dehydrogenase activity. As a result, the TPF formation is generally higher in samples with a pH value higher than 7.

2.4.3 Quantitative analysis of aerobic microbial diversity

Total viable counts in faecal samples were mostly in the range of 10^8 cells per gram dry weight and each faecal sample contained all the targeted hydrolase-producers (Table 5). The viable counts and number of hydrolase-producers per gram dry weight in the soil samples were always at least 1 to 2 log lower than the counts in the corresponding faecal samples and showed clear differences in the proportions of specific hydrolase-producers (Fig 3). Gong (2007) reported microbial plate counts for cattle dung of about 10^{11} cfu/g (dry weight) with proteolytic bacteria present at around 10^7 to 10^8 cfu/g for both lipolytic and amylolytic and 10^9 cfu/g for cellulolytic microorganisms. Microbial numbers in the Nguni cow faeces in this study were lower than these data on abundances. However, there were similarities in diversity, with the exception of cellulolytic microorganisms which were of higher abundance in the previous study (Gong, 2007) compared to the findings of this study. The difference in

numbers may be due to difference in incubation times as plates were incubated for 24-48 hours in this study, whilst plates were incubated for 1-5 days in the study conducted by Gong (2007). Furthermore, the differences in diet and the adaptation of the Nguni cow to poorer diet compared to other cattle breeds (Tada *et al.*, 2013) may have contributed to the differences between the two. Although giraffe faeces showed the highest viable counts for all microorganisms tested in this study, metabolic activity (dehydrogenase and hydrolase) was not the highest which might be due to the differences between the ruminant digestive system and the hindgut system of the zebra and the resulting differences between the microbial communities present therein.

Faecal samples from impala and giraffe had similar proportions of hydrolase-producers (Fig 1). Both impala and giraffe had proportions of 92% and 96% respectively for amylase producers, protease producer proportions of 91% and 93% respectively and almost the same percentage of esterase positive producers (17 and 18%).

Zebra had the highest proportion of esterase-producers (74%) and the lowest number of protease and cellulase producers (18 and 21%) amongst the three ungulates. In comparison, Nguni faeces had a similar distribution, with more esterase producers (63%) and low proportions of cellulose degraders (22%), which is similar to zebra.

When comparing Tables 4 and 5, it appears that high hydrolytic activity relates with high viable counts. Nguni faeces had a high total viable count but displayed lower hydrolytic and dehydrogenase activity compared to other ungulates, which could be due to lower proportions of hydrolase-producers and different digestive systems in the animals. Findings on the cellulolytic microbial abundance of the Nguni cow in particular are consistent with previous reports in the literature where cellulolytic microorganisms were found to be highly abundant within the rumen but when in competition with other specialists, they were found to be present in the lowest proportions (Witten and Richardson, 2003). The Nguni cow had lower viable counts than those reported by Gong (2007), however the percentage distribution of diversity in hydrolase producers was similar.

Furthermore, the apparent differences in microbial colony counts and diversity within faeces from different herbivores (Table 5) can be attributed to their different feeding habits. Zebras and cattle are grazers (Odadi *et al*., 2011) whilst giraffe is a browser and impala is a mixed feeder (grazer and browser) (Sponheimer *et al*., 2005).

There is an interesting similarity in the proportions of the specific hydrolytic microorganisms found in the Nguni cow and zebra faeces (Fig 3), with more relative esterase positives than other hydrolase-producers, whilst giraffe and impala have higher relative proportions of other hydrolase-producers and lower esterase positives. The grazing method requires feeding on grasses more than any other forage and has been found to be lengthier than browsing (Udén and Van Soest, 1982). The grasses have been found to be less nutritional than browses and the time taken for grazing is longer than that taken by browsers. Zebra faeces, however, displayed a higher hydrolytic activity than Nguni cow faeces (Table 4) and this may be due to the difference in their digestive systems. Cows are ruminants and grazers. This puts 3 factors into play; their size, digestion time and preferred grasses. As ruminants, their digestion time is longer than that of non-ruminants and due to their large size, grazing time is extended further. Moreover, the ruminant digestive system implies that a lot of biogenic polymers, e.g. cellulose, are more efficiently digested in the foregut. Adequate digestion in the foregut is facilitated by anaerobic microorganisms. Movement of cud to the hindgut is usually for secondary digestion of other polymers. As a result, lower aerobic microbial numbers and hydrolase-producers can be expected in cow faeces, but more cellulose degraders such as *Clostridia* spp. as they are anaerobic (Mackie, 2002).

Grass diets of cows and zebras are similar, though zebra possess an advantage to feed on greater forage variety and for shorter periods as a hindgut fermenter. (Odadi *et al*., 2011). Although findings in this study indicate that relative proportions of hydrolase-producers are similar due to similar feeding habits, the hydrolytic activities and microbial loads were not. This could be due to the difference in digestive tracts and time of digestion. Hydrolytic microorganisms from cattle have been found to form biofilms on forage substrates within the rumen and require varied co-cultures to efficiently hydrolyse polymeric substrates (McSweeney *et al*., 1999). When comparing the data for cattle and zebra, it appears that there are higher numbers of viable microorganisms in equine faeces than in bovine faeces possibly due to shorter retention time (Odadi *et al*., 2011). This results in insufficient polymer hydrolysis, since digestion occurs within the hindgut, which means forage is usually defecated without being properly hydrolysed. As a result, more microorganisms are found in zebra faeces (Mackie, 2002) than in cattle faeces after defecation, which was indicated by the results in this study. The zebras in this study were out in the wild and therefore probably had a greater variety in their diet as opposed to the Nguni cow that was kept at the farm.

Impala and giraffe appear to be similar regarding the relative hydrolase producer proportions. As ruminants, they have a more complex digestive system than zebra and a more diverse diet as browsers. It appears that the impala in this study might have fed on a diet similar to the giraffe, which would compose of more browse forage than grasses, resulting in similar hydrolase producer proportions. Their smaller body size means that digestion time is shorter compared to cows and faecal microbial numbers may be greater due to that (Gordon, 2003).

The presence of more esterase producers in zebra and Nguni cow faeces might be due to the varying lipid content (Hadley and Rosen, 1974), which varies in different plant species. The presence of proteases was expected as this enzyme class is present in the majority of heterotrophic microorganisms. The cellulose and amylase positive microbes could also be expected based on the high amounts of cellulose and starch in leaves and grasses (Ben-Shahar and Coe, 1992). Apart from the hydrolase enzymes targeted in this study, other enzymes which are of interest for industry (Kirk *et al*., 2002) such as xylanases and hemicellulases are typically detected in faecal samples of ungulates (Fon and Nsahlai, 2012).

The interactions between microbes within the intestinal systems of these herbivores are similar to anaerobic bioreactors digesting cellulosic material, with *Ruminococcus* spp. being documented as an active anaerobic cellulose hydrolyser found in artificial and natural systems (Morrison *et al*., 2009).

This study used a culture-based approach to determine numbers of specific hydrolase producers present in different samples. An interesting alternative approach is to employ metagenomics to determine the presence of genes encoding for hydrolytic enzymes (Morrison *et al*., 2009).

The findings in this chapter demonstrate that faecal matter might be a useful source for the isolation of microbial hydrolases based on the observed metabolic activity in the samples as well as the presence of polymer hydrolysing microorganisms. Zebra, giraffe and impala faeces appeared more active in this regard than cattle and soil samples, with higher numbers of microbial hydrolysers and higher hydrolytic activity.

2.5 References

Adam, G. & Duncan, H. 2001. Development of a rapid and sensitive method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils. *Soil Biology and Biochemistry,* 33**,** 943-951.

Artan, R., Cook, D.F., Dadour, I.R., Ali, D.N. 1996. Effect of diet on the excretion profile of ivermectin in cattle faeces. *International Journal for Parasitology,* 3**,** 291-295.

Battin, T. J. 1997. Assessment of fluorescein diacetate hydrolysis as a measure of total esterase activity in natural stream sediment biofilms. *Science of The Total Environment,* 198**,** 51-60.

Ben-Shahar, R. & Coe, M. J. 1992. The relationships between soil factors, grass nutrients and the foraging behaviour of wildebeest and zebra. *Oecologia,* 90**,** 422-428.

Bhaskar, N., Benila, T., Radha, C. & Lalitha, R. G. 2008. Optimization of enzymatic hydrolysis of visceral waste proteins of Catla (*Catla catla*) for preparing protein hydrolysate using a commercial protease. *Bioresource Technology,* 99**,** 335-343.

Blackburn, T. H. & Hobson, P. N. 1962. Further studies on the isolation of proteolytic bacteria from the sheep rumen. *Journal of General Microbiology,* 29**,** 69-81.

Bozic, N., Ruiz, J., Lopez-Santin, J. & Vujcic, Z. 2011. Optimization of the growth and α-amylase production of *Bacillus subtilis* IP 5832 in shake flask and laboratory fermenter batch cultures. *Journal of the Serbian Chemical Society,* 76**,** 965-972.

Cherry, J. R. & Fidantsef, A. L. 2003. Directed evolution of industrial enzymes: an update. *Current Opinion in Biotechnology,* 14**,** 438-443.

Chrzanowski, T. H., Crotty, R. D., Hubbard, J. G. & Welch, R. P. 1984. Applicability of the fluorescein diacetate method for detecting active bacteria in freshwater. *Microbial Ecology,* 10**,** 179-185.

Clarke, J. M., Gillings, M. R., Altavilla, N. & Beattie, A. J. 2001. Potential problems with fluorescein diacetate assays of cell viability when testing natural products for antimicrobial activity. *Journal of Microbiological Methods,* 46**,** 261-267.

De Garine-Wichatitsky, M., Fritz, H., Gordon, I. J. & Illius, A. W. 2004. Bush selection along foraging pathways by sympatric impala and greater kudu. *Oecologia,* 141**,** 66-75.

Doi, R. H. 2008. Cellulases of mesophilic microorganisms: cellulosome and noncellulosome producers. *Annals of the New York Academy of Science,* 1125**,** 267-279.

Fon, F. N. & Nsahlai, I. V. 2012. The *in vitro* fermentation of maize stover as affected by faecal bacteria obtained from ungulates. *South African Journal of Animal Science* 42**,** 478-482.

Ghaly, A. E. & Mahmoud, N. S. 2007. Effects of tetrazolium chloride concentration, O_2, and cell age on dehydrogenase activity of *Aspergillus niger*. *Applied Biochemistry and Biotechnology,* 136**,** 207-222.

Gong, C. 2007. Microbial safety control of compost material with cow dung by heat treatment. *Journal of Environmental Sciences,* 19**,** 1014-1019.

Gordon, I. J. 2003. Browsing and grazing ruminants: are they different beasts? *Forest Ecology and Management,* 181**,** 13-21.

Green, V. S., Stott, D. E. & Diack, M. 2006. Assay for fluorescein diactetate hydrolytic activity: Optimization for soil samples. *Soil Biology and Biochemistry,* 38**,** 693-701.

Gupta, R., Beg, Q. K. & Lorenz, P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology,* 59**,** 15-32.

Hadley, E. B. & Rosen, R. B. 1974. Carbohydrate and lipid contents of Celmisia plants in alpine snowbank and herbfield communities on Rock and Pillar Range, New Zealand. *American Midland Naturalist,* 91**,** 371-382.

Hartemink, A.E. 2006a. Soil fertility decline: definitions and assessment. *In* Lal, R. (ed) *Encyclopaedia of Soil Science,* 1618-1621. Netherlands.

Hartemink, A.E. 2006b. Assessing soil fertility decline inthe tropics using soil chemical data. *Advances in Agronomy,* 89**,** 179-225.

Horikoshi, K. 1999. Alkaliphiles: some applications of their products for biotechnology. *Microbiology and Molecular Biology Reviews,* 63**,** 735-750.

International Standard Organization, 2005. ISO 10390 Soil quality - determination of pH. 2nd ed. Switzerland: *International Organization for Standardization.*

Ishikawa, M., Robertson, A. J. & Gusta, L. V. 1995. Comparison of viability tests for assessing cross-adaptation to freezing, heat and salt stresses induced by abscisic acid in bromegrass (*Bromus inermis* Leyss) suspension cultured cells. *Plant Science,* 107**,** 83-93.

Juergensmeyer, M. A., Nelson, E. S. & Juergensmeyer, E. A. 2007. Shaking alone, without concurrent aeration, affects the growth characteristics of *Escherichia coli*. *Letters in Applied Microbiology,* 45**,** 179-183.

Keesing, F. 1998. Impact of ungulates on the demography and diversity of small mammals in central Kenya. *Oecologia,* 116**,** 381-389.

Kirk, O., Borchert, T. V. & Fuglsang, C. C. 2002. Industrial enzyme applications. *Current Opinion in Biotechnology,* 13**,** 345-351.

Laho, T., Váradyová, Zora, Mihaliková, K. & Kišidayová, S. 2012. Fermentation capacity of faecal microbial inocula of Przewalski horse, Kulan, and Chapman zebra and polysaccharide hydrolytic activities of faecal microbial constituents (ciliates and bacteria) of Kulan and Chapman zebra. *Journal of Equine Veterinary Science,* 33**,** 143-149.

Langeroodi, H. S. & Semnani, A. 2009. Behaviour of esters in blend and its possible applications. *African Journal of Pure and Applied Chemistry,* 3**,** 241-246.

Lundgren, B. 1981. Fluorescein diacetate as a stain of metabolically active bacteria in soil. *Oikos,* 36**,** 17-22.

Mackie, R. I. 2002. Mutualistic fermentative digestion in the gastrointestinal tract: diversity and evolution. *Integrative and Comparative Biology,* 42**,** 319-326.

Maitin, V., Kavitha, R. & Umesh-Kumar, S. 2001. Properties of an extracellular amylase purified from a *Bacillus* species. *World Journal of Microbiology and Biotechnology,* 17**,** 823-826.

Maki, M., Leung, K. T. & Qin, W. 2009. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *International Journal of Biological Sciences,* 5**,** 500-516.

Maranon, E., Castrillon, L., Fernandez, J. J. & Fernandez, Y. 2006. Anaerobic mesophilic treatment of cattle manure in an upflow anaerobic sludge blanket reactor with prior pasteurization. *Journal of the Air & Waste Management Association,* 56**,** 137-143.

Mcsweeney, C. S., Dalrymple, B. P., Gobius, K. S., Kennedy, P. M., Krause, D. O., Mackie, R. I. & Xue, G. P. 1999. The application of rumen biotechnology to improve the nutritive value of fibrous feedstuffs: pre- and post-ingestion. *Livestock Production Science,* 59**,** 265-283.

Mikula, A., Niedzielski, M. & Rybczynski, J. J. 2006. The use of TTC reduction assay for assessment of *Gentiana* spp. cell suspension viability after cryopreservation. *Acta Physiologiae Plantarum,* 28**,** 315-324.

Moran, J. 2005. *Tropical dairy farming: feeding management for small holder dairy farmers in the humid tropics,* Melbourne, CSIRO Publishing.

Morrison, M., Pope, P. B., Denman, S. E. & Mcsweeney, C. S. 2009. Plant biomass degradation by gut microbiomes: more of the same or something new? *Current Opinion in Biotechnology,* 20**,** 358-363.

Mosher, J. J., Levison, B. S. & Johnston, C. G. 2003. A simplified dehydrogenase enzyme assay in contaminated sediment using 2-p-iodophenyl-3 p-nitrophenyl-5-phenyl tetrazolium chloride. *Journal of Microbiological Methods,* 53**,** 411-415.

Odadi, O. W., Jain, M., Van Wieren, S. E., Prins, H. H. T. & Rubenstein, D. I. 2011. Facilitation between bovids and equids on an African savanna. *Evolutionary Ecology Research,* 13**,** 237-252.

Pellew, R. A. 1984. Food consumption and energy budgets of the giraffe. *Journal of Applied Ecology,* 21**,** 141-159.

Ray, R. R. 2011. Microbial isoamylases: an overview. *American Journal of Food Technology,* 6**,** 1-18.

Sadhu, S., Saha, P., Mayilraj, S. & Maiti, T. K. 2011. Lactose-enhanced cellulase production by *Microbacterium* sp. isolated from faecal matter of Zebra (*Equus zebra*). *Current Microbiology,* 62**,** 1050-1055.

Sanchez-Porro, C., Martin, S., Mellado, E. & Ventosa, A. 2003. Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. *Journal of Applied Microbiology,* 94**,** 25-300.

Schnurer, J. & Rosswall, T. 1982. Fluorescein diacetate as a measure of total microbial activity in soil and litter. *Applied and Environmental Microbiology,* 43**,** 1256-1261.

Shehu, B., Abubakar, U. I. & Ismail, N. 2012. Anaerobic digestion of cow dung for biogas production *ARPN Journal of Engineering and Applied Sciences,* 7**,** 169-172.

Sponheimer, M., Grant, C. C., De Ruiter, D. J., Lee-Thorp, J. A., Codron, D. M. & J. C. 2005. Assessing diet in savanna herbivores using stable carbo isotope ratios of faeces. *Koedoe,* 48**,** 115-124.

Stevenson, I. L. 1959. Dehydrogenase activity in soils. *Canadian Journal of Microbiology,* 5**,** 229-235.

Swisher, R. & Carroll, G. C. 1980. Fluorescein diacetate hydrolysis as an estimator of microbial biomass on coniferous needle surfaces. *Microbial Ecology,* 6**,** 217-226.

Tada, O., Muchenje, V. & Dzama, K. 2013. Preferential traits for breeding Nguni cattle in low-input in-situ conservation production systems. *SpringerPlus,* 2**,** 195. Accessed [23 May 2014].

Tergendy, R. P., Nagy, J. G. & Martin, B. 1967. Quantitative measurement of bacterial growth by reduction of tetrazolium salts. *Applied Microbiology,* 15**,** 954-955.

Trevors, J. 1984. Effect of substrate concentration, inorganic nitrogen, O2 concentration, temperature and pH on dehydrogenase activity in soil. *Plant and Soil,* 77**,** 285-293.

Udén, P. & Van Soest, P. J. 1982. Comparative digestion of timothy (*Phleum pratense*) fibre by ruminants, equines and rabbits. *British Journal of Nutrition,* 47**,** 267-272.

Varel, V. H., Fryda, S. J. & Robinson, I. M. 1984. Cellulolytic bacteria from pig large intestine. *Applied and Environmental Microbiology,* 47**,** 219-221.

Vasileva-Tonkova, E. & Galabova, D. 2003. Hydrolytic enzymes and surfactants of bacterial isolates from lubricant-contaminated wastewater. *Zeitschrift für Naturforschung* 58**,** 87-92.

Von Mersi, W. & Schinner, F. 1991. An improved and accurate method for determining the dehydrogenase activity of soils with iodonitrotetrazolium chloride. *Biology and Fertility of Soils,* 11**,** 216-220.

Wanandy, S., Brouwer, N., Liu, Q., Mahon, A., Cork, S., Caruso, P., Vemulpad, S. & Jamie, J. 2005. Optimisation of the fluorescein diacetate antibacterial assay. *Journal of Microbiological Methods,* 60**,** 21-30.

Wardle, D. A. & Parkinson, D. 1990. Comparison of physiological techniques for estimating the response of the soil microbial biomass to soil moisture. *Soil Biology and Biochemistry,* 22**,** 817-823.

Witten, G. Q. & Richardson, F. D. 2003. Competition of three aggregated microbial species for four substrates in the rumen. *Ecological Modelling,* 164**,** 121-135.

Ziemer, C. J., Kerr, B. J., Weber, T. E., Arcidiacono, S., Morrison, M. & Ragauskas, A. 2012. Effects of feeding fiber-fermenting bacteria to pigs on nutrient digestion, faecal output and plasma energy metabolites. *Journal of Animal Science,* 90**,** 4020-4027.

3. Storage and seasonal effects on microbial properties in indigenous ungulate faecal matter

3.1 Introduction

Scientific analysis of environmental samples usually requires laboratory analyses and therefore some form of storage after sample collection. Depending on when a sample is collected or how long it is kept after collection, storage and seasonal effects may influence the microbiological composition of that sample (Wu and Ma, 2001).

Drying (Peoples and Koide, 2012) and refrigeration or freezing (Rist *et al*., 2013) are examples of conventional sample storage methods. Depending on the type of assessment being conducted, samples are usually stored at room temperature, cooled to 4°C or frozen at temperatures as low as -20°C (Peoples and Koide, 2012). When assessing the microbiology of environmental samples, storage conditions need to be considered as a factor in the findings over time.

Studies have been reporting on the impact of storage on the microbiological composition of samples such as compost soils (Wu and Ma, 2001) and rumen contents (Dehority and Grubb, 1980). Storage was found to have an effect after 90 days of storage in the case of compost soils and recommended storage for microbial analysis was 0°C or refrigeration at 2-4°C (Wu and Ma, 2001). The impact of storage on soil bound microbial activity was found to be greater at 37°C compared to a more gradual decrease at 4°C, (Brohon *et al*., 1999) while DNA amplification success rates from faecal matter samples of tropical ungulates were found to decrease over four weeks of storage at -23°C (Soto-Calderon *et al*., 2009). Another study analysing the microbial diversity in soil and faecal samples showed only a slight impact in the microbial diversity and burden after two weeks of storage at room temperature(Lauber *et al*., 2010). In light of this information, changes in microbial abundance and diversity due to storage over time need to be considered.

Soil bound properties such as enzyme activity were found to fluctuate with time in a previous study, with recommendations to assess enzyme activity as quickly as possible and be mindful of different storage conditions for different assays (DeForest, 2009). However, temperatures of 4°C and -20°C did not produce major differences in the samples over 21 days of storage (DeForest, 2009). An earlier study reported a decrease in microbial burden over the first three months of

storage at 2°C (Stenberg *et al*., 1998). Samples used for enzyme activity and estimation of microbial burden are usually stored without alteration like air drying or they are refrigerated to preserve the sample properties (Wallenius *et al*., 2010). Samples used for fungal succession analysis are typically stored at room temperature and are usually air dried (Cázares and Trappe, 1994; Dix and Webster, 1994).

In addition to storage, the season in which samples are collected is also an important factor. Seasonal changes may also have an effect on the microbial properties of a sample at a given time. A study on the seasonal effects on microbiological properties of soil on crop farms found that microbial biomass carbon and enzyme activity differed with seasonal changes (Ullah *et al*., 2013). An earlier study also reported seasonal changes in microbial dehydrogenase activity but a fairly constant microbial biomass (Rogers and Tate III, 2001). Conversely, microbial pathogen numbers fluctuated in estuaries and sediment over different seasons (Lipp, 2001). In the case of ungulates, feeding habits during different seasons affected dietary preferences of these animals (Hansen *et al*., 1985; McNaughton, 1985) as well as the decomposition of faecal matter after defecation (Omaliko, 1981). Although there are various studies looking into seasonal effects on microbial abundance and activity of soil as well as the effects of storage, there is no literature available reporting on the microbiological changes of zebra, giraffe and impala faecal matter in different seasons. This information is vital in the analysis of these samples and for the purposes of this study.

The study objectives were therefore to compare hydrolytic activity and microbial diversity of zebra, giraffe and impala faecal samples in summer (rainy season) and winter (dry season). In addition, the effects of faecal sample storage at 4°C and 30°C on microbial diversity and hydrolytic activity of zebra, giraffe and impala faeces was examined over four weeks.

3.2 Materials and methods

3.2.1 Sample collection

Fresh faeces from zebra, giraffe and impala were collected at the Bisley Park Nature Reserve (29°39'44"S 30°23'25"E) in Pietermaritzburg through 2011 and 2012 and specifically on the 7th of June 2011 (winter) and 9th of January 2012 (summer) at 9am. Soil samples around faecal collection points were collected as controls. All soil and faeces samples were collected in clean

plastic bags. Samples to be stored at 4°C were kept on ice during transportation, while samples for storage at 30°C were kept at ambient temperature. Samples were subsequently stored at 4°C and 30°C under laboratory conditions for four weeks.

3.2.2 Hydrolytic activity assay

Fresh samples collected throughout 2011 and 2012 were assayed for hydrolytic activity as well as the samples collected specifically on the 7th of June 2011 and the 9th of January 2012 for the analysis of storage and seasonal effects. The FDA assay was carried out in duplicate Erlenmeyer flasks containing 50 ml 60 mM sodium phosphate buffer pH 7.6, to which 500 µl of 4.8 mM fluorescein diacetate in acetone and 1 g of fresh environmental sample material was added. Flasks were incubated in a water bath at 30°C for 1.5 hours either at 150 rpm or statically in the dark. The reaction was terminated with addition of 2 ml acetone. The supernatant was clarified by centrifugation at 20000 x g for 5 minutes. Absorbance of samples was measured at 490 nm using a Shimadzu UV 1240 spectrophotometer. Fluorescein concentrations in samples were determined from calibration curves based on concentrations of 0, 1.75, 2.5, 3.75 and 5.0 µg per ml. Hydrolytic activity was reported in µg of fluorescein formed per gram dry weight per hour of incubation. Flasks containing environmental sample and buffer without FDA were prepared similarly and used to correct for background absorbencies, whilst phosphate buffer with FDA served as a control for abiotic cleavage.

3.2.3 Moisture content estimation

Moisture content measurements of samples were established through drying 1 g of samples in a drying oven at 100°C for 48 hours. Samples were then cooled to room temperature and reweighed. Based on the dry weight obtained, moisture content was estimated as percentages.

3.2.4 Estimation of microbial abundance and diversity

Viable counts were determined weekly for samples stored at both 4°C and 30°C for four weeks in January 2012. Viable counts of heterotrophic aerobes were determined with spread plating onto PC agar. Decimal dilutions of environmental samples (ranging from 10^{-3} to 10^{-8}) were established by initially adding 10 g of each sample material to 90ml (10^{-1}) peptone water (8.5 g NaCl, 0.5 g peptone per litre, pH 7.2) followed by homogenisation at 150 rpm for 15 minutes and subsequent decimal dilutions up to 10^{-8}. Samples (100 µl) from each decimal dilution were

then spread-plated in triplicate onto plate count agar (PC agar) (5 g tryptone, 2.5 g yeast extract, 1 g glucose, 14 g agar per litre, pH 7.2); Tween 80 agar (5 g peptone, 3 g meat extract, 10 ml Tween 80, 100 mg $CaCl_2.2H_2O$, 15 g agar per litre, pH 7.2); Skim milk agar (10 g skim milk powder, 3 g meat extract, 5 g NaCl, 2 g Na_2HPO_4, 15 g agar, 0.05 g bromothymol blue per litre, pH 7.2); Carboxy-methylcellulose agar (CMC agar) (2 g $NaNO_3$, 1 g K_2HPO_4, 0.6 g $MgSO_4$, 0.6 g KCl, 2 g carboxy-methylcellulose sodium salt, 0.2 g peptone,17 g agar per litre, pH 7.2) and Starch agar (3 g beef extract, 10 g soluble starch, 12 g agar per litre, pH 7.2). The colony counts were established after 24-48 hour incubation at 30°C. CMC and Starch agar plates were flooded with Gram's iodine to test for cellulase and amylase positive colonies respectively which displayed a clear halo; a positive protease reaction on skim milk agar was displayed by a change of colour from green to midnight blue due to casein hydrolysis; esterase positive colonies on Tween 80 agar produced calcium oleate which precipitates around the colonies. Only plates with colony numbers in the range of 10-300 per plate were considered for weighted means calculations.

3.2.5 Fungal detection and cultivation

Swab samples from areas of fungal growth present on faeces incubated at 30°C after three weeks were streaked out onto potato dextrose agar (Merck) and cultured at 30°C for 72 hours. The growth was then microscopically examined. The isolated fungus was sub-cultured in nutrient broth (Merck) at 25°C at 150 rpm for 48 hours.

1ml of the inoculated broth was harvested by centrifugation at 20000 x g for 10 minutes and washed with 0.9 % saline solution for spore counts. Spores were counted using the Helber type (Marienfeld, Germany) counting chamber and Motic (BA 310) phase contrast microscope at 400x magnification. One ml of broth was also harvested for FDA assay analysis. To test whether the fungi produced hydrolytic activity, 1 ml of spores from the broth were harvested through centrifugation at 20000 x g for 10 minutes and washed with 0.9 % saline solution. The spores were re-dissolved in 20 ml saline to a density of $2.6x10^6$ spores per ml. 10 ml of this suspension was spiked onto 5 g autoclaved zebra faeces, which was then stored at 30°C and another 10 ml was spiked onto 5 g autoclaved zebra faeces which was stored at 4°C. The FDA assay was used to assess hydrolytic activity in samples after 1 week of incubation at 4°C and 30°C as specified

under section *3.2.2*. Protein estimation of the fungal biomass was done according to Spector (1978) after alkaline hydrolysis (1 M NaOH) for 15 minutes at 95°C.

3.3 Results

3.3.1 Seasonal effects on hydrolytic activity and microbial diversity in faecal and soil samples

The FDA assay was conducted on fresh samples collected over January 2011 to January 2012. Figure 4 shows the mean hydrolytic activity determined for indigenous faecal samples, and matching soil controls collected in 2011 and 2012 from four different sampling dates in different seasons. All samples incubated under shaker conditions were higher in hydrolytic activity than those incubated statically. Zebra faeces had the highest mean activity (1107 µg x g^{-1} x h^{-1}) over the year followed by giraffe and impala, with means of 677 and 685 µg of fluorescein per gram released per hour respectively. Soil control samples (shaken and static) produced hydrolytic activity never exceeding 200 µg fluorescein released per gram dry weight and hour. The hydrolytic activity present in the faecal samples of all three animals based on the mean annual hydrolytic activity was consistently higher than that of control soils.

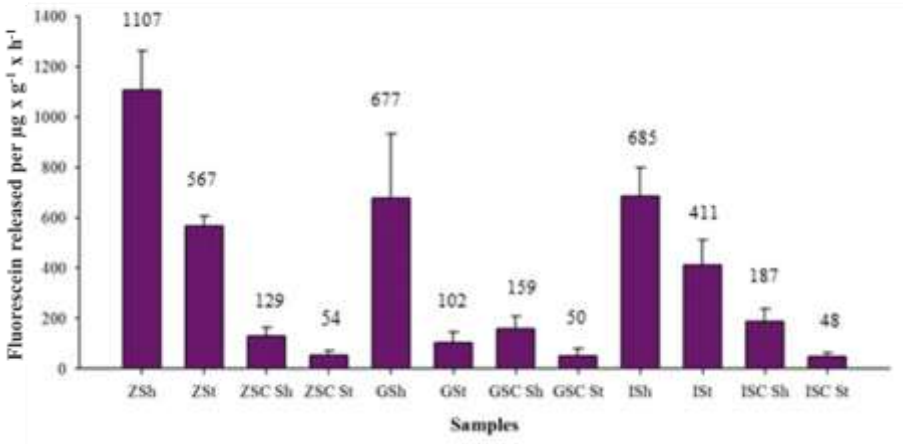


Figure 4: Mean hydrolytic activity of faecal and soil samples over the four seasons in 2011 and 2012. The error bars shown indicate the standard error. The activity in the y-axis is expressed as µg x g^{-1} x h^{-1}

Key: ZSh: Zebra shaker; ZSt: Zebra static; GSh: Giraffe shaker; GSt: Giraffe static; ISh: Impala shaker; ISt: Impala static; ZSC-Sh: Zebra soil control shaker; ZSC-St: Zebra soil control static; GSC-Sh: Giraffe soil control shaker; GSC-St: Giraffe soil control static; ISC-Sh: Impala soil control shaker; ISC-St: Impala soil control static. The mean hydrolytic activity is calculated from samples taken on four different dates in the dry and rainy seasons.

The diversity of hydrolytic microorganisms of faecal samples as well as viable counts were compared for winter (June 2011) and summer (January 2012) (Fig 5 and 6). Each pie chart represents the proportions of different hydrolase producers present in a sample, relative to the total viable counts established on plate count agar. Viable counts were slightly higher in summer than in winter in all faecal samples analysed. Zebra faeces had the highest proportions of esterase producing microorganisms in both seasons, whilst giraffe had the highest proportions of cellulase producers. The overall distribution of hydrolytic microbial diversity in impala and giraffe faeces was similar in summer. In winter (the dry season), the distribution of hydrolytic microbial diversity was similar for impala and zebra faeces. Soil controls differed in diversity and microbial counts but no apparent seasonal trend was noted (Fig 7 and 8).

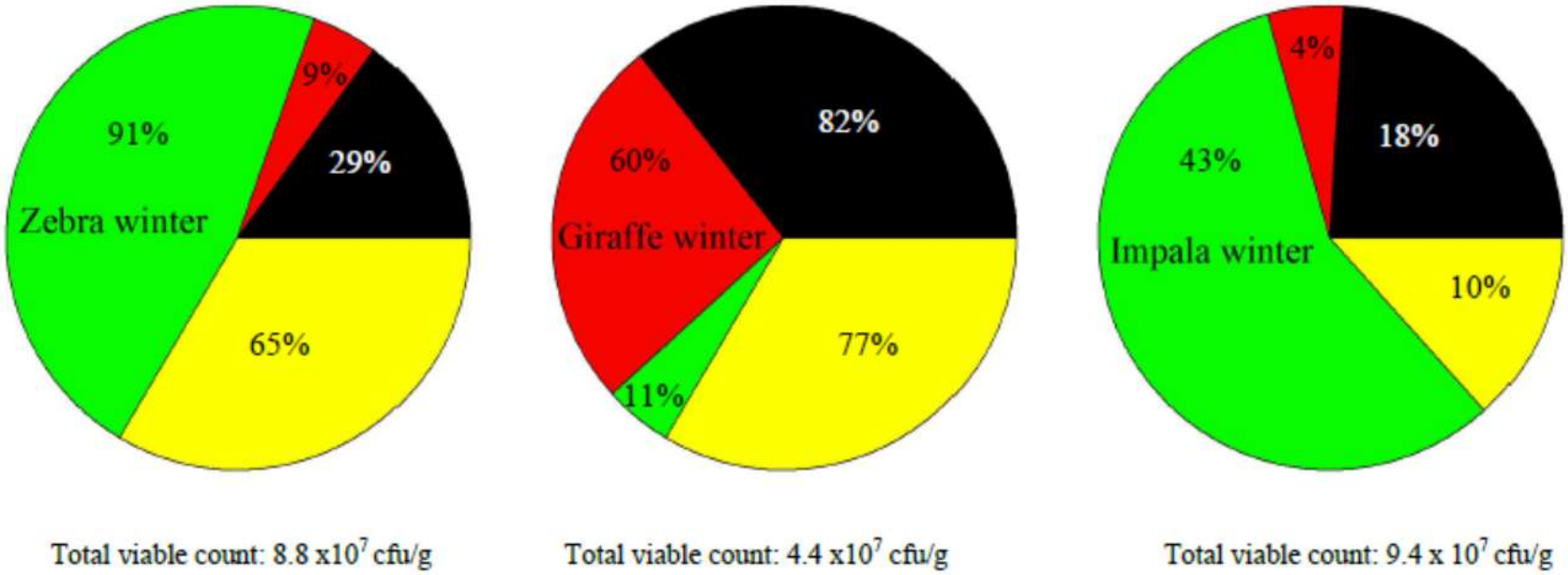


Figure 5: Relative proportions of different hydrolytic microorganisms and viable counts in fresh zebra, giraffe and impala faeces in winter (June 2011).

Green: esterase-producers; red: protease-producers; black: amylase-producers; yellow: cellulase-producers: viable count in cfu per gram dry weight

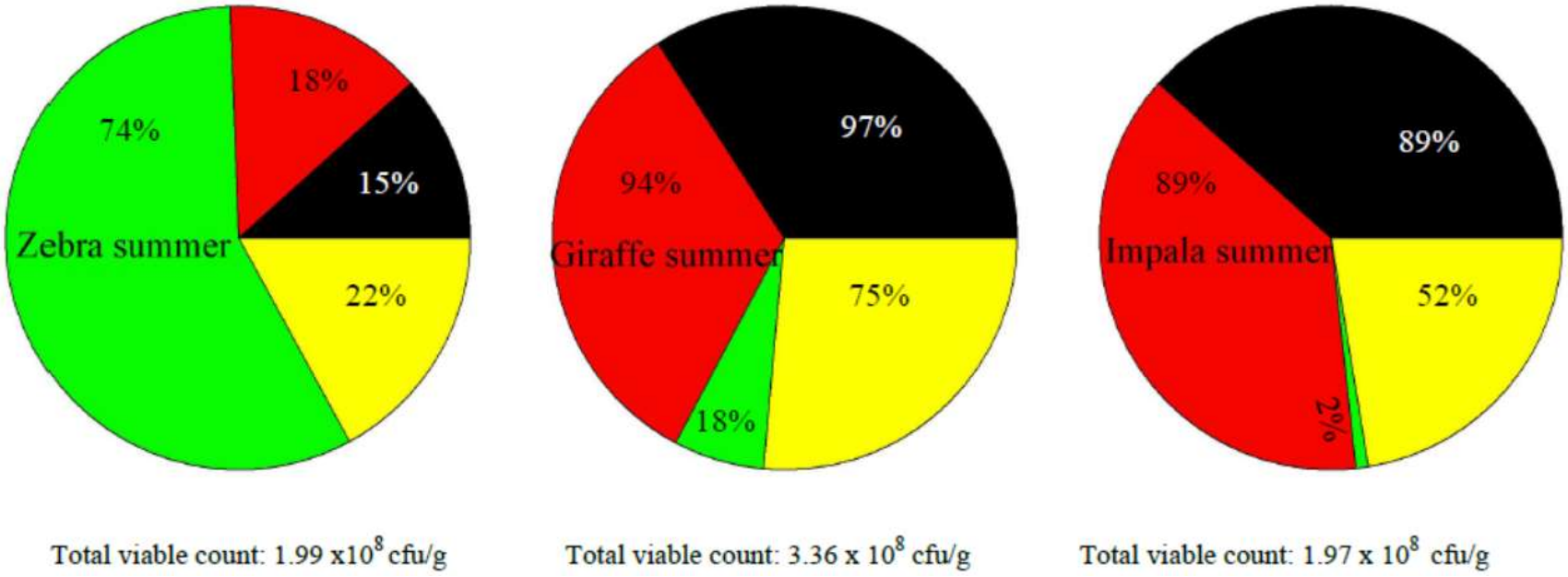


Figure 6: Relative proportions of different hydrolytic microorganisms and viable counts of fresh zebra, giraffe and impala faeces in summer (January 2012)

Green: esterase-producers; red: protease-producers; black: amylase-producers; yellow: cellulase-producers: viable count in cfu per gram dry weight

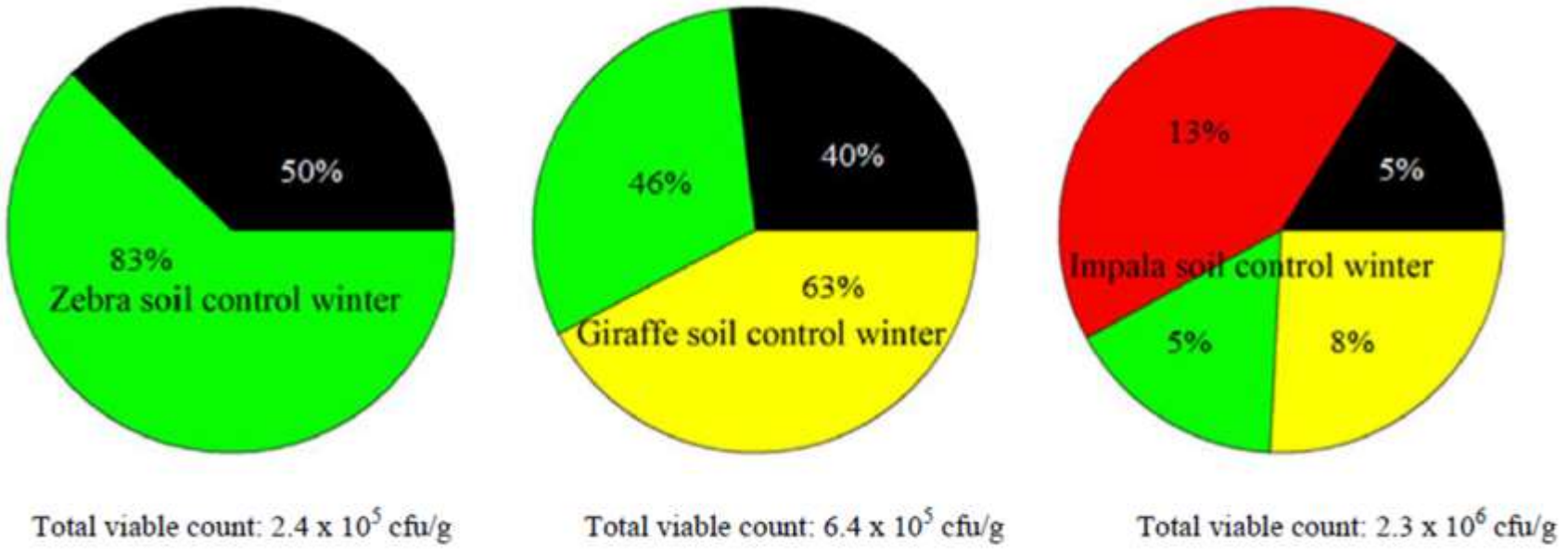


Figure 7: Relative proportions of different hydrolytic microorganisms and viable counts in soil control samples in winter (June 2011)

Green: esterase-producers; red: protease-producers; black: amylase-producers; yellow: cellulase-producers: viable count in cfu per gram dry weight

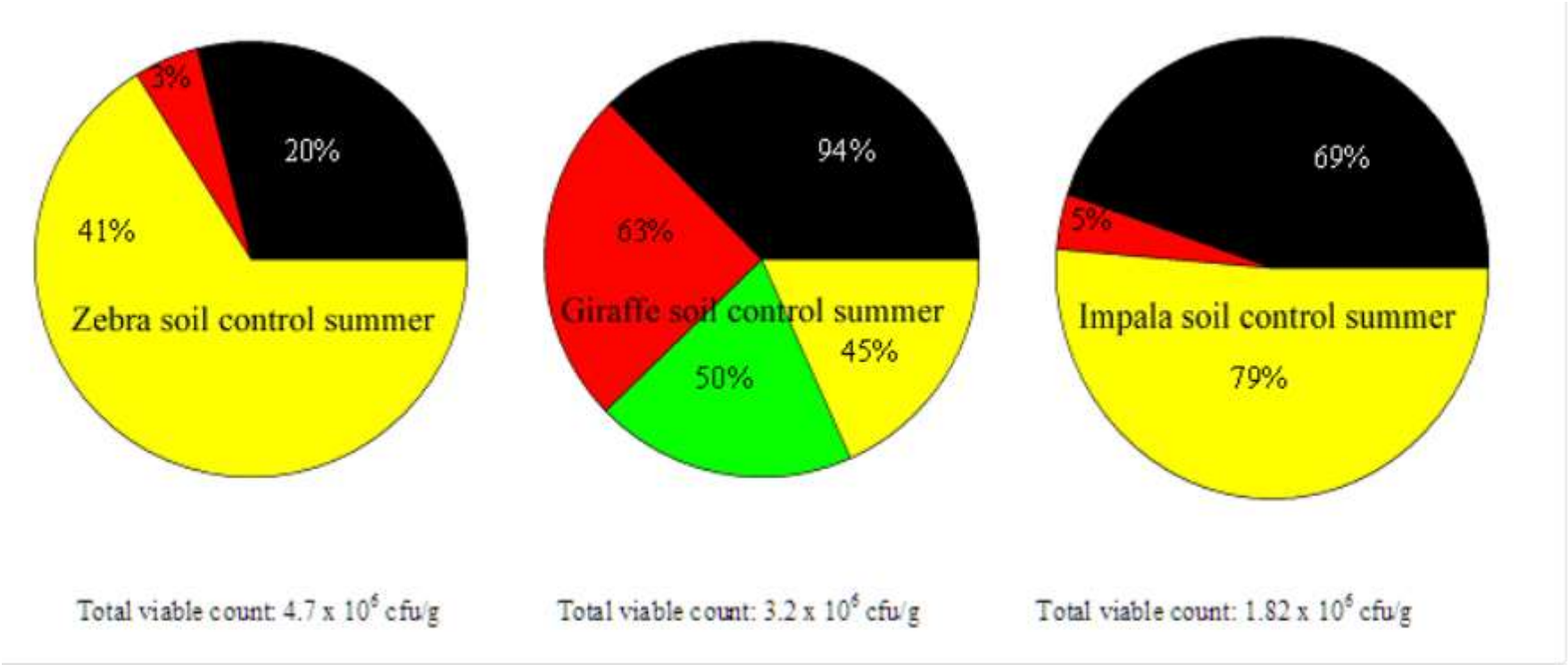


Figure 8: Relative proportions of different hydrolytic microorganisms and viable counts of soil control samples in summer (January 2012)

Green: esterase-producers; red: protease-producers; black: amylase-producers; yellow: cellulase-producers: viable count in cfu per gram dry weight

Hydrolytic activities of samples in summer and winter were also compared (Fig 9) and found to be lower in summer than in winter for all samples, although microbial numbers were higher in summer (Fig 5and 6). Hydrolytic activity measurements for soil control samples were also recorded and found to have a similar trend (Appendix A).

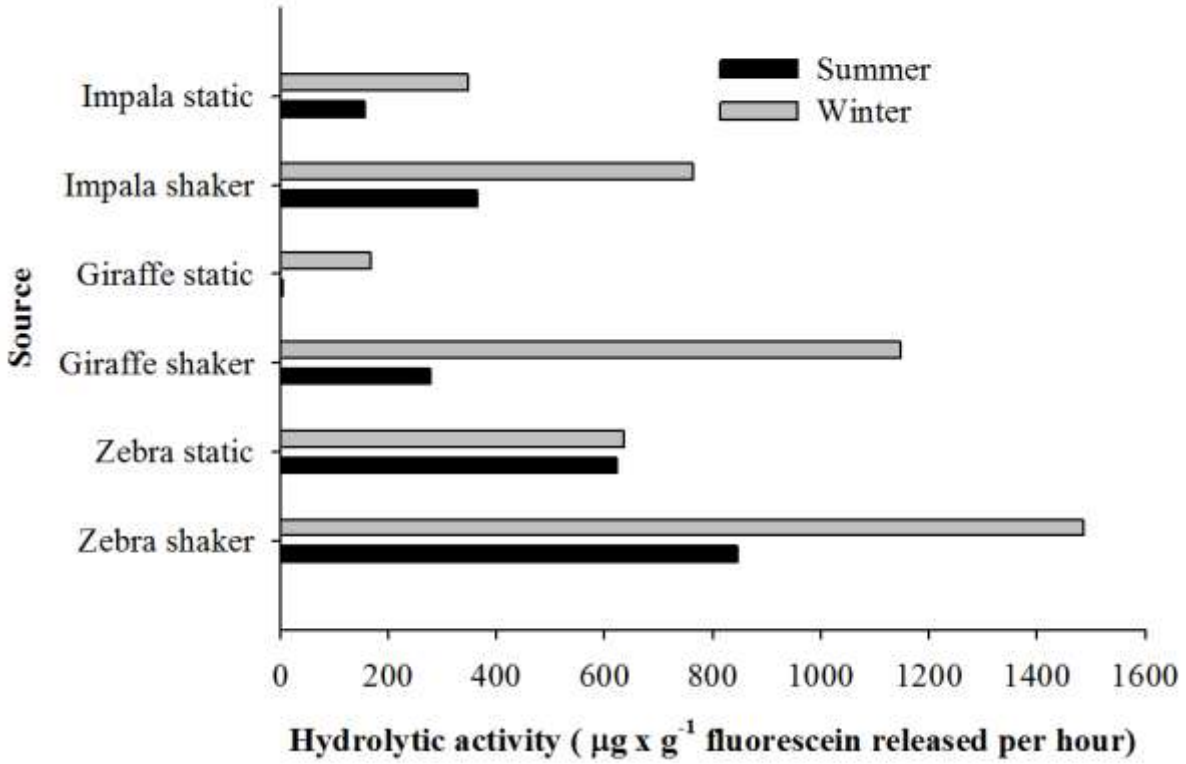


Figure 9: Comparison of seasonal hydrolytic activity in faecal samples (dry weight) incubated under shaker and static conditions. The data shown are the means of duplicate measurements of hydrolytic activity.

3.3.2 Effects of storage on microbial abundance and diversity

As a means to investigate whether hydrolytic activity fluctuates during storage at two different temperatures (4°C and 30°C), the FDA assay was performed using fresh samples collected in the winter (June 2011) and the summer (January 2012) season. Zebra faecal matter and the corresponding soil control were selected as representatives to illustrate the changes in hydrolytic activity at weekly intervals at both storage temperatures in winter (Fig 10). There is a marked decrease in the hydrolytic activity in the second week of storage, with an increase in the third week and another drop in the fourth week of storage. The activity is

highest in the first week of storage and mostly lowest in the second week, this trend was observed in the other faecal and soil samples as well (Appendix A).

The initial activity (Week 1, 7th June) in most samples was lower at 30°C storage in comparison to that measured in the first week at 4°C storage (Fig 10). There was a reduction in sample hydrolytic activity in the second week with an increase in the third week at both storage temperatures. In the 3rd and 4th week of storage, faecal matter samples developed a white fungal-like growth on the surfaces. The growth was more abundant in samples stored at 30°C. When microscopically examined (not shown), the presence of fungal growth was confirmed as fungal mycelium exhibiting septate hyphae and conidia were present. No other factor except fungal growth could be visually identified as the causative agent of the increased activity in the 3rd week. This increased activity was observed on soil samples as well, though no visual changes were evident for these samples.

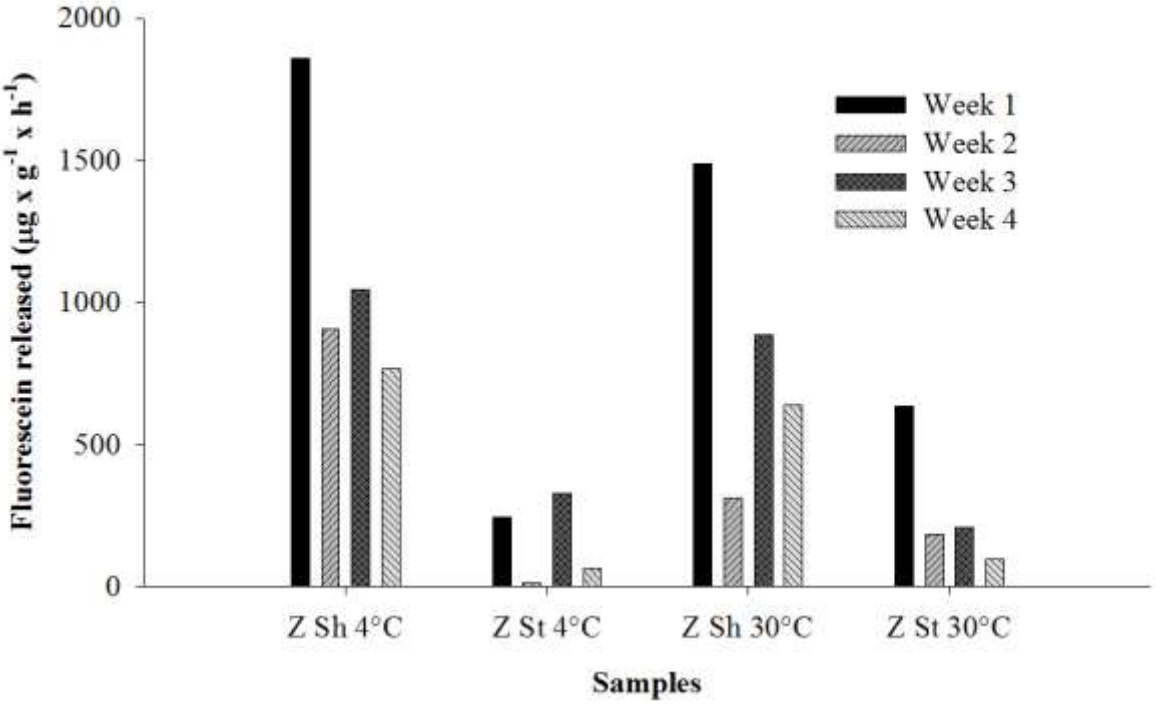


Figure 10: Hydrolytic activity of zebra faecal samples collected in winter (June 2011) upon storage at 4°C and 30°C for four weeks. All data shown are the means of duplicate measurements.

ZSh: Zebra shaker; ZSt: Zebra static

Hydrolytic activity of the fungal biomass was determined with FDA and was found to be 41 and 40 µg of fluorescein per mg of protein released per hour under shaker and static conditions respectively. This indicated that fungal biomass present on the faeces may have contributed to the overall hydrolytic activity measured in faecal matter.

To verify the possible contribution of fungal biomass to hydrolytic activity and to determine the effects of storage temperature, autoclaved zebra faeces (5 g) were inoculated with fungal spores by adding 10 ml suspension containing $2.6x10^6$ conidia per ml and incubated at 4°C and 30°C. Hydrolytic activity of the spiked faeces was measured after one week of incubation (Table 6). Shaking resulted in higher hydrolytic activity than when performing the FDA assay under static conditions, corresponding with previously seen trends in the assay (Fig 4). Hydrolytic activity (shaking) was greater after 30°C incubation in comparison to 4°C, where the fungal growth was less abundant and was not as visible as on samples stored at 30°C.

Table 6: Hydrolytic activity in conidia spiked zebra faeces after 1 week incubation at 4°C and 30°C

Sample	Fluorescein released (µg x g^{-1} x h^{-1})
SF 4 °C shaker	78
SF 4 °C static	70
SF 30 °C shaker	572
SF 30 °C static	18

SF: spiked zebra faeces ($2.6x10^5$ conidia added per gram dry weight). The data shown are the mean of duplicate measurements

Additionally, the weekly hydrolytic activity was compared with changes in viable plate counts in samples collected in January 2012 over the four week incubation period at 4°C and 30°C. Zebra faeces stored at 4°C provided a representation of the change in microbial number and diversity over four weeks (Fig 11). There was an apparent gradual decrease in total viable counts and hydrolase-producers over storage time, with relative proportions of hydrolase-producers remaining steady, apart from the decrease of cellulase producers in week four, which reduced the numbers below the detection limit (<10000 cfu/g). Decreases in microbial diversity were slower in faecal samples than in soil samples (Appendix A).

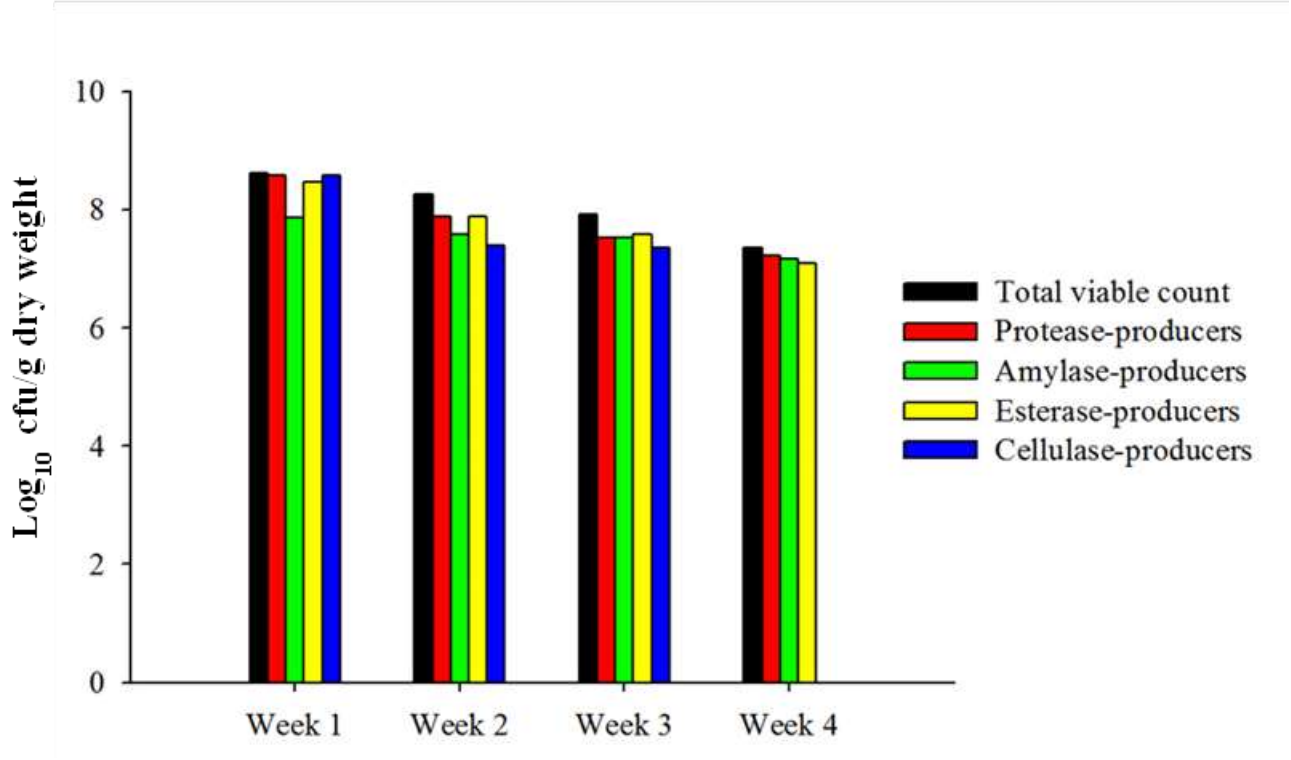


Figure 11: Changes in microbial counts and diversity of hydrolytic microorganisms in zebra faeces collected in summer (January 2012) over 4 weeks storage at 4°C

Colony counts (cfu/g dry weight) were determined in triplicate and calculated as weighted mean

The decrease in microbial abundance and diversity regarding hydrolytic microorganisms was more evident in samples stored at 30°C than samples stored at 4°C (Fig 12). Zebra faeces stored at 30°C were used to represent the changes occurring in other samples (Appendix A) stored at the same temperature. Microbial load and diversity decreased faster at this temperature than at 4°C and after two weeks of storage, the counts for some hydrolase producers were below the detection limit (<10000 cfu/g). In the zebra faecal sample specifically, counts for protease and amylase producers decreased to below 10000 cfu/g. The differences in microbial abundance between Fig 11 and 12 shows that lower storage temperature seemed to be more favourable for maintaining microbial diversity and number.

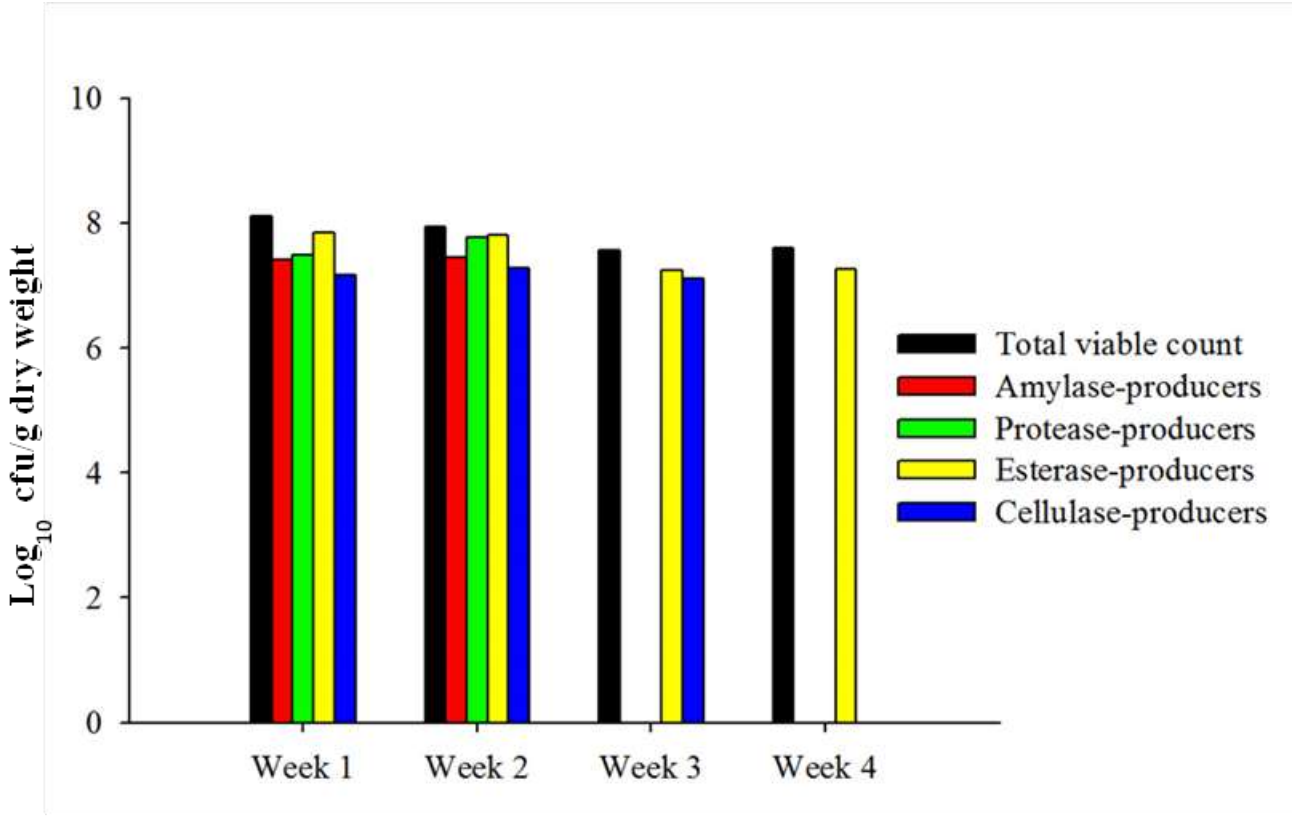


Figure 12: Changes in microbial counts and diversity of hydrolytic microorganisms in zebra faeces collected in summer (January 2012) over 4 weeks storage at 30°C.

Colony counts (cfu/g dry weight) were determined in triplicate and calculated as weighted mean

3.4 Discussion

Faecal samples in this study were compared with soil collected from the same area to analyse whether the hydrolytic activity present in faecal matter defecated by indigenous herbivores is higher than that in surrounding soil. This showed that the hydrolytic activity and microbial abundance in faeces were unique and not influenced by surrounding soil after defecation.

3.4.1 Seasonal effects on hydrolytic activity and microbial abundance of samples

Hydrolytic activity measurements of faecal samples collected in different seasons provided an idea of the mean hydrolytic activities over the year (Figure 4). Hydrolytic activity was higher for all samples incubated under shaker conditions, confirming that shaken samples facilitate higher fluorescein release (Schnurer and Rosswall, 1982).

Samples collected in June (winter) produced a higher hydrolytic activity than samples collected in January (summer) (Figure 9). According to a study conducted in Nigeria on cattle dung decomposition by Omaliko (1981), dung decomposition occurred faster in dry seasons

(winter) than in wet seasons (summer) due to tropical termites *Isoptera* and *Coleoptera* spp., which are found in South Africa as well. In addition, higher and faster fungal colonisation was evident in the wet (summer) seasons than in dry (winter) seasons (Omaliko, 1981; Pietikäinen *et al*., 2005), which is similar to findings in this study, where visible fungal growth occurred faster in samples collected in summer than in winter.

Hydrolytic activity in all samples was higher in winter than in summer. Higher hydrolytic activity in winter (Figure 9) did not necessarily correlate with higher microbial numbers (Figure 5 and 6), with ungulates having total viable counts lower in winter than in summer. This may be due to the rate of dung decomposition being higher in winter (Omaliko, 1981), thus affecting microbial properties of faeces faster after defecation, than in summer. Additionally, higher temperatures and humidity have been found to increase bacterial numbers and respiration rates, which might also explain the slightly higher microbial numbers in summer.

Zebra faeces produced the highest mean hydrolytic activity throughout the seasons followed by impala and giraffe (Fig 4 and 9) The mean hydrolytic activity of giraffe and impala was nearly the same (Fig 4) whilst giraffe produced a clearly higher hydrolytic activity in winter than impala (Fig 9). A study measuring seasonal fibrolytic activity of herbivores found zebra and impala to produce the highest activity in both summer and winter seasons (Fon and Nsahlai, 2012) which supports the findings of this study for zebra faecal matter. The high hydrolytic activity for zebra may be due to their digestive system as hindgut fermenters, resulting in higher numbers of metabolically active microorganisms being found in their faeces after defecation than in ruminants (Mackie, 2002).

Forage quality and variety was found to be higher in wet seasons than in dry seasons (McNaughton, 1985) and defecation rates of cattle were higher during wet seasons than in dry seasons (Omaliko, 1981). According to a study by van der Waal *et al.* (2011), large herbivore faeces were found to be good phosphorous and nitrogen soil fertilisers. As a result, the higher defecation rates could in turn be a cause of greater forage quality, apart from rainfall. This creates a cycle of higher defecation and good forage quality in wet seasons, which is the summer season in this area. However these findings do not match the lower hydrolytic activities detected for summer samples in this study.

The proportions of microbial hydrolase producer groups in zebra and impala (Figure 5) were similar in winter and this matches evidence from a study in Kenya which suggested that impala and zebra have similar diets in dry seasons, with a preference for grasses like *Themeda triandra*, which is generally abundant in savannahs (Hansen *et al*., 1985). However, in summer (wet season, Fig 6) impala's pattern of hydrolase producer proportions is similar to that of giraffe, suggesting that impala may have a higher preference for shrubbery when it is available than for grasses, thus having a diet similar to giraffe in the wet season. These findings concurred with previous publications that suggest impala has a dietary preference towards browse fodder than to grasses, when it is available (Wrench *et al*., 1997; van der Waal *et al*., 2011).

The findings in this study indicate that higher forage quality and higher defecation rates did not correlate with higher hydrolytic activity in faecal samples, though microbial viable counts as expected were higher in the wet season than in the dry season (Figure 5and 6). Microbial numbers have been found to be higher in cattle manure in summer seasons (Miller *et al*., 2003), supporting the findings in this study, although it is not clear whether it is due to elevated temperatures increasing microbial numbers, since the hydrolytic activity measured was higher in winter (lower microbial numbers).

Hydrolytic activity is a potential measure of the microbial activity present and has been used to estimate microbial abundance and diversity in environmental samples (Lundgren, 1981; Adam and Duncan, 2001), thus one may expect higher hydrolytic activity in samples with higher microbial numbers. However, this was not the case in this study; the hydrolytic activity measured provided an overview of the potential enzyme activity in the faeces but did not correlate with viable count data, this could be due to the fact that each colony forming microorganism may not have been hydrolytically active. To produce a more accurate comparison, other seasonal factors that may influence metabolic activity in faeces need to be considered in future studies.

Relative proportions of hydrolase-producers in giraffe faeces were similar in winter and summer (Figure 5 and 6), except for the clearly higher relative percentage of protease producers in summer than in winter. This could be due to the consistence in dietary intake of the giraffe; as a tall ungulate there is no competition for food in tall trees (Leuthold, 1978) and they were found to feed mostly on acacia plants in wet and dry seasons (Hansen *et al.*, 1985). However, the quality of the foliage will differ between these seasons due to lesser rain

and therefore lower growth of fresh foliage in plants. This would explain the observed trend that microbial abundances were lower in winter than in summer.

3.4.2 Effects of storage on hydrolytic activity

Over time, hydrolytic activities were found to fluctuate in stored samples, displaying a decrease in activity after 2 weeks of storage in most of the samples analysed. This prompted the investigation of the effects of storage on the samples. The FDA assay was chosen for this analysis as it is fast, sensitive and specific for hydrolytic enzymes. Samples were subjected to higher (30°C) and lower (4°C) temperature storage, in an effort to somewhat mimic environmental and rumen temperatures. The samples were assessed weekly for activity. This was done with samples collected in the months of June (South African Winter) and January (South African Summer) to observe whether collection in different seasons affects the faecal sample properties. Activities for samples from winter season (June) stored at 4°C and 30°C (Figure 10) over four weeks did not decrease steadily but fluctuated over time. All the samples displayed a similar fluctuation under both shaker and static conditions of the FDA assay. Along with this, faeces were observed macroscopically and found to have fungal growth developing on them. The visible growth was white and fluffy, becoming thicker as weeks progressed, appearing more prominently in giraffe faecal samples. It was observed in samples at 30°C as well as at 4°C, with the growth increasing faster in samples kept at 30°C. The same growth was observed in the summer months which is not unexpected on microbiological grounds as herbivore faecal matter has various properties (moisture, pH, nutrients) that make it a favourable substrate for microorganisms after defecation (Dix and Webster, 1994). As a result, various studies have been conducted on the succession of coprophilous organisms on faecal material of ruminants and herbivores like cows, sheep, rabbits and grouse (Richardson, 2000, Nannipieri *et al*., 2003).

The fungal growth pattern was then compared to the observed fluctuations in hydrolytic activity. Overall, there appeared to be a drop in the hydrolytic activity after the first week of incubation. Activity then increased on the third week and dropped afterwards. This could have been due to the onset of fungal growth that typically occurs on faecal samples after 2 to 3 weeks of storage (Dix and Webster, 1994) like in this study. At higher temperatures there seemed to be a faster growth of the fungi than at a lower temperature as visually detected on the stored samples. This could be due to the higher metabolic and growth rates that higher temperatures enable, as generally most fungi thrive better under higher humidity and

temperatures. Fungal succession has been reported to occur on faecal samples over storage time and they are believed to be first colonised by *Zygomycetes* followed by *Ascomycetes* and then *Basidiomycetes*. *Ascomycetes* were the fungi found to be present in the droppings samples when they were analysed after 2-3 weeks incubation. *Zygomycetes* are usually succeeded by the class *Ascomycetes* within 2 days after defecation (Dix and Webster, 1994).

Upon conduction of the experiment, the initial hydrolytic activity of fresh samples was found to be the highest. A drop in activity during the second week of storage, with subsequent increase in the third week and a final drop in the fourth week were noted. This could be due to the contribution of fungal growth on the faeces as further analysis in this study established that the isolated fungus shows hydrolytic activity. The drop in activity in the last week could be a result of genuine strain on the microorganisms within the faeces, due to changes in the environment from the effects of storage. This is supported by a decrease in microbial abundance as well. Apart from fungal interference, the dynamics of microbial metabolic changes within faecal or soil samples cannot be fully explained without more detailed monitoring of the exact processes occurring within the faeces. However, there seems to be a relation between fungal growth, storage and the changes in microbial hydrolytic activity. It must be noted however, that other processes could be contributing to the changes in activity such as competition for substrate, decreased aeration and change in moisture content (Nannipieri *et al*., 2003).

3.4.3 Changes in microbial burden and diversity with storage

Changes in microbial hydrolase producers and total viable counts over time of storage were monitored weekly. Zebra faecal matter and the matching soil were used as representative samples (Fig 11 and 12). Hydrolytic activity and viable counts of the other soil and faecal samples was also measured weekly (Appendix A). Total viable counts in samples decreased steadily over time, however, the proportions of hydrolase producers did not change concomitant with the decrease in numbers. Viable counts of soil controls were much lower than faecal samples. In addition, cellulolytic microorganisms may take longer to develop colonies on agar plates due to the complexity of the substrate. Cellulose is a difficult substrate to degrade and is usually broken down by co-cultures (Halsall and Gibson, 1985). Incubation times for viable count measurements of all hydrolase producers including cellulolytic microorganisms were kept constant (24-48 hours) in light of the fact to safeguard identical experimental parameters for all the hydrolase producers. This could mean that cellulose

positive microorganisms may have been under estimated in that respect, though the decrease in detection of cellulolytic microorganisms, as storage time increases, reflects the effects of storage.

In most of the samples, protease producing microorganisms were lower in abundance, compared to amylase and esterase producers, which were present in higher relative proportions (Appendix A). The overall picture emerging from this analysis is that storage of samples does affect microbial numbers, diversity and activity. The fluctuation observed in week 2 of hydrolytic activity measurements was not evident in the viable count measurements; this may be due to higher sensitivity of the FDA assay or suggests that the fluctuation of activity was not directly related to changes in microbial abundance. Fungal growth was not observed on direct plate counts as it is believed that the incubation time (24-48 hours) was not sufficient for accurate enumeration of hyphal growth on the plates. In addition to the fact that most microbes cannot be cultured under laboratory conditions, there may be other factors contributing to the changes in activity seen in the storage experiments. Nannipieri *et al*. (2003) describes the difficulty in obtaining an accurate idea of the interactions in soil systems and the inability to attain the exact compositions of the microbial ecosystem using a variety of methods. The plate count method was done specifically to select for aerobic microorganisms that could perform the required hydrolysis of the various polymers for isolation purposes, as well as to get a brief idea of microbial abundance and diversity in relation to the changes in activity. The obtained results show that the FDA activity assay can be used to assess samples qualitatively and indicates interferences and changes in a sample. Coprophilous fungi, microbial competition, decreased aeration, changes in temperature and the depletion of nutrients could be factors that contribute to these changes (Renella *et al*., 2002; Nannipieri *et al*., 2003; Maranon *et al*., 2006).

Faecal samples that have not been dried and are kept in their natural state should be stored at low temperatures and analysed as soon as possible. Recommended temperatures are 2-4°C for analysis of samples for more than 2 weeks, although changes in the sample will even occur over that period (Wu and Ma, 2001). According to Dehority and Grubb (1980), refrigeration (0°C) was found to produce inconsistencies in samples and a sudden increase in microbial numbers after 8 hours (Dehority and Grubb, 1980). A more recent study by Lauber *et al.* (2010) also stresses that samples should be analysed as soon as possible and that unrefrigerated soil and faecal samples may not be useful for microbial analysis.

In conclusion, the observed seasonal effects on faecal matter and soil samples were in agreement with some previous reports, though more research is required to explore factors such as the specific diets of the ungulates in Bisley Park and forage availability in different seasons. Although the exact interaction between microbial diversity, ungulate diet and seasonal influences may not be clear, this study indicates that seasonal effects exist and provides a starting point for further research in this aspect. In analysing the effects of storage, it is recommended that fresh faecal samples should be kept at 2-4°C and analysed as soon as possible, preferably within the first week of collection.

3.5 References

Adam, G. & Duncan, H. 2001. Development of a rapid and sensitive method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils. *Soil Biology and Biochemistry,* 33**,** 943-951.

Brohon, B., Delolme, C. & Gourdon, R. 1999. Qualification of soils through microbial activities measurements influence of the storage period on int-reductase, phosphatase and respiration. *Chemosphere,* 38**,** 1973-1984.

Cázares, E. & Trappe, J. M. 1994. Spore dispersal of ectomycorrhizal fungi on a glacier forefront by mammal mycophagy. *Mycologia,* 86**,** 507-510.

DeForest, J. L. 2009. The influence of time, storage temperature, and substrate age on potential soil enzyme activity in acidic forest soils using MUB-linked substrates and l-DOPA. *Soil Biology and Biochemistry,* 41**,** 1180-1186.

Dehority, B. A. & Grubb, J. A. 1980. Effect of short-term chilling of rumen contents on viable bacterial contents. *Applied and Environmental Microbiology,* 39**,** 376-381.

Dix, N. J. & Webster, J. 1994. *Fungal ecology,* London, Chapman & Hall Ltd.

Fernández-Segovia, I., Escriche, I., Fuentes, A. & Serra, J. A. 2007. Microbial and sensory changes during refrigerated storage of desalted cod (*Gadus morhua*) preserved by combined methods. *International Journal of Food Microbiology,* 116**,** 64-72.

Fon, F. N. & Nsahlai, I. V. 2012. Seasonality of fibrolytic enzyme activity in herbivore microbial ecosystems. *African Journal of Biotechnology,* 11**,** 13123-13130.

Halsall, D. M. & Gibson, A. H. 1985. Cellulose decomposition and associated nitrogen fixation by mixed cultures of *Cellulomonas gelida* and *Azospirillum* species or *Bacillus macerans*. *Applied and Environmental Microbiology,* 50**,** 1021-1026.

Hansen, R. M., Mugambik, M. M. & Bauni, S. M. 1985. Diets and trophic ranking of ungulates of the northern Serengeti. *Journal of Wildlife Management,* 49**,** 823-829.

Lauber, C. L., Zhou, N., Gordon, J. I., Knight, R. & Fierer, N. 2010. Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. *FEMS Microbiology Letters,* 307**,** 80-86.

Leuthold, W. 1978. Ecological Separation among browsing ungulates in Tsavo East National Park, Kenya. *Oecologia,* 241-252.

Lipp, E. K., Kurz, R., Vincent, R., Rodriguez-Palacios, C., Farrah, S.R., Rose, J.B. 2001. The effects of seasonal variability and weather on microbial faecal pollution and enteric pathogens in a subtropical estuary. *Estuaries,* 24**,** 266-276.

Lundgren, B. 1981. Fluorescein diacetate as a stain of metabolically active bacteria in soil. *Oikos,* 36**,** 17-22.

Mackie, R. I. 2002. Mutualistic fermentative digestion in the gastrointestinal tract: diversity and evolution. *Integrative and Comparative Biology,* 42**,** 319-326.

Maranon, E., Castrillon, L., Fernandez, J. J. & Fernandez, Y. 2006. Anaerobic mesophilic treatment of cattle manure in an upflow anaerobic sludge blanket reactor with prior pasteurization. *Journal of the Air & Waste Management Association,* 56**,** 137-143.

Mcnaughton, S. J. 1985. Ecology of a grazing ecosystem: the Serengeti. *Ecological Monographs,* 55**,** 259-294.

Miller, J. J., Beasley, B. W., Yanke, L. J., Larney, F. J., Mcallister, T. A., Olson, B. M., Selinger, L. B., Chanasyk, D. S. & Hasselback, P. 2003. Bedding and seasonal effects on chemical and bacterial properties of feedlot cattle manure. *Journal of Environmental Quality,* 32**,** 1887-1894.

Nannipieri, P., Ascher, J., Ceccherini, M., Landi, L., Pietramellara, G. & Renella, G. 2003. Microbial diversity and soil functions. *European Journal of Soil Science,* 54**,** 655-670.

Omaliko, C. P. E. 1981. Dung deposition, breakdown and grazing behaviour of beef cattle at two seasons in a tropical grassland ecosystem. *Journal of Range Management,* 34**,** 360-362.

Peoples, M. S. & Koide, R. T. 2012. Considerations in the storage of soil samples for enzyme activity analysis. *Applied Soil Ecology,* 62**,** 98-102.

Pietikäinen, J., Pettersson, M . & Bååth, E. 2005. Comparison of temperature effects on soil respiration and bacterial and fungal growth rates *FEMS Microbiology Ecology,* 52**,** 49-58

Renella, G., Landi, L. & Nannipieri, P. 2002. Hydrolase activities during and after the chloroform fumigation of soil as affected by protease activity. *Soil Biology and Biochemistry,* 34**,** 51-60.

Richardson, M. J. 2000. Diversity and occurence of coprophilous fungi. *Mycological Research,* 105**,** 387-402.

Rist, M., Muhle-Goll, C., Görling, B., Bub, A., Heissler, S., Watzl, B. & Luy, B. 2013. Influence of freezing and storage procedure on human urine samples in NMR-based metabolomics. *Metabolites,* 3**,** 243-258.

Rogers, B. F. & Tate III, R. L. 2001. Temporal analysis of the soil microbial community along a toposequence in Pineland soils. *Soil Biology and Biochemistry,* 33**,** 1389-1401.

Schnurer, J. & Rosswall, T. 1982. Fluorescein diacetate as a measure of total microbial activity in soil and litter. *Applied and Environmental Microbiology,* 43**,** 1256-1261.

Soto-Calderon, I. D., Ntie, S., Mickala, P., Maizels, F., Wickings, E. J. & Anthony, N. M. 2009. Effects of storage type and time on DNA amplification success in tropical ungulate faeces. *Molecular Ecology Resources,* 9**,** 471-479.

Spector, T. 1978. Refinement of the Coomassie blue method of protein quantitation. *Analytical Chemistry,* 86**,** 142-146.

Stenberg, B., Johansson, M., Pell, M., Sjödahl-Svensson, K., Stenström, J. & Torstensson, L. 1998. Microbial biomass and activities in soil as affected by frozen and cold storage. *Soil Biology and Biochemistry,* 30**,** 393-402.

Ullah, R., Lone, M., Ullah, K., Mehdi, S. & Qazi, M. 2013. Effect of cropping system and seasonal variation on soil microbial biomass and enzymatic activities in arid soils. *Journal of Animal and Plant Sciences,* 23**,** 493-499.

Van Der Waal, C., Kool, A., Meijer, S. S., E., K., Heitkoning, I. M. A., De Boer, W. F., Van Langevelde, F., Grant, R. C., Peel, M. J. S., Slotow, R., De Knegt, H. J., Prins, H. H. T. & De Kroon, H. 2011. Large herbivores may alter vegetation structure of semi-arid savannas through soil nutrient mediation. *Oecologia,* 165**,** 1095-1107.

Wallenius, K., Rita, H., Simpanen, S., Mikkonen, A. & Niemi, R. M. 2010. Sample storage for soil enzyme activity and bacterial community profiles. *Journal of Microbiological Methods,* 81**,** 48-55.

Wrench, J. M., Meissner, H. H. & Grant, C. C. 1997. Assessing diet quality of African ungulates from faecal analyses: the effect of forage quality, intake and herbivore species. *Koedoe,* 40**,** 125-136.

Wu, L. & Ma, L. Q. 2001. Effects of sample storage on biosolids compost stability and maturity evaluation. *Journal of Environmental Quality,* 30**,** 222-228.

4. Isolation and characterisation of faecal isolates with hydrolase activity

4.1 Introduction

The discovery of microbes that possess useful hydrolases is a profitable aspect of industrial and environmental microbiology. Industrial fermentation is estimated to have grossed roughly $1.5 billion since the year 2000 (Kirk *et al*., 2002). Less than 5 of the largest enzyme companies in the world have been commercially utilising more than 10 bacterial enzymes sourced naturally in industrial fermentation processes (Kirk *et al*., 2002). The hydrolytic enzymes largely being utilised are primarily for detergents, bakery and oil-production based processes.

The main fermentation methods employed in industrial production for the production of hydrolases are submerged fermentation (SMF) and solid state fermentation (SSF) (Latifian *et al*., 2007). SMF has primarily been employed due to the ability to control fermentation parameters easily, but is not as cost effective as SSF. Due to the applicability of wastes as substrates in SSF, it is now being considered, despite its drawback of poor parameter control, as a cost-effective alternative that aids in pollution reduction. Studies have shown that product yield is more substantial and costs are reduced by 78% under SSF than SMF reaction conditions. The poor parameter control in SSF fermentations requires hydrolases that possess thermo-stability, alkalotolerance and ideally halotolerance to maintain hydrolytic activity under changing process conditions within the fermenter (Couto and Sanromán, 2006). This can be achieved by mutagenesis or genetic engineering of existing hydrolases or screening for hydrolases that are more stable from microbial isolates.

Examples of widely used hydrolases are proteases, amylases, cellulases, and esterases (Underkofler *et al*., 1958). All of these hydrolases have been isolated from the genera *Bacillus* and *Pseudomonas*, although these are mostly used for the extraction of amylase enzymes (Ray, 2011).

Hydrolytic enzyme properties of microbial isolates from soil and other environmental sources have been well documented (Jaeger *et al*., 1996; Vasileva-Tonkova and Galabova, 2003). Faecal matter from domestic herbivores is among the sources that have been explored for potential hydrolytic isolates, due to their microbial abundance (Moriarty and Gilpin, 2014). In the case of South African wild ungulates, so far only a strain of *Myxococcus* spp. and *Microbacterium* spp. were isolated from zebra (*Equus burchelli*) faeces showing cellulolytic

capabilities (Sadhu *et al*., 2011; Memela and Schmidt, 2013). This indicates the potential of faeces as a source for the isolation of microbial isolates producing hydrolases with biotechnological capacity.

The study objectives were (i) to isolate and taxonomically characterise hydrolase positive bacteria from zebra, giraffe and impala faeces (ii) to assess the ability of isolates to utilise polymers such as cellulose, ester lipids, starch and protein over time (iii) to determine selected hydrolytic enzyme activities in crude extracts and compare these to documented enzymatic activities to verify potential industrial applicability.

4.2 Materials and methods

4.2.1 Screening and isolation of hydrolase-possessing micro-organisms

Serial dilutions of zebra, giraffe and impala faeces in peptone water (8.5 g NaCl, 0.5 g peptone (Merck) per litre) were spread-plated onto Plate count agar (PC) (5 g tryptone, 2.5 g yeast extract, 1 g glucose, 14 g agar per litre, pH 7.2); Tween 80 agar (5 g peptone, 3 g meat extract, 10 ml Tween 80, 100 mg $CaCl_2$ x $2H_2O$, 15 g agar per litre, pH 7.2); Skim milk agar (10 g skim milk powder, 3 g meat extract, 5 g NaCl, 2 g Na_2HPO_4, 15 g agar, 0.05 g bromothymol blue per litre, pH 7.2); Carboxy-methylcellulose (CMC) agar (2 g $NaNO_3$, 1 g K_2HPO_4, 0.6 g $MgSO_4$, 0.6 g KCl, 2 g carboxy-methylcellulose sodium salt, 0.2 g peptone,17 g agar per litre, pH 7.2) and Starch agar (3 g beef extract, 10 g soluble starch, 12 g agar per litre, pH 7.2). Colony counts were established after 24-48 hour incubation at 30°C. CMC and starch plates were flooded with Gram's iodine (1 g iodine, 2 g potassium iodide, 300 ml distilled water) solution to test for amylase positive colonies (which displayed a clear halo); protease positives turned plates from green to midnight blue due to casein hydrolysis which in turn alkalised the medium; esterase/lipase positives produced calcium oleate precipitate around colonies. 1% Congo red (1g Congo red (Sigma-Aldrich) in 100ml distilled water) was also used as an alternative on selected CMC agar plates. Plates were flooded with Congo red and left to stand for 10 minutes. The stain was then carefully decanted and plates were then rinsed with 1M sodium chloride solution for 10 minutes. Zones of hydrolysis were displayed by a clear halo around colonies.

Isolates able to utilise any of the four polymeric substrates (CMC, Tween 80, Starch and Skim milk) were selected and sub-cultured onto nutrient agar (Merck) as pure cultures for further analysis. Master cultures of pure isolates were prepared from overnight nutrient broth

(Merck) cultures incubated at 25°C and at 150rpm. A volume of 800 µl of broth was added to 200 µl of glycerol and stored at -20°C. 12 isolates from the four polymeric substrates which were re-culturable onto nutrient agar as pure isolates were selected for further analysis. The 12 isolates were assigned names and numbers according to their source and appearance. All isolates from zebra faecal samples were given numbers, followed by abbreviations for their colony colour on nutrient agar plates either "w" for white or "c" for cream. Isolates from giraffe faeces begin with "G" and isolates from impala begin with "I" followed by abbreviations for their colour or description.

4.2.2 Microscopy of unknown isolates

Samples of overnight nutrient agar cultures of unknown isolates were Gram-stained and examined by bright field microscopy (Zeiss Primo star). Gram stains were performed on the cultures at different stages of growth between overnight to two week old cultures to verify the results. The KOH test (Gregersen, 1978) was conducted in addition to Gram stains. Endospore stains were also conducted on Gram positive isolates (Schaeffer and Fulton, 1933). In addition, motility of isolates was determined microscopically (Zeiss Primo star) by preparing hanging drops from overnight cultures grown on nutrient agar at 30°C. Colonies from two selected isolates (11w and GW) were grown overnight at 30°C on nutrient agar and analysed by environmental scanning electron microscopy (ESEM). A Philips XL130 ESEM with Lab6 gun was used at optimally adjusted vacuum, temperature and humidity levels.

4.2.3 Biochemical tests

Catalase, oxidase, indole, methyl red, Voges-Proskauer and citrate utilisation tests were performed according to Bergey's Manual (Bergey and Holt, 1994). Fructose, galactose, glucose, lactose, maltose, sucrose and xylose utilisation were tested by growing the isolates in 1% of the individual sugars in mineral salts medium The mineral salts medium used was made up by adding 50 ml stock solution 1 (56g Na_2HPO_4 x $2H_2O$ and 20g KH_2PO_4 per litre), 20 ml stock solution 2 (25 g $(NH_4)_2SO_4$, 5 g $MgCl_2$ x $6H_2O$, 2.5 g $Ca(NO_3)$ x $4H_2O$ per litre) and 500 µl stock solution 3 (5 g EDTA, 3g Fe_2SO_4, 0.03g $MnCl_2$ x $4H_2O$, 0.05g $Co(II)Cl_2$ x $6H_2O$, 0.01 g $CuCl_2$ x $2H_2O$, 0.02 g, $NiCl_2$ x $6H_2O$, 0.03 g Na_2MoO_2, 0.05 g $ZnSO_4$ x $7H_2O$, 0.02 g H_3BO_3 per litre), made up to 1 L with distilled water and adjusted to a final pH of 7.4. Isolates were also grown on gelatine (3 g beef extract, 5 g peptone, 120 g gelatine per litre, pH 7.2) agar and on all the polymeric substrates (CMC, Tween 80, Starch and skim milk) to assess their abilities to hydrolyse these polymers.

4.2.4 MALDI-TOF MS analysis of cell extracts

Isolates were identified using MALDI-TOF MS analysis as reported (Gemmell and Schmidt, 2013). Ethanol extraction of cell biomass was done based on the manufacturer's protocol (Bruker Daltonics, Bremen, Germany).

4.2.5 Polymerase chain reaction (PCR) based amplification of the 16S rRNA gene and sequence analysis

The forward primer fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer rP2

(5' -ACGGCTACCTTGTTACGACTT-3') (Weisburg *et al*., 1991) were used for hot-start PCR based amplification of the 16S rRNA gene of all isolates.

Fresh single colonies were dissolved in 100 µl of sterile TE buffer in an Eppendorf tube and lysed by 2 cycles of boiling (10 min at 100°C) and subsequent freezing in liquid nitrogen (10 min). Tubes were thawed and centrifuged at 10000 x g for 5 minutes and the supernatant was used for PCR. Amplification of the 16S rRNA gene was done according to the procedure suggested by Weisburg *et al.* (1991).

The positive control used was *Escherichia coli (*ATCC8739) and the negative control was deionised water. PCR products were analysed on 2% agarose gels (2 g agarose in 100 ml TAE buffer (40 mM Tris, 20 mM acetic acid, and 1m M EDTA, pH 8.0)) and stained with 1 µl SYBR Safe DNA Gel stain solution). A 1 Kb DNA molecular size ladder (Promega) was used and the gel was run for 45 minutes at 100V. The gel was documented using a gel imager (G:Box, Syngene, UK) with Genesnap (version 7.09) to assess whether the amplification products were of the correct size (i.e. 1500 bp). Sequencing of PCR products was done by Inqaba Biotech (Pretoria, South Africa) and the sequences were compared to sequences deposited in GenBank (http://blast.ncbi.nlm.nih.gov). Phylogenetic trees using appropriate type strain and out-group sequences obtained from RDP (ribosomal database project, www.rdp.cme.msu.edu) were generated, based on sequence alignments established with Clustal W and the neighbour joining method using MEGA 6 (Tamura *et al.*, 2013) with 500 times resampling. The topology of the trees was verified through the maximum likelihood method for comparison.

4.2.6 Carbon source assimilation and growth curves

Growth curves for the 12 selected isolates were conducted by growing the isolates in mineral salts medium with specific polymers (1% Tween 80, 1% starch, 1% casein, 1% CMC) added

as the sole carbon source and measuring the optical density (OD) at 600nm over time using a UV Mini-1240 Shimadzu spectrophotometer and sterile mineral salts medium as the blank.

Cultures were prepared for growth experiments by culturing the 12 individual isolates in nutrient broth for 48 hours at 150 rpm at 30°C followed by sub-culturing onto mineral salts agar with 1% of one of the specific polymers each as sole carbon source at 25°C for 4 days. Mineral salts agar plates without added carbon source served as controls. In the case of CMC, 0.5% peptone was added in the pre-cultured cells to assist growth. The isolates were then grown in mineral salts medium with 1% carbon source for 4 days at 150 rpm at 25°C. The cells were then harvested by centrifugation at 10000 x g, washed with 0.9% saline and re-suspended in mineral salts medium. Mineral salts medium was prepared as specified in section *4.2.3*. Growth experiments were started by inoculating 100 ml of mineral salts medium containing 1% carbon source with up to 1 ml of cell suspension to an initial OD_{600} of 0.05-0.2. Incubation temperature was 25°C for all growth curves.

4.2.7 Cell counts

A higher initial optical density was observed in mineral salts medium containing 1% skim milk in comparison to the other carbon sources. As a result, cell counts of skim milk cultures were conducted after 24 hours for a representative isolate to confirm that the increased optical density was due to bacterial proliferation. The cell counts were conducted with a bacterial counting chamber (Helber type, Marienfeld, Germany) at 400x magnification using a phase contrast microscope (Motic BA310, China).

4.2.8 Preparation of cell lysate and supernatants

Isolates that grew best on specific polymers as carbon sources based on the growth experiments were used for enzyme analysis. One litre Erlenmeyer flasks containing 250ml of mineral salts medium and 1% organic polymer substrate were inoculated with the selected isolates and incubated at 25°C for 72 hours at 150 rpm. Cells were harvested by centrifugation at 10000 x g for 10 minutes and washed three times with 0.9% saline and the supernatant was also kept. Cells were re-suspended in 2 ml 50 mM phosphate buffer (pH 7.4) and lysed with 2 g sea sand (Merck) and liquid nitrogen for 20 minutes in a cooled mortar and pestle. The mixture was centrifuged for 10 minutes at 10000 x g to remove cell debris. The supernatant was kept and centrifuged again for 1 hour at 20000 x g. This was then stored as the cell lysate to be used for enzyme assays. Supernatants from cultures were also kept for

analysis. Both cell lysates (the crude extract used in enzyme assays) and culture supernatants were stored at -20°C. All enzyme activity measurements were conducted in duplicate.

4.2.9 Protein estimation

If required, cells were lysed with 1M NaOH at 95°C for 10 minutes and cooled on ice for 10 minutes. Protein estimation was then done through the Bradford assay (Bradford, 1976) using bovine serum albumin (BSA, Sigma Aldrich, South Africa) as protein standard.

4.2.10 Amylase activity assay

Amylase activity was determined through a 3,5 dinitrosalicylic (DNS) acid enzyme assay (Ghose, 1987). The assay was performed on isolate 7w which grew best with starch as the sole carbon source. A standard curve was constructed using glucose. The assay was amended for bacterial strains by using 1 ml 0.1 M Tris-HCl buffer at pH 7 (instead of citrate buffer at pH 4.8 as recommended for the fungus *Trichoderma*) with 200 µl of aqueous soluble starch (1%) solution and 800 µl crude enzyme extract added to a final volume of 2 ml. The assay was conducted at 37°C and 50°C on cell lysate and supernatant for 15 minutes. The reaction was arrested with addition of 3 ml of DNS (50mM) reagent added after incubation. Enzyme activity was determined spectrophotometrically (UV Shimadzu 1240) at 540 nm based on the amount of glucose produced in 15 minutes, which was quantified via the colour change from orange to red based on a calibration curve established using glucose.

4.2.11 Cellulase activity assay

The filter paper assay (50 mg) as described by Ghose (1987) was employed to measure cellulase activity of isolate IW. In addition, the DNS assay was also employed and conducted similarly to the amylase assay (section *4.2.10*) with the cell lysate and supernatant of isolate IW from impala. However, 200 µl of aqueous cellulose (1%) solution was used instead of starch solution and with only one incubation period of one hour.

4.2.12 Esterase activity assay

p-Nitrophenylacetate (*p*-NPA) and fluorescein diacetate were used as substrates to determine esterase activity. In the *p*-NPA assay, the reaction was carried out in a final volume of 1 ml containing 2.5 µmol of *p*-NPA and 200 µl of cell lysate of isolate 7w in 27 mM of phosphate buffer (pH 7.4). In the FDA assay, the reaction was carried out in a final volume of 1 ml as above but with 200 µl of lysate and 5 µmol FDA. Enzyme activity was determined spectrophotometrically by measuring the increase in absorbance over 60-120 seconds at

405 nm for ρ-NPA and at 490 nm for FDA. Both reactions were conducted at 30°C. Molar extinction coefficients used to determine activity for ρ-NPA and FDA hydrolysis were 18500 (Bowers *et al.*, 1980) and 88000 (Klonis and Sawyer, 1996) cm^{-1} x M^{-1} respectively.

4.2.13 Protease activity assay

Protease activity was measured for two isolates (9c and 11w were selected since a lot of the isolates grew well on skim milk as sole carbon source) was determined using azocasein (Sigma-Aldrich) as a protease substrate. Five ml of azocasein stock solution (10 mg per ml in phosphate buffer (50 mM, pH 7.4)) was added to 0.5 ml of cell lysate and incubated at 37°C for 20 minutes. Protease activity was measured at 400 nm as described by Kühn and Fortnagel (1993). The phosphate buffer (50 mM, pH 7.4) was used as a negative control.

4.3 Results

4.3.1 Characterisation of unknown bacterial isolates

All of the randomly selected 12 aerobic isolates were Gram positive, of which 9 were rod-shaped and 3 were coccoid (Table 7). The presence of endospores in isolate 7w as well as the other Gram positive rod shaped isolates indicated that these belong to the genus *Bacillus*.

Table 7: Colony morphology, Gram reaction and light microscopic cell morphology of unknown isolates

Isolate	Gram reaction	Motility	Colony Morphology
4w	Gram positive endospore forming rods	Motile	Off-white, circular flat
6w	Gram positive endospore forming rods	Motile	Transparent, slimy circular
7w	Gram positive endospore forming rods	Motile	Off-white, flat, circular, dull
9c	Gram positive endospore forming rods	Motile	Cream-white, flat, shiny, circular
11w	Gram positive endospore forming rods	Motile	Off-white, circular, flat
19c	Gram positive endospore forming rods	Motile	Cream-white, flat, irregular
G1	Gram positive endospore forming rods	Motile	White, flat, irregular
GF	Gram positive endospore forming rods	Motile	Flat, off-white, irregular, dull
GW	Gram positive endospore forming rods	Motile	Raised, watery, slimy, transparent
IW	Gram positive cocci in clusters	Not motile	Small, white, shiny, circular
IY	Gram positive cocci in clusters	Not motile	Shiny, bright yellow, raised, circular
IY 2/03	Gram positive cocci in clusters	Not motile	Light yellow, circular raised

Additional testing of the isolates was employed using basic characterisation tests (Table 8). Three of the Gram positive isolates that displayed microscopic properties matching those of

Bacillus spp showed positive results for both the methyl red and Voges Proskauer test which can be the case in some *Bacillus* strains (Nicholson, 2008). All of the unknown isolates were unable to utilise citrate as a carbon source and unable to produce tryptophanase, which is also common in *Bacillus* isolates. All of the isolates were catalase positive and only three were oxidase negative. All 12 isolates were negative for the KOH test, confirming Gram reaction results.

Table 8: Biochemical tests of unknown isolates after 48 hour incubation at 37°C

Isolate	VP Test	M R	Indole	Citrate	Oxidase	Catalase
4w	+	+	-	-	+	+
6w	-	-	-	-	-	+
7w	+	-	-	-	+	+
9c	-	+	-	-	-	+
11w	+	-	-	-	+	+
19c	+	+	-	-	+	+
G1	-	+	-	-	+	+
GF	+	+	-	-	+	+
GW	+	-	-	-	+	+
IW	+	-	-	-	-	+
IY	-	+	-	-	+	+
IY 2/03	-	-	-	-	+	+

Key: VP: Voges-Proskauer, MR: Methyl red); "+" positive; "-" negative.

Further testing was done on the isolates to characterise them according to their usage of different sugars as specified in Table 9. Gas was produced by all the isolates recorded as positive. Isolates IW, IY and IY 2/03 were only positive for glucose utilisation, whilst no gas or acid was produced from the other sugars. In comparison, all the rod-shaped isolates were able to utilise glucose and at least one other sugar.

Table 9: Carbohydrate utilisation tests of unknown isolates

Isolate	Fructose	Galactose	Glucose	Lactose	Maltose	Sucrose	Xylose
4w	+	-	+	-/+	+	+	-
6w	+	-	+	-	-	-	-
7w	+	-	+	-	-	+	-
9c	+	-	+	-	-	+	+
11w	+	-	+	+	-	+	-
19c	-	-	+	-	-	+	-
G1	-	-	+	-	-	+	-
GF	-	-	+	-/+	-	+	-
GW	-	-	+	-	-	+	-
IW	-	-	+	-	-	-	-
IY	-	-	+	-	-	-	-
IY 2/03	-	-	+	-	-	-	-

-/+: gas produced with slight colour change +: yellow colour with gas produced -: red colour with no gas produced.

The isolates were then analysed for their ability to hydrolyse different polymer substrates of interest to select for the specific hydrolases (Table 10). Most of the isolates with the exception of IY hydrolysed three or more polymers. The ability of all the isolates to utilise skim milk and starch as substrates shows that proteases and amylases are produced by these isolated bacteria. Gelatine is also utilised via proteases and it was therefore not unexpected that most of the microbes that could utilise skim milk are also able to utilise gelatine. Cellulase activity was expected since these bacteria were isolated from animals with a forage diet, which is rich in cellulose material and should therefore select for microorganisms targeting cellulose. Nine out of twelve isolates hydrolysed Tween 80 and all the isolates showed the presence of at least two different hydrolase types.

Table 10: Hydrolysis of selected polymer substrates according to agar based analysis by the unknown isolates

Isolate	Skim milk	Cellulose	Starch	Tween 80	Gelatine
4w	+	+	+	+	+
6w	+	+	+	-	-
7w	+	+	+	+	+
9c	+	+	+	+	+
11w	+	+	+	+	+
19c	+	+	+	-	-
G1	+	+	+	+	+
Gf	+	+	+	+	+
GW	+	+	+	+	+
IW	+	+	+	+	-
IY	+	-	+	-	-
IY 2/03	+	+	+	+	-

The following figure 13 demonstrates a positive result for hydrolase activity on CMC agar by isolate 7w.

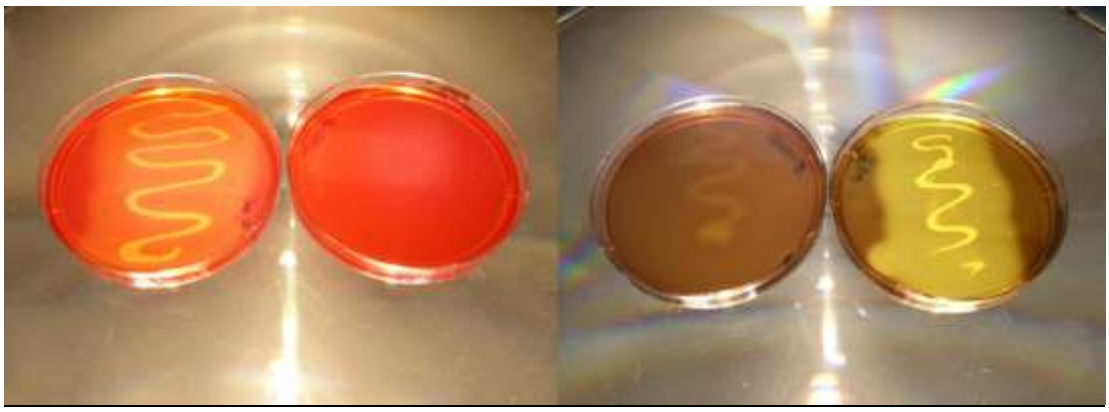


Figure 13: Cellulose hydrolysis by isolate 7w on CMC agar plates stained with Grams iodine (right) and 0.1% Congo Red stain (left) after 48 hour incubation. *E. coli* served as a negative control.

The results from all the tests were used to provisionally assign the isolates taxonomically using Bergey's manual of determinative bacteriology (1994) (Table 11). Not unexpectedly, the closest match for the Gram positive endospore forming rods was the genus *Bacillus* with

no conclusive species identification possible for most of them based on the limited number of characteristics assessed. The closest presumptive match for the isolated Gram positive cocci was the genus *Micrococcus* with no identification possible at species level.

To enable a more reliable taxonomic assignment of the isolates, further testing of the isolates was conducted via MALDI-TOF MS analysis. The highest scores of the isolates and their closest matches in the Bruker spectral database are presented in Table 11. The scores between 2.3 and 3 indicate a high probability of species level identification whilst scores between 2 and 2.299 indicate probable species identification and secure genus identification. Scores between 1.7 and 1.999 indicate probable genus identification. Most of the isolates were securely identified up to genus level based on the MALDI-TOF MS results except for isolates 6w, G1 and GF. Most of the isolates from zebra and giraffe appear to be from the genus *Bacillus* whilst those from impala appear to be isolates from the genus *Arthrobacter*, although the identification for isolates 6w, GF and G1 is not reliable at genus level. The MALDI-TOF MS findings confirm the initial results for the rod shaped endospore forming Gram positives as members from the genus *Bacillus*.

Table 11: MALDI-TOF MS results of unknown isolates, obtained through analysis of ethanol extracts and closest isolate match based on Bergey's manual of determinative bacteriology.

Isolate	Highest Score	MALDI-TOF MS Closest species match	Bergey's manual closest match based on biochemical tests
4w	2.292	*Bacillus licheniformis*	*Bacillus cereus*
6w	1.947	*Bacillus valismortis*	*Bacillus* spp.
7w	2.362	*Bacillus subtilis*	*Bacillus pumilis*
9c	2.166	*Bacillus cereus*	*Bacillus* spp.
11w	2.31	*Bacillus licheniformis*	*Bacillus* spp.
19c	2.21	*Bacillus subtilis*	*Bacillus polymyxa*
G1	1.98	*Bacillus valismortis*	*Bacillus* spp.
GF	1.807	*Bacillus mojavensis*	*Bacillus* spp.
GW	2.013	*Bacillus mojavensis*	*Bacillus* spp.
IW	2.35	*Arthrobacter chlorophenolicus*	*Micrococcus* spp.
IY	2.307	*Arthrobacter gandavensis*	*Micrococcus* spp.
IY 2/03	2.096	*Arthrobacter gandavensis*	*Micrococcus* spp.

Samples of overnight colonies of two randomly selected isolates, (11w and GW) were selected for further analysis by electron microscopy to verify the cell morphology observations made by light microscopy. Both isolates had been assigned to the genus *Bacillus* based on biochemical tests and MALDI-TOF MS analysis. Both isolates were rod-shaped confirming the earlier findings and measured about 1.3 (11w) and 1.7µm (Gw) in length.

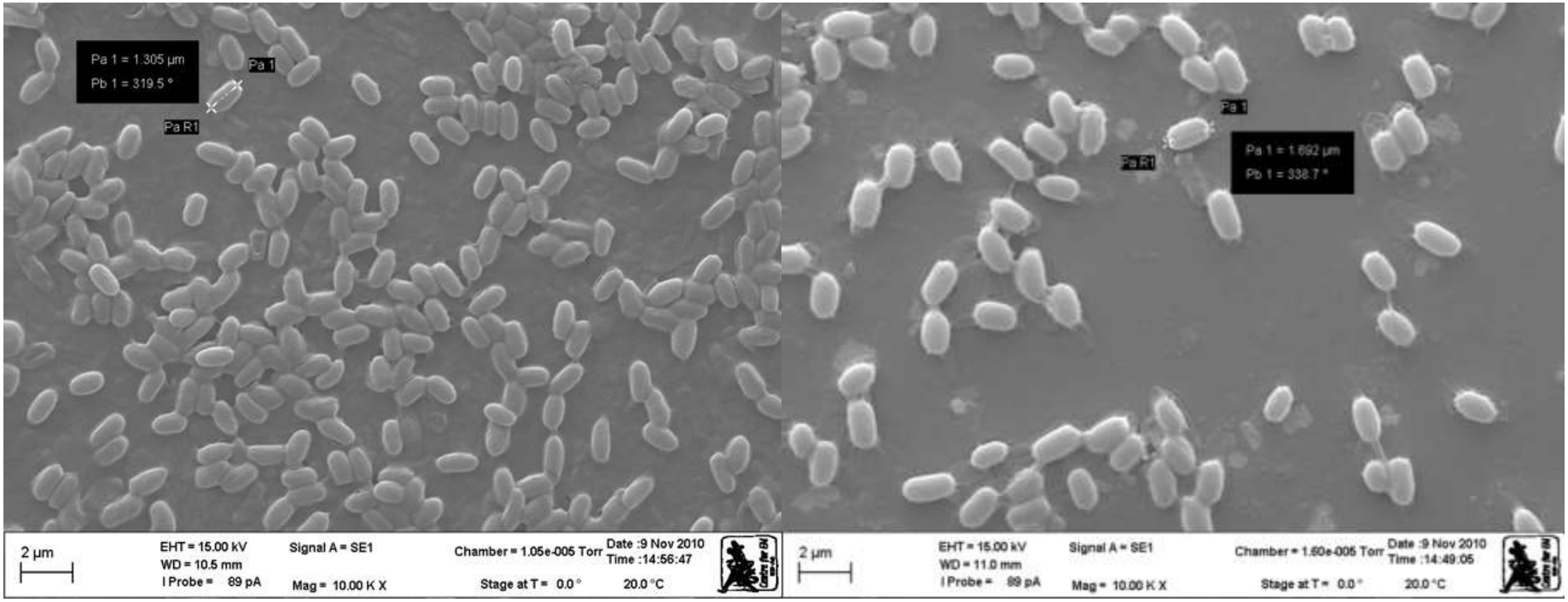


Figure 14: Scanning electron micrographs of cells of isolates 11w (left) and GW (right) from colonies grown overnight on nutrient agar at 30°C.

Isolates were further identified through sequence analysis of the amplified 16S rRNA gene. The 16S rRNA gene sequences of the isolates were compared to sequences deposited in NCBI-Genbank by using Megablast. The best matches based on sequence similarity and the E-values are

shown in table 12. The results confirmed for most the isolates the most probable assignment at genus level based on the MALDI-TOF MS analysis with the exception of isolate IY 2/03, which was not securely identified at genus level, with a 90% similarity to *Arthrobacter* and IY, which showed a 96% sequence similarity to the genus *Clavibacter*.

Table 12: Comparison of the 16S rRNA gene sequences of isolates to sequences deposited in GenBank.

Isolate	GenBank accession number	Description	Percentage similarity	E-Value
4w	NC 009725.1	*Bacillus amyloliquefaciens* FZB42, complete genome	99%	0.0
6w	NC 000964.3	*Bacillus subtilis* subsp. *subtilis* str. 168 chromosome, complete	99%	0.0
7w	NC 000964.3	*Bacillus subtilis* subsp. *subtilis* str. 168 chromosome, complete genome	99%	0.0
9c	NC 004722.1	*Bacillus cereus* ATCC 14579, complete genome	100%	0.0
11w	NC 006322.1	*Bacillus licheniformis* DSM 13= ATCC 14580 chromosome complete genome	99%	0.0
19c	NC 004722.1	*Bacillus cereus* ATCC 14579, complete genome	99%	0.0
G1	NC 009725.1	*Bacillus amyloliquefaciens* FZB42	99%	0.0
GF	NC 014639.1	*Bacillus atrophaeus* 1942 chromosome, complete genome	99%	0.0
GW	NC 00964.3	*Bacillus subtilis* subsp. *subtilis* str. 168 chromosome complete genome	99%	0.0
IW	NC 011886.1	*Arthrobacter chlorophenolicus* A6 chromosome, complete genome	99%	0.0
IY	NC 009480.1	*Clavibacter michiganensis subsp. michiganensis* NCPPB 382 chromosome, complete genome	96%	0.0
IY2/03	NC 008711.1	*Arthrobacter aurescens* TC1, complete genome	90%	0.0

Accessed 21/11/2014

To assess the phylogenetic relationship of the different *Bacillus* spp. isolates (filled circles), a phylogenetic analysis was conducted with selected *Bacillus* spp. type strains reference sequences (open circles) and with *Lactobacillus* as an out group (filled square).

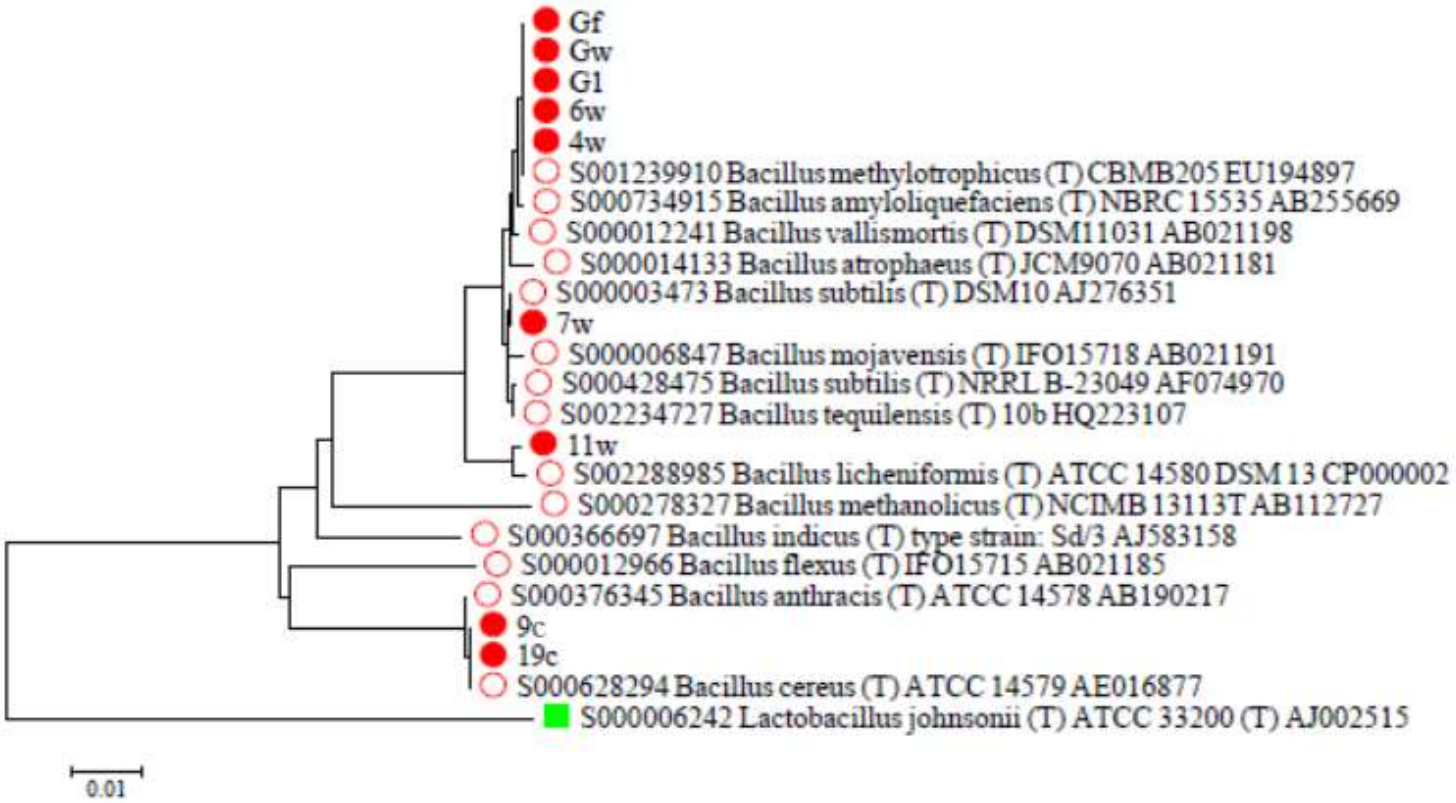


Figure 15: Phylogenetic affiliation of the isolates 4w, 6w, 7w, 9c, 11w, 19c, G1, GF and GW (dark circles) based on the comparison of their 16S rRNA gene sequence with selected 16S rRNA gene sequences for selected type strains of the genus *Bacillus* (open circles). The alignment of selected sequences and the construction of the tree are specified in the Materials and Methods (4.2.5). The scale bar represents one estimated change per 100 nucleotides. *Lactobacillus johnsonii* was used as an out group (square).

Phylogenetic assignment of two isolates IW, and IY 2/03 was done using *Arthrobacter* type strain sequences and *Propionibacterium* as the out group (Fig 16).

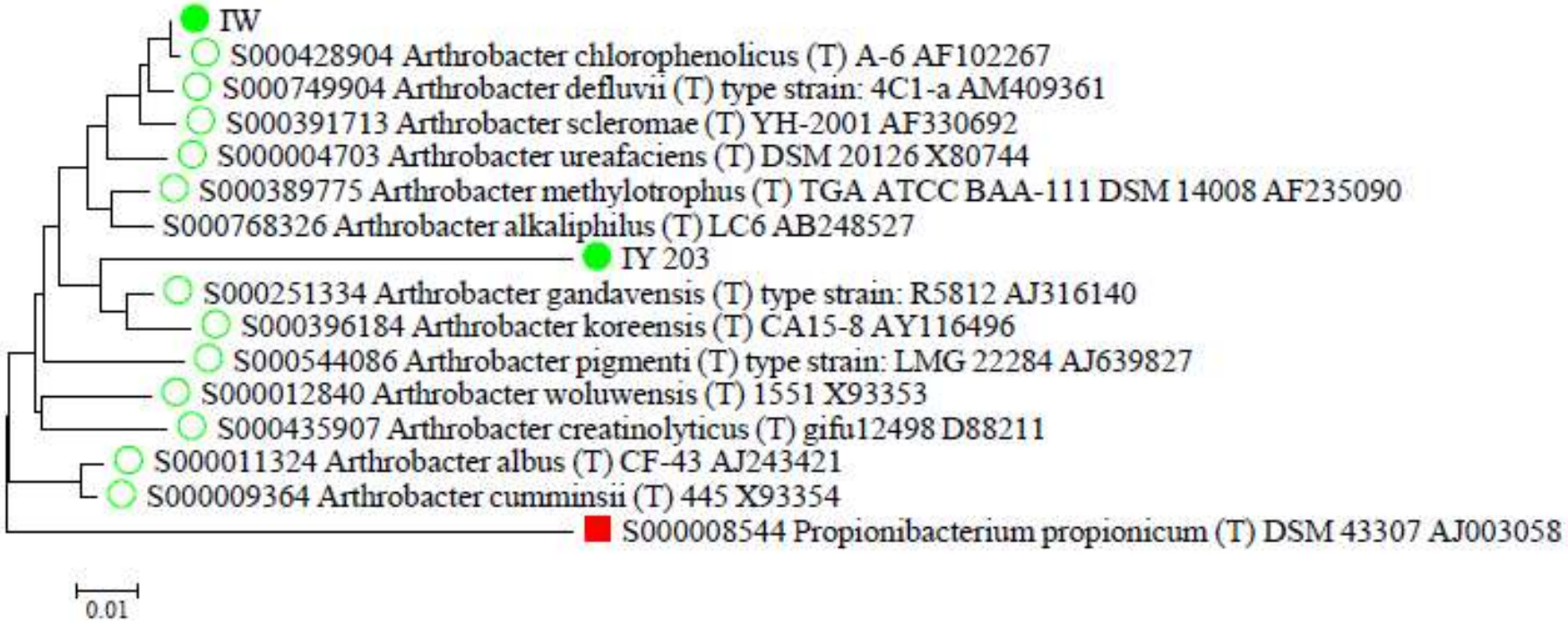


Figure 16: Phylogenetic affiliation of the isolates IW and IY 2/03 (dark circles) based on the comparison of their 16S rRNA gene sequence with selected 16S rRNA gene sequences for type strains of the genus *Arthrobacter* (open circles). The alignment of selected sequences and the construction of the tree are specified in the Materials and Methods (4.2.5). The scale bar represents one estimated change per 100 nucleotides. *Propionibacterium propionicum* was used as an out group (filled square).

The phylogenetic relationship of isolate IY (filled diamond) was conducted with selected type strains (open diamonds) reference sequences from different genera, based on 16S rRNA gene sequence analysis similarities, with *Propionibacterium* as an out group (filled square) (Fig 16).

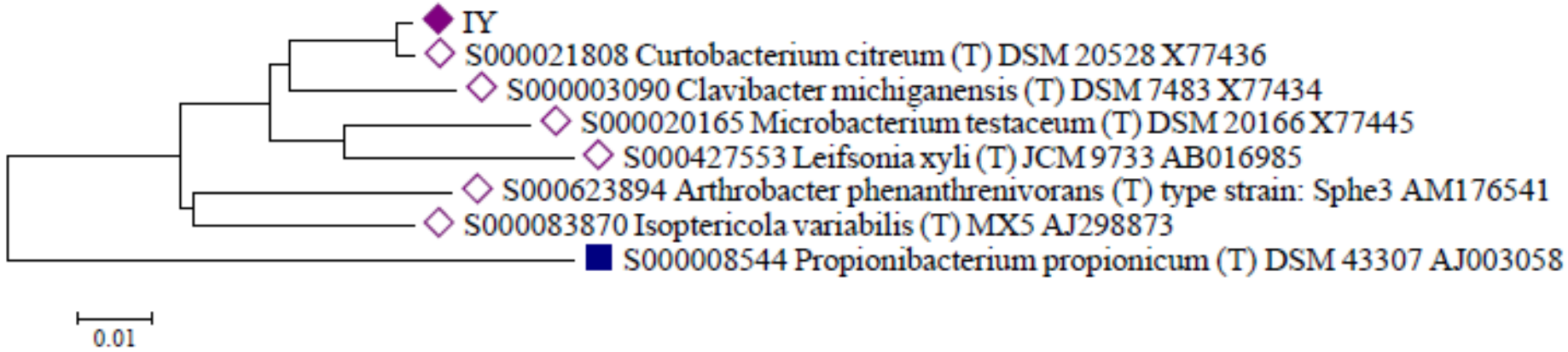


Figure 17: Phylogenetic affiliation of isolate IY (dark diamond) based on the comparison of their 16S rRNA gene sequence with selected 16S rRNA gene sequences for type strains of the genus *Clavibacter, Leifsonia, Microbacterium, Arthrobacter, Isoptericola* and *Curtobacterium* (open diamonds). The alignment of selected sequences and the construction of the tree are specified in the Materials and Methods (4.2.5). The scale bar represents one estimated change per 100 nucleotides. *Propionibacterium propionicum* was used as an out group (filled square).

4.3.2 Use of polymers as sole carbon sources

Once the isolates were characterised, their ability to utilise the various polymers as a sole carbon source was analysed via growth curves. This involved the measurement of biomass increase (OD 600 nm) of individual isolates over time in mineral salts medium containing only the polymer of interest as source of carbon. Bacteria were pre-cultured in the same medium to induce enzyme production before being subjected to growth curve analysis. The isolates producing the highest OD_{600} within the incubation period were selected as the best growing isolates. Although all 12 isolates were able to utilise starch in the screening tests (Table 10), only 10 showed increasing biomass over time based on optical density measurements in mineral salts medium. As a result, only these 10 isolates were used for growth experiments (Fig 18). Isolate 7w, a *Bacillus* sp., grew best in comparison to all the other isolates, reaching a maximum optical density of about 1.3 at 600 nm after 5 days, with a doubling time of about 19 hours incubation (Fig 18).

The control was inoculated with cells from isolate 7w, to the same starting optical density as used in the growth curves but lacking starch as a sole carbon source. The optical density data over time show that there was no increase in biomass over time, confirming that biomass increase was due to hydrolysis and subsequent utilisation of starch.

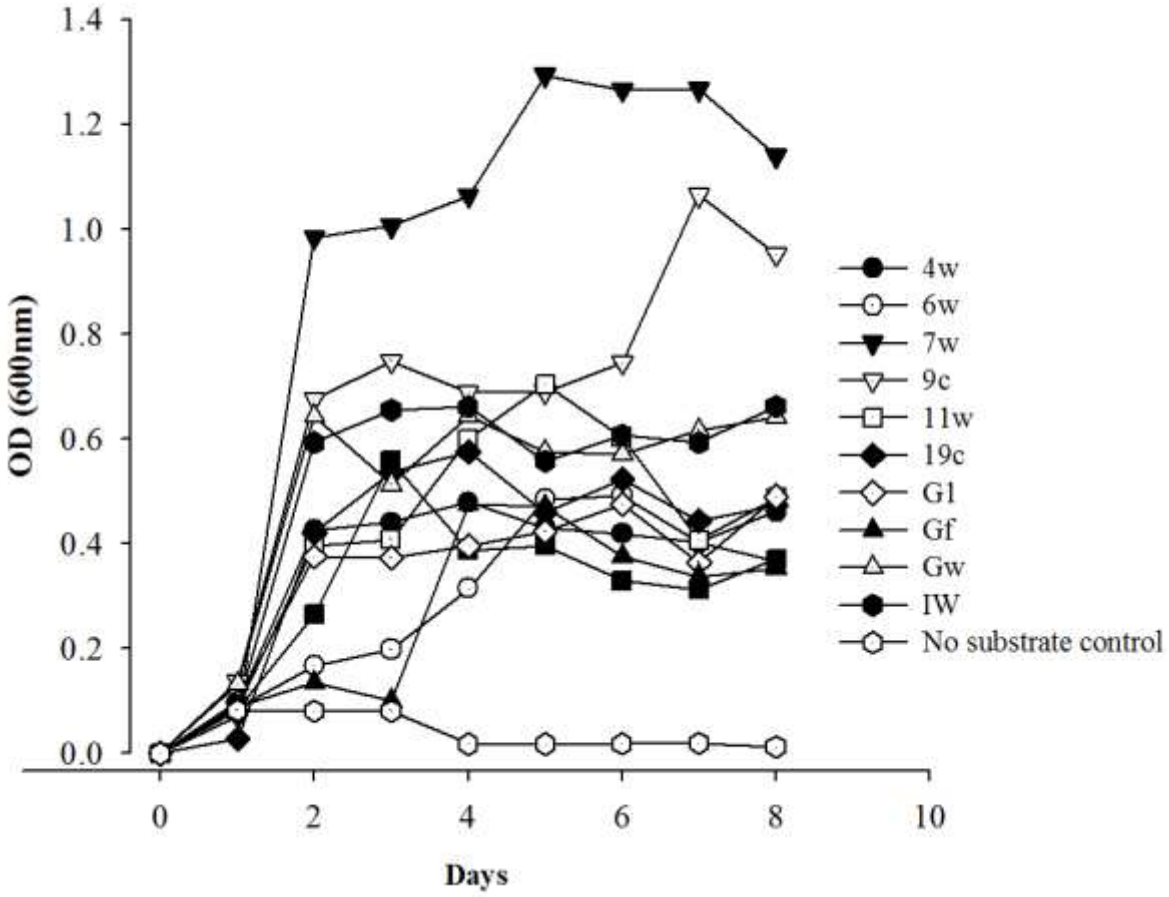


Figure 18: Growth of 10 selected isolates on starch as sole carbon and energy source over 8 days at 25°C and at 150 rpm

Only 4 (7w, 9c, 11w, IY 2/03) of the 8 isolates screened as positive for Tween hydrolysis (Table 10) were able to grow on Tween 80 as the sole carbon source in mineral salts medium based on OD_{600} measurements over time (Fig 19), with 3 out of the 4 Tween 80 utilising isolates reaching stationary phase after 12-15 hours, and isolate IY 2/03 reaching stationary phase after 18 hours. Isolate 7w and IY 2/03 showed doubling times of about 3.5 and 3 hours respectively and the highest biomass concentration. The "no substrate control" consisted of isolate 7w in mineral salts medium without any carbon source confirming again that increase in biomass was due to utilisation of Tween 80.

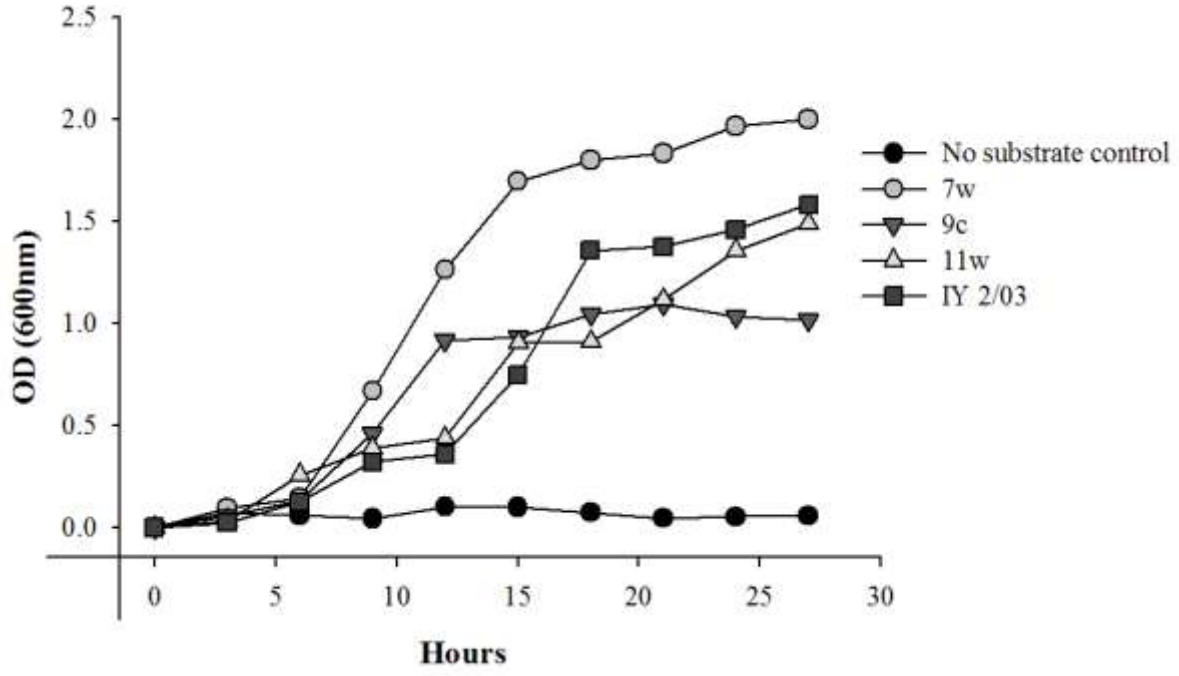


Figure 19: Growth of 4 selected isolates on Tween 80 as sole carbon and energy source over 27 hours at 25°C and at 150 rpm

Growth on skim milk as the sole carbon source (Fig 20) showed biomass formation for all isolates and that the stationary growth phase was not reached after 30 hours for all isolates. Isolate 9c was selected as the best growing isolate based on its doubling time (5.7 hours) and the highest final biomass reading at OD_{600}, with isolate 11w having a similar doubling time of 5.7 hours.

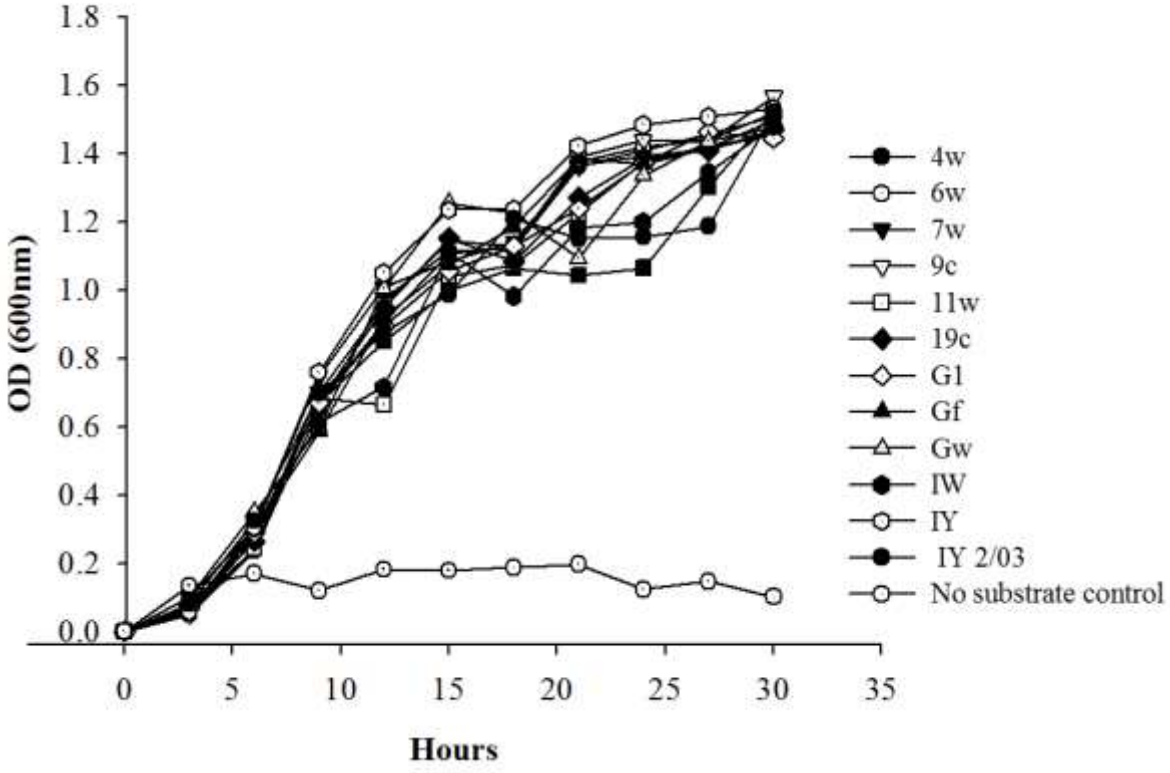


Figure 20: Growth of isolates on skim milk as sole carbon and energy source over 30 hours at 25°C and at 150 rpm.

To verify the utilisation of CMC in mineral salts medium (Fig 21), the starting optical density (600nm) was adjusted to 0.1 but could not be accurately measured over time as cells began to form clumps in the medium. As a result, changes in protein concentration were measured instead. Protein concentrations of each of the selected isolates were measured on Day 0 and Day 7 of the 7 day incubation period. Controls without substrate had lower protein concentrations in comparison to the isolates that had cellulose as a carbon source. Although 11 isolates were able to hydrolyse cellulose in the screening tests (Table 10), isolate 19c did not show any increase in OD_{600} measurements during growth experiments. The increase in protein concentration within 7 days indicated that isolates were utilising CMC as a sole carbon source. The highest protein concentration after 7 days was detected in isolate IW; which was then selected for further enzymatic analysis.

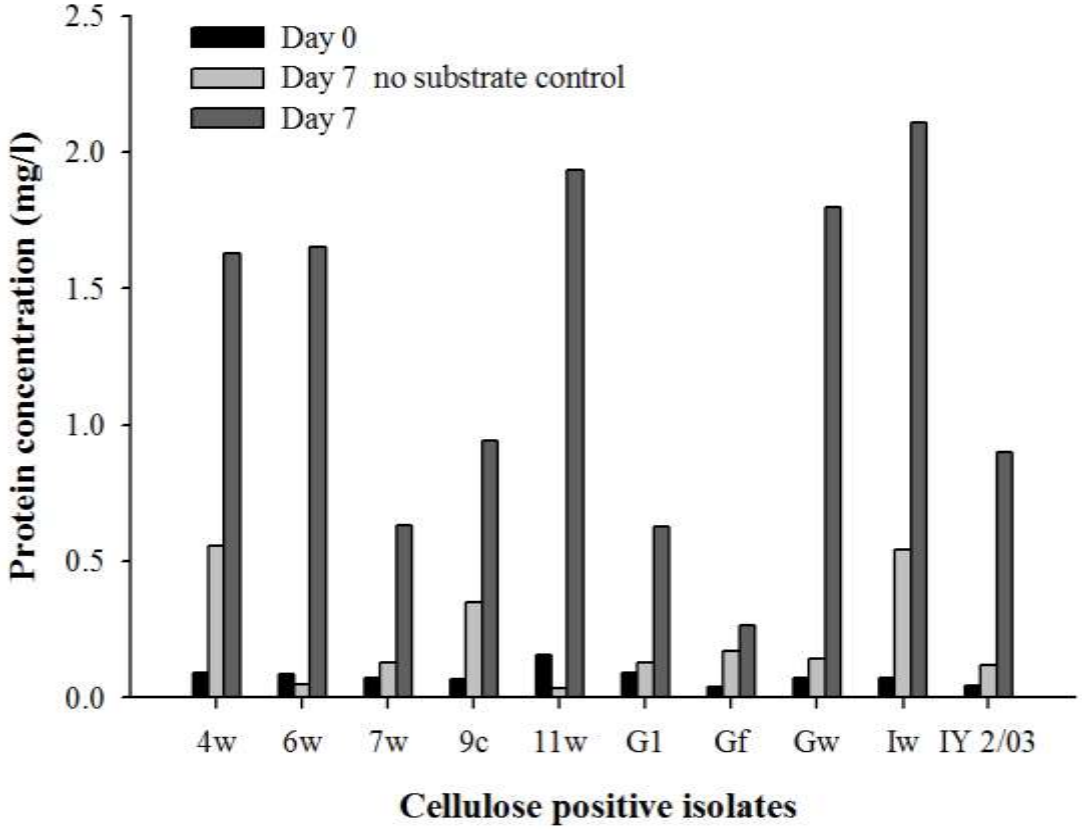


Figure 21: Protein concentration in cultures of selected isolates at day 0 and at day 7 when grown with CMC as the sole carbon and energy source at 25°C and 150 rpm.

4.3.3 Enzyme activity assays

To limit the determination of specific hydrolytic enzyme activities to a manageable number given that 12 isolates were available, only a few isolates were selected for each hydrolytic enzyme to be tested based on the highest optical density at the end of the incubation period and smallest doubling time or highest yield in protein concentration.

4.3.3.1 Specific amylase activity

The specific activity for amylase of isolate 7w was measured (Table 13) in the supernatant and crude cell extract. As expected, the specific amylase activity was higher in the crude cell extract than in the supernatant. Given the measured protein concentration of 4.16 mg/ml in the cell lysate and 0.51 mg/ml in the supernatant, the difference in enzyme activity might be due to a lower concentration of amylase in the supernatant as no further concentration treatment like precipitation and column chromatography was done. The incubation

temperature that yielded the highest specific enzyme activity was found to be 50°C for cell lysate with a specific activity of 160 nmol x min^{-1} x mg^{-1}.

Table 13: Specific amylase activity detected in cell lysate and supernatant of isolate 7w after growth on starch, using the DNS assay.

Crude enzyme source	Assay temperature	Specific amylase activity (nmol x min^{-1} x mg^{-1})
Cell lysate	37°C	87
Supernatant	37°C	36
Cell lysate	50°C	160
Supernatant	50°C	25

All results are means of two independent experiments

4.3.3.2 Specific cellulase activity

The specific cellulase activity of isolate IW was assessed after growth on mineral salts medium supplemented with 1% of CMC. Cellulolytic activity was only found in cell lysate of isolate IW at 37°C with a specific activity of 297 nmol x min^{-1} x mg^{-1}. The hydrolytic activity at 50°C was below the detection limit with the obtained enzymatic activity being lower than 1 nmol x mg^{-1} x min^{-1}.However, no activity (below 1 nmol x mg^{-1} x min^{-1}) could be measured using culture supernatant and filter paper as an assay substrate at both temperatures.

Table 14: Specific cellulase activity detected in cell lysate and supernatant of isolate IW after growth on cellulose, using the DNS assay.

Crude enzyme source	Temp (°C)	Specific cellulase activity (nmol x min^{-1} x mg^{-1})
Cell lysate	37	297
Supernatant	37	<1
Cell lysate	50	<1
Supernatant	50	<1

All results are means of duplicate independent experiments

4.3.3.3 Specific esterase activity

Specific esterase activity in crude extract from isolate 7w was assayed using *p*-nitrophenylacetate (*p*-NPA) and fluorescein diacetate (FDA) as substrates (Table 15). The specific esterase activity obtained with *p*-NPA as assay substrate was higher than that obtained with FDA as assay substrate.

Table 15: Specific esterase activity determined in crude extract of isolate 7w after growth on Tween 80.

Assay substrate	Specific activity (nmol x min^{-1} x mg^{-1})
p-NPA	1431
FDA	325

All results are means of duplicate independent experiments

Azocasein was used to measure protease activity in crude extracts of isolate 9c and 11w, which were grown on skim milk as a sole carbon and energy source (Table 16). The specific protease activity in crude extract of isolate 9c was about 9 fold higher than that established for isolate 11w.

4.3.3.4 Specific protease activity

Table 16: Specific protease activity detected in crude extracts of isolate 9c and 11w after growth on skim milk.

Cell lysate source	Specific activity (nmol x min^{-1} x mg^{-1})
9c	933
11w	100

All results are means of duplicate independent experiments.

4.4 Discussion

4.4.1 Microbiological characterisation of isolates

Twelve randomly selected faecal microbial isolates with the ability to hydrolyse cellulose, starch, tween 80 and skim milk were characterised and targeted for subsequent enzyme analysis. The initial steps in the identification of these selected isolates involved their Gram stain reaction, cell morphology and colony morphology (Table 7). The Gram stain has been described as the primary step in the identification and classification of cells in bacteriology (Bartholomew and Mittwer, 1952). As many Gram positive species stain Gram variable or even Gram negatively after being exposed to starvation or heating (Bartholomew and Mittwer, 1952), in this study, overnight cultures of the isolates were used for Gram staining to obtain reliable stains. It is generally recommended that isolates be stained at different stages of growth to determine the validity of the Gram reaction obtained for unknown cells (Bartholomew and Mittwer, 1952), which was done in this study and confirmed all that the isolates were indeed Gram positive. The Gram stain results were confirmed by the KOH test

which lyses the cell wall of the Gram negative bacteria thereby liberating a viscous thread of DNA from the cell but not from the thicker cell wall of Gram positive bacteria. (Gregersen, 1978, Halebian *et al*., 1981). All the tested isolates were negative for the KOH test, supporting the Gram stain findings.

Part of the initial analysis was observation of colony morphologies of each isolate on nutrient agar plates. With the advancement of technology, colony morphology has moved from being one of the essential characterisation methods to a useful complementary technique, applied for the detection of typical colony morphologies usually on differential and selective media (Sousa *et al*., 2013). However, colony morphology has been found to change based on the age of the isolates as well as the type of medium they are cultured on. Colony morphology is also reflective of stress and mutations within an isolate but adds meaningful value when the typical colony morphology of an isolate is known. The colony morphology of most of the isolates analysed in this study showed typical characteristics as described for *Bacillus* species such as fluffy colonies and round watery colonies which was reported by Roberts (1935), Nazina *et al.* 2001 and Di Franco *et al*. (2002).

The isolates were all Gram positive with 3 of the isolates exhibiting coccus shaped cells while the other 9 formed rod-shaped cells (Table 7). Isolate 4w and 7w displayed growth forms similar to those described for *Bacillus* species, such as visible swarming on plates after 48 hour incubation (Shapiro, 1995).

A number of biochemical tests were performed on the isolates as a means to achieve further taxonomic classification (Table 8). All the isolates were unable to produce tryptophanase (indole test) which supports that they are Gram positive isolates, as these are less able to utilise amino acids compared to gram negative isolates (Bartholomew and Mittwer, 1952; Logan and Berkeley, 1984). As expected, all 12 isolates were catalase positive given that they were all able to grow aerobically. No testing for facultative anaerobes was done. Tables 9 and 10 showed that the isolates utilised some simple sugars (e.g. glucose and fructose) as well as complex polymers (e.g. cellulose and starch). Table 10 further showed that most of the selected isolates were able to hydrolyse more than one of the targeted polymeric substrates used.

In this study, the hydrolysis of cellulose was demonstrated primarily by using iodine instead of Congo red staining as recommended by Kasana *et al.* (2008). The excellent visibility of

hydrolysis zones using this approach is demonstrated in figure 13 for strain 7w. Not only was there an increased visibility when using Gram's iodine, but the waiting time was reduced as well. Preliminary tests in this study compared both the Congo red and Grams iodine stain and found that the Congo red stain took 20 minutes and required rinsing with sodium chloride, as opposed to 5 minutes of staining with Grams iodine. Staining with Grams iodine also enabled enumeration of cellulose hydrolysing colonies in earlier screening tests. This supports the earlier findings of Kasana *et al.* (2008) that Grams iodine is a more efficient stain for the detection of cellulose hydrolysis.

Based on cell and colony morphological data and selected biochemical test results (Table 7 to 10), the isolates were assigned to presumptive taxonomic ranks based on the key characteristics reported for the genus *Bacillus* which are endospore-formation, aerobic/facultatively anaerobic growth, presence of catalase, inability to utilise citrate, polymer hydrolysis, indole test and Gram stain (Bartholomew and Mittwer, 1952; Vary, 1994; Slepecky and Hemphill 2006, Logan *et al.,* 2009).

The rod shaped isolates were grouped into the *Bacillus subtilis* group II of the genus *Bacillus* based on their biochemical test results, such as the hydrolysis of starch and casein, gelatine liquefaction (with the exception of 6w and 19c) and motility which is typical for members of this taxonomic group (Nazina *et al.*, 2001; Reva *et al.*, 2001; Saman *et al.*, 2010). The isolation of Bacillus spp. from the faeces of wild ungulates in this study is in line with reports for faeces from other animals such as insects (Stahly *et al.,* 2006), cattle (Wu *et al.,* 2006), horses (Altayar and Sutherland, 2006) and seahorses (Balcázar *et al.,* 2010) yielding *Bacillus* spp. isolates. The ability of the isolates to hydrolyse different polymers tested demonstrates the potential of faecal matter from indigenous animals as a source for microbial hydrolytic enzymes for biotechnological applications, with the genus *Bacillus* widely known for industrial applications (Reva *et al.,* 2001).

The rod shaped Gram positive isolates were all assigned to the genus *Bacillus* based on cell morphologies and biochemical test results. However, due to the high biochemical similarity of many *Bacillus* species (Bergey and Holt, 1994; Reva *et al.,* 2001; Koeppel *et al.,* 2008; Logan and De Vos, 2009), identification to species level is not always reliable with biochemical tests. For example, results for isolate 7w showed that it is unable to utilise citrate and hydrolyses starch, which does not enable a reliable assignment to *Bacillus pumilis*. However, the additional analysis of the 16S rRNA gene sequence as well as MALDI-TOF

MS analysis confirmed the assignment of all the rod shaped endospore forming isolates to the genus *Bacillus*.

Three of the twelve isolates (IW, IY and IY 2/03) were mostly present in coccoid form when analysed by light microscopy, which explained the presumptive assignment to the genus *Micrococcus* based on Bergey's manual (Table 11) However, 16S rRNA gene sequence analysis of these isolates (Table 12) showed one isolate similarity to the genus *Arthrobacter* (IW), and two isolates were not securely identified to genus level (IY and IY 2/03) while MALDI-TOF MS analysis classified all three isolates into the genus *Arthrobacter*. *Arthrobacter* species are generally characterised as a group of coryneform, aerobic bacteria that are rod-shaped. Cells can be characterised either as rod or coccoid depending on the age of cultures analysed. In a previous study by Jones and Keddie (2006), the cell morphology for *Arthrobacter globiformis* was coccoid after 6 hours of growth and rod-shaped after 12 to 24 hours. Cells morphed into coccoid to rod and back to coccoid cells over a period of 3 days (Jones and Keddie, 2006). The three isolates (IW, IY and IY 2/03) monitored in this study were assessed from overnight cultures and mostly appeared in the coccoid form. This bacterial genus is often confused with *Corynebacterium* and *Brevibacterium* species, due to their similarities in colony morphology and enzyme activity in biochemical tests (Funke *et al*., 1996). Isolates IY and IY 2/03 matched previous findings reported for *Arthrobacter* isolates, with no motility, catalase positive and unable to utilise simple sugars. *Arthrobacter* strains have also been reported to have no amyloytic capability. The isolates in this study were catalase positive and were capable of utilising more than one of the polymers. This is contrary to a previous report on the genus by Jones and Keddie (2006) but has been reported for certain isolates before (John and Hampel, 1992).

4.4.2 MALDI-TOF MS

The use of mass spectrometry in the identification of unknown bacterial isolates is a potential alternative to traditional biochemical identification methods. Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is a method of bacterial identification that generates cellular fingerprints based on mass fragments related mostly to ribosomal proteins to identify bacteria at genus or even species level (Ruelle, 2004; Hausdorf *et al*., 2012). MALDI-TOF MS was successfully used in this study to assign the unknown bacterial isolates based on ethanol extracts to genus level (Table 11). Ethanol

extraction is necessary to penetrate the thick peptidoglycan cell wall in order to obtain measurable proteins and peptides (Smole *et al*., 2002).

In environmental microbiology, MALDI-TOF MS is currently not as common as biochemical and molecular identification methods but can be accurate in its identification of isolates given that they are contained in the mass spectral library used for taxonomic identification. However, these libraries are primarily targeting clinical microorganisms, which has been noted as a drawback when identifying unknown environmental isolates (Vargha *et al*., 2006). MALDI-TOF MS analysis of whole cells is a more recent approach and reproducible results are not always obtained. This is due to factors such as pre-analysis preparation methods, the matrix used and the number of cells spotted (Williams *et al*., 2003). The isolates used in this study were analysed by extracting biomass from overnight colonies with ethanol. Despite the variations in methods, taxonomic identification by MALDI-TOF MS has been found to be an alternative that can be equally efficient as PCR based identification (Hausdorf *et al*., 2012). However, in this study based on 16S rRNA gene sequence analysis isolate IY was not securely assigned to genus level, whilst based on MALDI-TOF MS it was placed into the genus *Arthrobacter*, showing that there can be inconsistencies between the two identification methods. Nevertheless, most of the isolates (9 out of 12) were reliably assigned to genus level and some (four) even to species level by MALDI-TOF MS (Table 11), with the exception of isolate 6w, G1 and GF, which were not reliably identified at genus level. Commonly, low scores are obtained if the isolates are not covered in the library database. Also, isolates that are closely related are usually scored only to genus level if the identification at species level is not reliable. Identification becomes complicated if there are many varied matches at genus and species level thereby yielding a potentially unreliable result (Khot *et al*., 2012). This may explain the assignment only to genus level for isolate 6w and GW. This might happen with *Bacillus* isolates as they are difficult to differentiate between closely related species (Logan and Berkeley, 1984) such as those within the *Bacillus cereus* group (Økstad and Kolstø, 2011). This may explain the assignment only to genus level for isolate 6w, GI and GF.

The expansion of existing MALDI-TOF MS library databases to incorporate more species has been reported as an ongoing challenge, however, it has been used to successfully classify environmental *Arthrobacter* isolates in previous studies (Vargha *et al*., 2006) and was successfully applied in this study with respect to one isolate (IW). Comparing taxonomic assignments based on biochemical tests and MALDI-TOF MS analysis, Table 11 shows that

with the exception of the Gram positive cocci (IY, IY 2/03 and IW), isolates were similarly assigned to the genus *Bacillus*. In fact, isolates that could not be assigned reliably to genus level via MALDI-TOF MS were also not clearly identified based on the analysis of morphological features and results obtained from biochemical tests.

4.4.3 Sequence analysis

PCR was used to amplify the 16S rRNA gene sequence and the taxonomical assignment of isolates based on sequence similarity scores obtained using GenBank was also compared to the results from MALDI-TOF MS and biochemical tests (Table 12). 16S rRNA gene sequencing is a well-established method of bacterial identification. This is based on the principle that the 16S rRNA gene of the prokaryotes contains highly conserved regions and can be used to analyse phylogenetic relations between prokaryotic species (Woese, 1987). Over the years this has led to the identification of many slow growing and poorly staining bacterial species (Drancourt and Raoult, 2005). The primers employed in this study which were published by Weisburg *et al* (1991), are well-established and have been used in previous studies for the characterisation of environmental isolates from animal dung (Memela and Schmidt, 2013), for characterisation of marine bacteria (Moxley and Schmidt, 2012) as well as for the assessment of bacterial diversity in the rumen of cows and sheep (Wood *et al*., 1998).

In this study, based on the suggested genus level similarity threshold for the 16S rRNA gene of 97% (Drancourt *et al.,* 2004), a10 of the isolates were identified to genus level. The low E-(expect) values indicate that the matches shown for the unknown isolates are highly "significant". E-values were reported to serve as confidence measure of sequence similarity, with a lower E-value indicating a higher likelihood and hence a higher confidence in isolate identification (Kerfeld and Scott, 2011).

In the case of the genus *Bacillus*, sequence based species assignment is not straightforward as even different Bacillus species can display a >99% sequence similarity (Clarridge, 2004; Logan *et al.,* 2009). Therefore isolates can usually only be assigned reliably to genus level and grouped based on their similarity into one of several *Bacillus* RNA groups within the genus *Bacillus*. For example, *Bacillus anthracis* and *Bacillus thurigenesis* have a 99% similarity in 16S rRNA gene sequence similarity (Sacchi *et al.,* 2002), although one is a human pathogen and the other is an insect pathogen, both can be distinguished phenotypically

via different colony morphologies and susceptibilities to antibiotics (Sacchi *et al.,* 2002). Specific biochemical tests and more importantly DNA-DNA hydridisation have to be performed to further differentiate the isolates and enable assignment to species level. Another method discussed for the differentiation of *Bacillus* isolates is ecotype stimulation (ES) which screens for differential protein-coding genes to enable species differentiation of *Bacillus* isolates (Koeppel *et al*., 2008).

In the case of *Arthrobacter,* 16s rRNA sequence similarity of 99% in combination with biochemical tests and DNA-DNA hybridisation has been accepted as a valid species identification approach in a recent publication (SantaCruz-Calvo *et al*., 2013). Isolate IW was assigned to species level with a 99% identity score as *Arthrobacter chlorophenolicus,* thereby matching the MALDI-TOF MS results. For isolate IY 2/03 only MALDI-TOF MS assigned this isolate to the genus *Arthrobacter*, with only a 90% similarity from 16S rRNA sequence analysis. However, in contrast, IY was classified as a *Clavibacter* species, a plant pathogen (Gartemann *et al.,* 2003), with a 96% similarity, which did not secure assignment to genus level while MALDI-TOF MS classified this isolate as a species of *Arthrobacter*. The high similarity between *Arthrobacter, Curtobacterium,* and *Micrococcus* has been documented in the comparison of coryneform clinical bacterial isolates (Adderson *et al*., 2008). The inability of all three isolates to liquefy gelatine after 48 hours as described in the biochemical tests does match in the descriptions of *Arthrobacter* species, where some species do not yield a positive result (Crocker *et al.,* 2002; Kim *et al.,* 2008). Further tests are necessary to securely identify isolate IY and IY 2/03, potentially including DNA-DNA hybridisation, which is more conclusive than the more limiting 16S rRNA gene sequence based assignment (Cho and Tiedje, 2001; Janda *et al*., 2005).

The isolates were identified to genus level, with a 99% similarity in most cases. Comparing the three identification methods, nine of the isolates were from the genus *Bacillus*, with no conclusive results on the species. The Gram positive cocci (IY and IY 2/03) were not conclusively identified from the three methods and appear to be *Arthrobacter* or *Clavibacter* species.

Combining different identification methods enabled in this study the assignment of 9 out of 12 isolates to the genus *Bacillus* with no conclusive result obtained at species level albeit a 99% sequence similarity was obtained in most cases. The non-endospore forming Gram positive cocci appear to be *Arthrobacter*/*Clavibacter* related species. The initial presumptive

assignment of these three isolates to the genus *Micrococcus* was based on a limited number of morphological and biochemical characteristics.

Phylogenetic analysis (Fig 15, 16 and 17) enabled a visual relation between the unknown isolates and reference strains. The *Bacillus* isolates were grouped closely to the *Bacillus subtilis*/ *Bacillus cereus* group, whilst the *Arthrobacter* species IY and IY 2/03 seem unique to the reference strains, with only isolate IW showing close relation to *Arthrobacter chorophenolicus* as identified previously. Isolate IY grouped closely to *Curtobacterium,* which is not surprising as certain *Clavibacter* species have been reclassified to *Curtobacterium* (Harris-Baldwin and Gudmestad, 1996) and *Leifsonia* (Haapalainen *et al.*, 2001) genera. The use of phylogenetic analysis enabled the establishment of the phylogenetic relation of the isolates in this study to known species based on 16S rRNA gene sequence comparisons. Similar phylogenetic analyses led to the identification and taxonomic assignment of lipolytic bacteria from oil enriched soils demonstrating that phylogenetics is a useful method in the identification of isolates (Narihiro *et al.*, 2014). The phylogenetic analysis of the 12 isolates (Fig 15, 16 and 17) illustrates the grouping of the isolates to known species from similar genera. Isolates Gf, GW G1, 6w and 4w all clustered closely to *Bacillus methylotrophicus*, with isolate 4w being closest to *Bacillus methylotrophicus* and *Bacillus amyloliquefasciens*, which it was identified as through 16S rRNA sequence analysis (Table 12). Isolate 7w grouped closely to *Bacillus mojavensis* and *Bacillus subtilis*, whilst isolate 11w grouped very closely to *Bacillus licheniformis*. Isolates 9w and 19c grouped closely to Bacillus cereus. Figure 15 matches the results in Table 12, although the grouping of isolates is different to the initial presumption based on biochemical tests. *Lactobacillus johnsonii*, although from the same phylum (*Firmicutes*), is clearly not closely related or clustered with any of the isolates as an out-group. In Figure 16, isolate IW is very closely clustered to *Arthrobacter chlorophenolicus*, which supports the results in MALDI-TOF-MS results (Table 11) and 16S rRNA gene sequence analysis (Table 12). Isolate IY was closely clustered with *Curtobacterium luteum*, whilst isolate IY 2/03 appears to be related to *Arthrobacter* but is not clustered closely to any specific species. *Propionibacterium propionicum,* which is also from the same phylum (*Actinobacteria*), was used as an out group. The significance in the clustering of each isolate to known species is that a further distance apart indicates dissimilarity. As a result, the distance between species can indicate the evolution or differences between them (Brocchieri, 2013).

4.4.4 Utilisation of polymeric substrates

Figures 18 to 21 depict the ability of some of the 12 isolates to grow on selected polymeric substrates as sole carbon sources. This essentially tests the ability of the isolates to utilise these compounds thereby indicating that hydrolases of interest are being produced. Based on the growth curves, the most promising isolates based on biomass yields obtained and shortest doubling times observed were selected for enzymatic activity analysis. As expected, the doubling times were directly related to biomass yield, with isolates exhibiting shorter doubling times usually producing higher biomass yields. In the case of the growth curves established (Fig 18-21); the increase in biomass of each isolate over time indicated their ability to assimilate the specific polymeric substrate tested as this was supplied as sole carbon and energy source in a mineral salts medium.

Most of the isolates that were positive for starch hydrolysis during complex nutrient agar based screening were unable to grow optimally on starch when supplied as sole carbon source by showing only slow growth that was not steadily increasing (Fig 18). Meat extract present in the agar used for screening provides essential amino acids (Ercolini *et al*., 2009) and the omission of it seems to have produced unfavourable conditions for most isolates. However, the experiments confirmed that the growth was due to initial starch hydrolysis and subsequent metabolism as starch was the only carbon source. Isolate 7w grew steadily and was selected as the best growing isolate on starch medium, with a doubling time of about 19 hours, compared to doubling times of 24-33 hours for the other isolates.

Selected esterase positive isolates grew well with Tween 80 as sole carbon source, displaying exponential growth and reaching stationery phase in less than 30 hours (Fig 19). All isolates showed doubling times between 3 and 3.5 hours. In addition, isolate 7w yielded the highest biomass after 30 hours. Tween 80 has been established to aid in microbial growth through its surfactant quality (Van Boxtel *et al*., 1990) and was reported to increase cellulase activity in fungal cultures (Reese and Maguire, 1969). As a surfactant, it has been reported to enhance the availability of nutrients to microorganisms through solubilising and emulsifying hydrocarbons (Rouse *et al.,* 1994). Tween 80 in 0.1 to 1% concentrations impacted the morphology *of Mycobacterium paratuberculosis* colonies, producing smoother, shinier colonies (Van Boxtel *et al*., 1990). In most cases, Tween 80 is used as a supplementary agent to enhance growth. In this study, isolates were grown on Tween 80 as a sole carbon and energy source. Microbial cultures can hydrolyse Tween 80 to produce oleate, which is then

used as a carbon source (Jacques *et al*., 1980). In addition, Tween 80 can increase the permeability of cell membranes thus enabling easier nutrient uptake as well as increased enzyme release (Reese and Maguire, 1969). Based on these properties of Tween 80, it is not surprising that the esterase positive isolates displayed exponential growth and short doubling times, with stationary phase typically reached in a little over 24 hours. As for starch, isolate 7w was selected as the best growing isolate on Tween 80.

Figure 20 shows that most of the isolates were able to grow exponentially on skim milk as a sole carbon source which is expected as skim milk contains fermentable carbohydrates and amino acids thus being an excellent substrate for heterotrophic proteolytic bacteria (Chen *et al.,* 2003). Average doubling times ranged from 5 to 6 hours. Isolate 9c grew best with the shortest doubling time of 5.7 hours and the highest biomass yield based on optical density. Proteolytic microorganisms have been sourced from sheep and found to hydrolyse liquid and powdered milk (Blackburn and Hobson, 1960).

Growth of isolates on cellulose as a sole carbon source was very slow (Figure 21). The changes in optical density from day to day were difficult to measure as cells appeared to clump within the medium, causing inaccurate OD_{600} measurements. As a result, biomass formation had to be estimated through protein concentration measurements which enabled the detection of biomass formation after 7 days incubation time. Cellulose has been described as complex substrate and microorganisms usually degrade it as co-cultures (Halsall and Gibson, 1985). Most cellulose media are supplemented with meat extract to enable the better growth of isolates on cellulose. The exclusion of meat extract in the cellulose containing mineral salts medium enabled the induction of cellulolytic enzymes for further enzymatic analysis and provided confirmation that growth was due to cellulose hydrolysis. Isolate IW was selected as the best growing isolate in cellulose medium based on the biomass yield obtained at the end of 7 days.

4.4.5 Enzyme activity assays: Amylase

Once the best growing isolates were identified, the specific enzyme activities were assessed. Amylolytic activity of isolate 7w and cellulolytic activity of isolate IW were analysed through the 3-5-dinitrosalicylic acid (DNS) enzyme assay. This assay measures enzyme activity through the formation of reducing sugars formed hydrolytically from the assay substrate (Miller, 1959). The reduction of DNS by the presence of these sugars enables the

indirect measurement of enzyme activity. DNS, which is orange in colour, is reduced to the red 3-amino-5-nitrosalicylic acid (ANS) under alkaline conditions by reducing sugars. The intensity of the colour is proportional to the quantity of reducing sugars released. The DNS assay has been widely used for the measurement of cellulase activity from filter paper (Ghose, 1987). It has also been used for the estimation of xylanase (Monisha *et al*., 2009) and amylase activities (Burhan *et al*., 2003; Khan and Priya, 2011). Although the assay has been reported to overestimate enzyme activities due to the instability of DNS at boiling temperatures, it has been accepted to produce more accurate measurements of cellulase activity when compared to the Nelson-Somgyi method (Gusakov *et al*., 2011). However, it has been found that different sugars produce different colour intensities (Saqib and Whitney, 2011), which confirms earlier findings (Miller, 1959). The assay as a result is more sensitive to disaccharides than to monosaccharides, which would explain the inconsistencies reported in the literature for enzyme measurements of amylase activity to a certain degree. Starch is hydrolysed to monomeric glucose sugars whilst cellulose is hydrolysed to cellobiose, a disaccharide sugar. As a result, cellulose activity is more accurately estimated than amylase activity (Saqib and Whitney, 2011). This means amylase activity may have been overestimated in this and other studies.

Isolate 7w cell lysate produced measurable amylase activity at 37° and 50°C, with higher activity at 50°C showing that the enzyme could be thermophilic. Previous studies have also reported an optimal amylase activity at 50°C for *Bacillus* species (Charbonneau *et al*., 2012). Although the amylase activity may be overestimated by the DNS assay, it can be compared to other reported amylase enzyme activities measured through the same assay. Tris-HCl buffer was used in this and previous studies instead of citrate buffer for the DNS assay in both the amylase and cellulase enzyme activity measurements, as a pH of 7 is better suited for bacterial cultures (Monisha *et al*., 2009) than the recommended citrate buffer for fungal cultures in other protocols (Ghose, 1998). In addition, the higher activity in cell lysate than in the supernatant confirms findings of a study done on ruminal bacteria amylases (Cotta, 1988), this could be due to the fact that the supernatant was not dialysed or concentrated prior to amylase enzyme activity measurements.

The specific amylase activity of isolate 7w was lower than previously reported enzyme activities of *Bacillus* species at 40°C (Kumari *et al*., 2013), with an amylase activity of 130 nmol x min^{-1} x mg^{-1} from the supernatant, which is almost 4-fold higher than the activity

obtained in isolate 7w supernatant, which was 36 nmol x min^{-1} x mg^{-1} at 37°C (Table 13). This could be due to the difference in incubation times used for the amylase assay, as Kumari *et al.* (2013) used 30 minutes instead of 15 minutes as in this study. Furthermore, these authors concentrated the enzyme present in the supernatants with ammonium sulphate. On the other hand, lower supernatant amylase activity was reported for *Bacillus dipsosauri*, at 3.48 nmol x min^{-1} x mg^{-1} using 2-chloro-4-nitrophenyl-α-maltotrioside (CNPG3) to estimate amylase activity. (Deutch, 2002). However, the cell lysate of 7w produced a much higher specific activity of 160 nmol x min^{-1} x mg^{-1} at 50°C, which was 9 fold lower than a previously reported specific α-amylase activity of 1470 nmol x min^{-1} x mg^{-1} albeit using precipitated crude enzyme extract of a *Bacillus subtilis* (MTCC 121) isolate at 40°C (Raul *et al.*, 2014). In addition, the enzyme activity present in cell lysate of isolate 7w displayed thermo-stability (50°C), as was previously reported of some *Bacillus* spp. amylase enzymes (Burhan *et al*., 2003). The thermal stability was however not confirmed over periods of time exceeding 15 minutes.

4.4.6 Cellulase

In the case of isolate IW, which was chosen as the best growing isolate, no measurable reducing sugars were detected after incubation at 50°C for 60 minutes with filter paper as a substrate. Cellulase activity (Table 14) was detected at 37°C. Isolate IW produced a specific cellulase activity of 297 nmol x mg^{-1} x min^{-1} in crude extracts, which was lower than the reports of cellulolytic activity from a *Micrococcus* strain isolated from zebra faeces, with 730 nmol x mg^{-1} x min^{-1} (Sadhu *et al*., 2011). Considerably higher cellulase activity was reported for *Bacillus licheniformis* with 6100 nmol x mg^{-1}x min^{-1} (Bischoff *et al*., 2006) which is considerably higher than the obtained activity in isolate IW. Interestingly, *Arthrobacter* strains isolated from sediments were found to be incapable of hydrolysing cellulose in a previous study (Crocker *et al*., 2002). Moreover, *Arthrobacter* has been utilised for its ability to produce cellulase-free xylan (Rajendran *et al*., 2008). Reports of a new *Arthrobacter* species from sewage, *Arthrobacter defluvii* , also documented it as unable to utilise cellobiose during screening tests (Kim *et al*., 2008) or to hydrolyse cellulose (Jones and Keddie, 2006). This indicates the novelty of the findings of a cellulase producing *Arthrobacter* species in this study. With cellulose being a fairly complex substrate, recommendations have been made in a recent study (Meng and Ragauskas, 2014) for increasing the accessibility of cellulose for microbial utilisation. Examples recommended include treating cellulose with heating, alkali

or ionic solutions to increase its bioavailability (Meng and Ragauskas, 2014). These findings indicate a variety of potential further investigations in optimising cellulose hydrolysis, even for cellulolytic isolates used in this study.

Based on these comparisons, it would appear that the amylase and cellulase enzymes from the selected isolates 7w and IW are not showing higher specific activities than those reported in other studies. However, the isolation in this study was done under mesophilic conditions and the thermo-stability or tolerance of the enzymes has not been explored beyond the DNS assay. Enzyme activities and yields in crude extracts or supernatants may be increased by optimising incubation conditions.

4.4.7 Esterase

Both *ρ*-NPA and FDA have been used for the screening of esterase activity (Lundgren, 1981, Hintner *et al*., 2005). *ρ*–NPA hydrolysis has been shown to follow first order reaction kinetics when tested with phosphate buffer at neutral pH (Goren and Fridkin, 1974), which is similar to the conditions in this study.

Microbial enzymes capable of hydrolysing a large number of substrates are classified as true lipases. A number of true lipases have been isolated from *Bacillus* species, however, they cannot be easily compared against each other (Arpigny and Jaeger, 1999). In the case of isolate 7w, there is no clarity on whether the esterase enabling hydrolysis of Tween is a true lipase or not as this would require additional experimental work. However, the measured specific activity of 1431 nmol x mg^{-1}x min^{-1} (Table 15) is higher than what has been reported in the case of two feruloyl esterases of *Dickeya dadantii*, a plant pathogen, which were 625 and 1140 nmol.mg^{-1}.min^{-1} (Hassan and Hugouvieux-Cotte-Pattat, 2011). Further comparison shows the esterase activity of isolate 7w to be about 100 times higher than the measured esterase activity of *Acinetobacter* utilising the same substrate, with a measured activity of 13 nmol x mg^{-1} x min^{-1} (Gottsching and Schmidt, 2007), but lower than the highest activity from a strain of *Rhodococcus*, which was 1880 nmol x mg^{-1} x min^{-1}(Hintner *et al*., 2005). Looking at the hydrolysis of FDA, the esterase activity measured is lower than that measured with *ρ* – NPA. This supports previous studies that *ρ*–NPA is a better esterase substrate than the more bulky FDA (Gershater *et al*., 2006). The esterase activity produced through FDA as a substrate was 325 nmol x mg^{-1} x min^{-1}, which was higher than the activity measured on a slightly different substrate fluorescein dibutyrate (FDB) where a thermostable *Bacillus*

stearothermophilus isolate yielded esterase activity of 0.11 nmol.mg^{-1}.min^{-1} (Simoes *et al*., 1997). The obtained activity indicates that the isolate 7w produced an activity within a range of activities reported for other bacterial isolates.

4.4.8 Protease

The protease activity of isolate 9c (Table 16) was determined with azocasein as a substrate and found to be about 9-fold higher than that of isolate 11w, which also grew well on skim milk. The obtained activity (933 nmol x mg^{-1}x min^{-1}) is almost 4-fold lower than the specific protease activity reported from disrupted rumen bacteria cells (3253 nmol x mg^{-1} x min^{-1}), which was measured with azocasein (Brock *et al*., 1982). However, the protease activity measured in that study was of a mixed culture, which means various enzymes may have been hydrolysing the substrate, whilst this study measured the specific protease activity of crude extract from one isolate. In addition, Shah *et al.,* (2010) isolated protease from *Bacillus cereus* with a specific protease activity of 232 nmol x mg^{-1} x min^{-1}, which is lower than the protease activity measured for isolate 9c, but higher than that of isolate 11w.

Overall, it appears that most of the enzymes produced in the isolates showed specific activities in a comparable range with previously reported activities for such enzymes. Isolation and purification of hydrolytic enzymes from these isolates may reveal novel or unique characteristics with potential for applications in industrial processes.

Further investigation of the microbial diversity including anaerobic prokaryotes within the studied faecal matter may reveal additional hydrolytically active strains with potential for biotechnology. Most of the isolates that were selected as the best growing isolates were from zebra faeces, which is interesting as zebra have been established to be quite efficient non-ruminant competitors to other ruminants in the wild (Estes, 1991).

The microorganisms analysed in this study as well as the hydrolytic enzymes explored support the previous findings of enzymes isolated from herbivores and their potential in industrial application. Additionally, further study is required in the analysis of other complex polymers, apart from the substrates employed in this study to verify whether these enzymes are suitable for certain industrial processes. A recent study addressed the importance of enzyme analysis on complex, insoluble substrates for lignocellulosic enzymes (Goacher *et al.,* 2014). Another good example of this is the use of carboxy-methyl cellulose (CMC), (which is a partially hydrolysed cellulose derivative) in assessing enzyme efficiency

(Karlsson *et al.*, 2002), whilst cellulosic material for industrial application may not always be in a partially hydrolysed state. Therefore further verification is required for enzyme efficiency to be applicable for industry or biotechnology.

4.4.9 Conclusion

Microbial aerobic isolates from zebra, giraffe and impala faecal matter were shown to possess a number of hydrolases which might have potential for biotechnological exploration. The 12 selected isolates were characterised using three different approaches and were found to be mostly from the genus *Bacillus* with one isolate assigned to the genus *Arthrobacter* and two isolates related to *Arthrobacter* and *Clavibacter*. All 12 of the isolates hydrolysed more than one polymeric substrate. The specific enzyme activities determined did not exceed activities reported in the literature. However, this study shows the potential of faecal matter from indigenous South African herbivores as a source for hydrolytically active microorganisms. Further investigation is required to evaluate its potential as a source for novel hydrolytic enzymes.

4.5 References

Adderson, E. E., Boudreaux, J. W., Cummings, J. R., Pounds, S., Wilson, D. A., Procop, G. W. & Hayden, R. T. 2008. Identification of clinical coryneform bacterial isolates: comparison of biochemical methods and sequence analysis of 16S rRNA and rpoB genes. *Journal of Clinical Microbiology,* 46**,** 921-927.

Altayar, M. & Sutherland, A. D. 2005 Bacillus cereus is common in the environment but emetic toxin producing isolates are rare. *Journal of Applied Microbiology,* 100**,** 7-14.

Balcázar, J. L., Pintado, J. & Planas, M. 2010. *Bacillus galliciensis* sp. nov., isolated from faeces of wild seahorses (*Hippocampus guttulatus*). *International Journal of Systematic and Evolutionary Microbiology,* 60**,** 892-895.

Bartholomew, J. W. & Mittwer, T. 1952. The Gram stain. Bacteriological Reviews, 16**,** 1-29.

Bergey, D. H. & Holt, J. G. 1994. *Bergey's Manual of Determinative Bacteriology,* Baltimore, USA, Williams & Wilkins.

Bischoff, K. M., Rooney, A. P., Li, X.-L., Liu, S. & Hughes, S. R. 2006. Purification and characterisation of a family 5 endoglucanase from a moderately thermophilic strain of *Bacillus licheniformis*. *Biotechnology Letters,* 28**,** 1761-1765.

Blackburn, T. H. & Hobson, P. N. 1962. Further studies on the isolation of proteolytic bacteria from the sheep rumen. *Journal of General Microbiology,* 29**,** 69-81.

Bowers, G., McComB, R. B., Christensen, R. & Schaffer, R. 1980. High-purity 4-nitrophenol: purification, characterisation, and specifications for use as a spectrophotometric reference material. *Clinical Chemistry,* 26**,** 724-729.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry,* 72**,** 248-254.

Brocchieri, L. 2013. Phenotypic and evolutionary distances in phylogenetic tree reconstruction. *Journal of Phylogenetics & Evolutionary Biology,* 1**,** 1-4.

Brock, F., Forsberg, C. & Buchanan-Smith, J. 1982. Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. *Applied and Environmental Microbiology,* 44**,** 561-569.

Burhan, A., Nisa, U., Gökhan, C., Ömer, C., Ashabil, A. & Osman, G. 2003. *E*nzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate *ANT-6*. *Process Biochemistry,* 38**,** 1397-1403.

Charbonneau, D. M., Meddeb-MouelhI, F., Boissinot, M., Sirois, M. & Beauregard, M. 2012. Identification of thermophilic bacterial strains producing thermotolerant hydrolytic enzymes from manure compost. *Indian Journal of Microbiology,* 52**,** 41-47.

Chen, L., Daniel, R. M. & Coolbear, T. 2003. Detection and impact of protease and lipase activities in milk and milk powders. *International Dairy Journal,* 13**,** 255-275.

Cho, J.-C. & Tiedje, J. M. 2001. Bacterial species determination from DNA-DNA hybridisation by using genome fragments and DNA microarrays. *Applied and Environmental Microbiology,* 67**,** 3677-3682.

Clarridge, J. E. 2004. Impact of 16S rRNA gene Sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews,* 17**,** 840-862.

Cotta, M. A. 1988. Amylolytic activity of selected species of ruminal bacteria. *Applied and Environmental Microbiology,* 54**,** 772-776.

Couto, S. R. & Sanromán, M. Á. 2006. Application of solid-state fermentation to food industry—A review. *Journal of Food Engineering,* 76**,** 291-302.

Crocker, F. H., Fredrickson, J. K., White, D. C., Ringelberg, D. B. & Balkwill, D. L. 2002. Phylogenetic and physiological diversity of *Arthrobacter* strains isolated from unconsolidated subsurface sediments. *Microbiology,* 146**,** 1295-1310.

Deutch, C. E. 2002. Characterisation of a salt-tolerant extracellular α-amylase from *Bacillus dipsosauri*. *Letters in Applied Microbiology,* 35**,** 78-84.

Di Franco, C., Beccari, E., Santini, T., Pisaneschi, G. & Tecce, G. 2002. Colony shape as a genetic trait in the pattern-forming *Bacillus mycoides*. *BMC Microbiology,* 2**,** 1-15.

Drancourt, M., Berger, P. & Raoult, D. 2004. Systematic 16S rRNA gene sequencing of atypical clinical isolates identified 27 new bacterial species associated with humans. *Journal of Clinical Microbiology,* 42**,** 2197-2202.

Drancourt, M. & Raoult, D. 2005. Sequence-based identification of new bacteria: a proposition for creation of an orphan bacterium repository. J*ournal of Clinical Microbiology,* 43**,** 4311-4315.

Drevinek, M., Dresler, J., Klimentova, J., Pisa, L. & Hubalek, M. 2012. *Ev*aluation of sample preparation methods for MALDI-TOF MS identification of highly dangerous bacteria. *Letters in Applied Microbiology,* 55**,** 40-46.

Ercolini, D., Russo, F., Nasi, A., Ferranti, P. & Villani, F. 2009. Mesophilic and psychrotrophic bacteria from meat and their spoilage potential in vitro and in beef. *Applied and Environmental Microbiology,* 75**,** 1990-2001.

Estes, R. D. 1991. *The behavior guide to African mammals: including hoofed mammals, carnivores, primates,* University of California Press.

Funke, G., Hutson, R. A., Bernard, K. A., Pfyffer, G. E., Wauters, G. & collins, M. D. 1996. Isolation of *Arthrobacter* spp. from clinical specimens and description of *Arthrobacter cumminsii sp. nov.* and *Arthrobacter woluwensis sp. nov. Journal of Clinical Microbiology,* 34**,** 2356–2363.

Gartemann, K., Kirchner, O., Engemann, J., Gräfen, I., Eichenlaub, R. & Burger, A. 2003. *Clavibacter michiganensis* subsp. *michiganensis*: first stepsin the understanding of virulence of a Gram-positive phytopathogenic bacterium. *Journal of Biotechnology,* 106**,** 179–191.

Gemmell, M. E. & Schmidt, S. 2013. Is the microbiological quality of the Msunduzi River (KwaZulu-Natal, South Africa) suitable for domestic, recreational, and agricultural purposes? *Environmental Science and Pollution Research,* 20**,** 1-12.

Gershater, M., Sharples, K. & Edwards, R. 2006. Carboxylesterase activities toward pesticide esters in crops and weeds. *Phytochemistry,* 67**,** 2561-2567.

Ghose, T. K. 1987. Measurement of cellulase activities. *Pure and Applied Chemistry,* 59**,** 257-268.

Goacher, R. E., Selig, M. J. & Master, E. R. 2014. Advancing lignocellulose bioconversion through direct assessment of enzyme action on insoluble substrates. *Current Opinion in Biotechnology,* 27**,** 123-133.

Goren, H. J. & Fridkin, M. 1974. The hydrolysis of *p*-Nitrophenylacetate in water. *European Journal of Biochemistry,* 41**,** 263-272.

Göttsching, A. & Schmidt, S. 2007. Productive degradation of the biocide benzylbenzoate by *Acinetobacter* sp. strain AG1 isolated from the River Elbe. *Research in Microbiology,* 158**,** 251-257.

Gregersen, T. 1978. Rapid method for distinction of gram-negative from Gram-positive bacteria. *European Journal of Applied Microbiology and Biotechnology,* 5**,** 123-127.

Gusakov, A. V., Kondratyeva, E. G. & Sinitsyn, A. P. 2011. Comparison of two methods for assaying reducing sugars in the determination of carbohydrase activities. *International Journal of Analytical Chemistry,* 2011**,** 1-4.

Haapalainen, M., Metzler, M. & Romantschuk, M. 2001. Extracellular Enzymes Produced by *Leifsonia* (*Clavibacter*) *xyli* subsp. *cynodontis*. *Plant Pathogenic Bacteria*, Sringer, Canada. 173-175.

Halebian, S., Harris, B., Finegold, S. M. & Rolfe, R. D. 1981. Rapid method that aids in distinguishing Gram-positive from Gram-negative anaerobic bacteria. J*ournal of Clinical Microbiology,* 13**,** 444-448.

Halsall, D. M. & Gibson, A. H. 1985. Cellulose decomposition and associated nitrogen fixation by mixed cultures of *Cellulomonas gelida* and *Azospirillum* species or *Bacillus macerans*. *Applied and Environmental Microbiology,* 50**,** 1021-1026.

Harris-Baldwin, A. & Gudmestad, N.C. 1996. I dentification of phytopathogenic coryneform bacteria using the biolog automated microbial identification system. *Plant Disease,* 80**,** 874-878.

Hassan, S. & Hugouvieux-Cotte-Pattat, N. 2011. Identification of two feruloyl esterases in *Dickeya dadantii* 3937 and induction of the major feruloyl esterase and of pectate lyases by ferulic acid. *Journal of Bacteriology,* 193**,** 963-970.

Hausdorf, L., Mund, T. K., Winzer, M., Cordes, C., Fröhling, A., Schlüter, O. & Klocke, M. 2012. Characterisation of the cultivable microbial community in a spinach-processing plant using MALDI-TOF MS. *Food Microbiology,* 34**,** 406-411.

Hintner, J., Fortnagel, P., Franke, S., Francke, W. & Schmidt, S. 2005. *C*atabolism of Mesamoll, a technical formulation of akylsulfonic phenyl esters, by two strains of *Rhodococcus rhodochrous*. *Research in Microbiology,* 156**,** 656-662.

Jacques, N. A., Hardy, L., Knox, K. W. & Wicken, A. J. 1980. Effect of Tween 80 on the morphology and physiology of *Lactobacillus salivarius* strain IV CL-37 grown in a chemostat under glucose limitation. *Journal of General Microbiology,* 119**,** 195-201.

Janda, J. M., Abbott, S. L., Bystrom, S. & Probert, W. S. 2005. Identification of two distinct hybridisation groups in the genus *Hafnia* by 16S rRNA gene sequencing and phenotypic methods. *Journal of Clinical Microbiology,* 43**,** 3320-3323.

John, E. & Hampel, W. A. 1992. Enzyme formation by a yeast cell wall lytic *Arthrobacter* species: formation of amylase. A*pplied Microbiology and Biotechnology,* 38**,** 214-219.

Jones, D. & Keddie, R. 2006. The genus *Arthrobacter*. *In:* Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. & Stackebrandt, E. (eds.) *The Prokaryotes.* Springer New York.

Karlsson, J., Momcilovic, D., Wittgren, B., Schülein, M., Tjerneld, F. & Brinkmalm, G. 2002. Enzymatic Degradation of carboxymethyl cellulose hydrolyzed by the endoglucanases Cel5A, Cel7B, and Cel45A from *Humicola insolens* and Cel7B, Cel12A and Cel45Acore from *Trichoderma reesei*. *Biopolymers,* 63**,** 32-40.

Kasana, R. C., Salwan, R., Dhar, H., Dutt, S. & Gulati, A. 2008. A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Current Microbiology,* 57**,** 503-507.

Kerfeld, C. A. & Scott, K. M. 2011. Using BLAST to Teach "E-value-tionary" Concepts. *PLoS Biology,* 92. [Accessed 26 November 2014]

Khan, J. A. & Priya, R. 2011. A study on partial purification and characterisation of extracellular amylases from *Bacillus subtilis*. *Advances in Applied. Science Research,* 2**,** 509-519.

Khot, P. D., Couturier, M. R., Wilson, A., Croft, A. & Fisher, M. A. 2012. Optimisation of Matrix-Assisted Laser Desorption Ionisation–Time of Flight Mass Spectrometry analysis for bacterial identification. *Journal of Clinical Microbiology,* 50**,** 3845-3852.

Kim, K. K., Lee, K. C., Oh, H. M., Kim, M. J., Eom, M. K. & Lee, J. S. 2008. *Arthrobacter defluvii sp. nov.,* 4-chlorophenol-degrading bacteria isolated from sewage. *International Journal of Systematic and Evolutionary Microbiology,* 58**,** 1916-1921.

Kirk, O., Borchert, T. V. & Fuglsang, C. C. 2002. Industrial enzyme applications. *Current Opinion in Biotechnology,* 13**,** 345-351.

Klonis, N. & Sawyer, W. H. 1996. Spectral properties of the prototropic forms of fluorescein in aqueous solution. *Journal of Fluorescence,* 6**,** 147-157.

Koeppel, A., Perry, E. B., Sikorski, J., Krizanc, D., Warner, A., Ward, D. M., Rooney, A. P., Brambilla, E., Connor, N. & Ratcliff, R. M. 2008. Identifying the fundamental units of bacterial diversity: a paradigm shift to incorporate ecology into bacterial systematics. *Proceedings of the National Academy of Sciences,* 105**,** 2504-2509.

Kühn, S. & Fortnagel, P. 1993. Molecular cloning and nucleotide sequence of the gene encoding a calcium-dependent exoproteinase from *Bacillus megaterium* ATCC 14581. *Journal of General Microbiology,* 139**,** 39-47.

Kumari, N., Jain, V. & Malhotra, S. 2013. Purification and characterisation of extracellular acidophilic α-amylase from *Bacillus cereus* MTCC 10205 isolated from soil. *African Journal of Microbiology Research,* 7**,** 5440-5448.

Latifian, M., Hamidi-Esfahani, Z. & Barzegar, M. 2007. Evaluation of culture conditions for cellulase production by two s mutants under solid-state fermentation conditions. *Bioresource Technology,* 98**,** 3634-3637.

Lisdiyanti, P., Suyanto, E., Gusmawati, N. F. & Rahayu, W. 2012. Isolation and characterisation of cellulase produced by cellulolytic bacteria from peat soil of Ogan Komering Ilir,South Sumatera. *International Journal of Environment and Bioenergy,* 3**,** 145-153.

Logan, N. A., Berge, O., Bishop, A. H., Busse, H.-J., De Vos, P., Fritze, D., Heyndrickx, M., Kämpfer, P., Rabinovitch, L. & Salkinoja-Salonen, M. S. 2009. Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria. *International Journal of Systematic and Evolutionary Microbiology,* 59**,** 2114-2121.

Logan, N. A. & Berkeley, R. C. W. 1984. Identification of *Bacillus* strains using the API system. *Journal of General Microbiology,* 130**,** 1871-1882.

Logan, N. A. & De Vos, P. 2009. *Bacillus In* De Vos, P., Garrity, G. M., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., Schleifer, K. H. & Whitman, W. B. (ed) *Bergey's Manual of Systematic Bacteriology*, 2nd ed. Vol 3**,**: *The Firmicutes* 21-128.

Memela, N. S. & Schmidt, S. 2013. Fishing with bait for bacterial predators in Zebra droppings – isolation and characterisation of a bacteriolytic *Myxococcus* strain. *In:* Mendez-Vilas, A. (ed.) *Microbial pathogens and strategies for combating them: science, technology and education* Spain: Formatex Research Centre.

Meng, X. & Ragauskas, A. J. 2014. *Recent advances in understanding the role of cellulose accessibility in enzymatic hydrolysis of lignocellulosic substrates*. *Current Opinion in Biotechnology,* 27**,** 150-158.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry,* 31**,** 426-428.

Monisha, R., Uma, M. V. & Murthy, V. K. 2009. Partial purification and characterisation of *Bacillus pumilus* xylanase from soil source. *Journal Of Science, Engineering and Technology,* 5**,** 137-148.

Moriarty, E. M. & Gilpin, B. J. 2014. Leaching of *Escherichia coli* from sheep faeces during simulated rainfall events. *Letters in Applied Microbiology,* 58**,** 569-575.

Moxley, K. & Schmidt, S. 2012. Isolation of a phenol-utilising marine bacterium from Durban Harbour (South Africa) and its preliminary characterisation as *Marinobacter* sp. KM2. *Water Science and Technology,* 65**,** 932-939.

Nazina, T. N., Tourova, T. P., Poltaraus, A. B., Novikova, E. V., Grigoryan, A. A., Ivanova, A. E., Lysenko, A. M., Petrunyaka, V. V., Osipov, G. A., Belyaev, S. S., & Ivanov, M. V. 2001. Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus, G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermoglucosidasius and G. thermodenitrificans*. *International Journal of Systematic and Evolutionary Microbiology,* 51**,** 433-446.

Narihiro, T., Suzuki, A., Yoshimune, K., Hori, T., Hoshino, T., Yumoto, I., Yokota, A., Kimura, N. and Kamagata, Y. 2014 The combination of functional metagenomics and an oil-fed enrichment strategy revealed the phylogenetic diversity of lipolytic bacteria overlooked by the cultivation-based method. *Microbes and Environments,* 29**,** 154-161.

Nicholson, W. L. 2008 The *Bacillus subtilis* ydjL (bdhA) gene encodes acetoin reductase/2,3-butanediol dehydrogenase. *Applied and Environmental Microbiology,*74**,** 6832-6838.

Økstad, O. A. & Kolstø, A. 2011. Genomics of *Bacillus* species. *In:* Wiedmann, M. & Zhang, W. (eds.) *Genomics of Foodborne Bacterial Pathogens.* Springer New York.

Rajendran, A., Selvaraj, V. & Thangavelu, V. 2008. Statistical optimisation and kinetic modeling of xylanase production by *Arthrobacter* sp. *Asia-Pacific Journal of Chemical Engineering,* 3**,** 347-353.

Raul, D., Biswas, T., Mukhopadhyay, S., Kumar Das, S. & Gupta, S. 2014. Production and partial purification of alpha amylase from *Bacillus subtilis* (MTCC 121) using solid state fermentation. *Biochemistry Research International***,** 2014**,** 1-5.

Ray, R. R. 2011. Microbial isoamylases: an overview. *American Journal of Food Technology,* 6**,** 1-18.

Reese, E. & Maguire, A. 1969. Surfactants as stimulants of enzyme production by microorganisms. *Applied Microbiology,* 17**,** 242-245.

Reva, O., Sorokulova, I. & Smirnov, V. 2001. Simplified technique for identification of the aerobic spore-forming bacteria by phenotype. *International Journal of Systematic and Evolutionary Microbiology,* 51**,** 1361-1371.

Roberts, J. L. 1935. A new species of the genus *Bacillus* exhibiting mobile colonies on the surface of nutrient agar. *Journal of Bacteriology,* 29**,** 229-237.

Ruelle, V., Moualij, B. E., Zorzi, W., Ledent, P., De Pauw, E. 2004. Rapid identification of environmental bacterial strains by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry,* 18**,** 2013-2019.

Sacchi, C. T., Whitney, A. M., Mayer, L. W., Morey, R., Steigerwalt, A., Boras, A., Weyant, R. S. & Popovic, T. Sequencing of 16S rRNA gene:a rapid tool for identification of *Bacillus anthracis*. *Emerging Infectious Diseases,* 8**,** 1117-1123.

Sadhu, S., Saha, P., Mayilraj, S. & Maiti, T. K. 2011. Lactose-enhanced cellulase production by Microbacterium sp. isolated from faecal matter of Zebra *(Equus zebra). Current Microbiology,* 62**,** 1050-1055.

Saman, S., Saman, S. & Slattery, P. 2010. Isolation of a potential new member of the *Bacillus cereus* group from snow covered soil. *Life Sciences and Medicine Research,* 2010: LSMR-18**,** 1-7.

Santacruz-Calvo, L., González-López, J. & Manzanera, M. 2013. *Arthrobacter siccitolerans sp. nov.,* a highly desiccation-tolerant, xeroprotectant-producing strain isolated from dry soil. *International Journal of Systematic and Evolutionary Microbiology,* 63**,** 4174-4180.

Saqib, A. A. N. & Whitney, P. J. 2011. *D*ifferential behaviour of the dinitrosalicylic acid (DNS) reagent towards mono-and di-saccharide sugars. *Biomass and Bioenergy,* 35**,** 4748-4750.

Schaeffer, A. B. & Fulton, M. D. 1933. A simplified method of staining endospores. *Science* (New York, NY), 77**,** 194-194.

Shah, K., Mody, K., Keshri, J. & Jha, B. 2010. Purification and characterisation of a solvent, detergent and oxidizing agent tolerant protease from *Bacillus cereus* isolated from the Gulf of Khambhat. *Journal of Molecular Catalysis B: Enzymatic,* 67**,** 85-91.

Shapiro, J. A. 1995. The significances of bacterial colony patterns. *BioEssays,* 17**,** 597-607.

Simoes, D., McNeill, D., Kristiansen, B. & Mattey, M. 1997. Purification and partial characterisation of a 1.57 kDa thermostable esterase from *Bacillus stearothermophilus*. *FEMS Microbiology Letters,* 147**,** 151-156.

Slepecky, R. A. & Hemphill, H. E. 2006. The genus Bacillus—non medical. *The Prokaryotes.* US: Springer.

Smole, S. C., King, L. A., Leopold, P. E. & Arbeit, R. D. 2002. Sample preparation of Gram-positive bacteria for identification by matrix assisted laser desorption/ionization time-of-flight. *Journal of Microbiological Methods,* 48**,** 107-115.

Sousa, A. M., Machado, I., Nicolau, A. & Pereira, M. O. 2013. Improvements on colony morphology identification towards bacterial profiling. *Journal of Microbiological Methods,* 95**,** 327-335.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution,* 30**,** 2725-2729.

Underkofler, L. A., Barton, R. R. & Rennert, S. S. 1958. Production of microbial enzymes and their applications. *Microbiological Process Report,* 6**,** 212-221.

Van Boxtel, R., Lambrecht, R. & Collins, M. 1990. Effects of colonial morphology and Tween 80 on antimicrobial susceptibility of *Mycobacterium paratuberculosis*. *Antimicrobial Agents and Chemotherapy,* 34**,** 2300-2303.

Vargha, M., Takáts, Z., Konopka, A. & Nakatsu, C. H. 2006. Optimization of MALDI-TOF MS for strain level differentiation of *Arthrobacter* isolates. *Journal of Microbiological Methods,* 66**,** 399-409.

Vary, P. S. 1994. Prime time for *Bacillus rnegaterium. Microbiology,* 140**,** 1001-1013.

Vasileva-Tonkova, E. & Galabova, D. 2003. Hydrolytic enzymes and surfactants of bacterial isolates from lubricant-contaminated wastewater. *Zeitschrift für Naturforschung* 58**,** 87-92.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology,* 173**,** 697-703.

Williams, T. L., Andrzejewski, D., Lay JR, J. O. & Musser, S. M. 2003. Experimental factors affecting the quality and reproducibility of MALDI TOF mass spectra obtained from whole bacteria cells. *Journal of the American Society for Mass Spectrometry,* 14**,** 342-351.

Woese, C. R. 1987. Bacterial evolution. *Microbiological Reviews,* 51**,** 221-271.

Wood, J., Scott, K. P., AVguštin, G., Newbold, C. J. & Flint, H. J. 1998. Estimation of the relative abundance of different *Bacteroides* and *Prevotella* ribotypes in gut samples by restriction enzyme profiling of PCR-amplified 16S rRNA gene sequences. *Applied and Environmental Microbiology,* 64**,** 3683-3689.

Wu, X., Walker, M., Vanselow, B., Chao, R. & Chin, J. 2006. Characterization of mesophilic bacilli in faeces of feedlot cattle. *Journal of Applied Microbiology,* 102**,** 872-879.

5. Conclusion

Findings in this study have confirmed that faecal matter can be used to source microbial hydrolytic enzymes that can potentially be used in industry. The analysis of hydrolytic activity and viable counts enabled an estimation of the diversity and hydrolytic potential within each sample. The microbiological properties of faeces were proven to be different from the surrounding soil and the properties of wild ungulate faeces differed from that of previously explored domestic ungulates.

The area of faecal analysis is common in clinical microbiology; however more research is required from an applied environmental microbiology perspective. The approaches employed in this study can be modified and extended to more specifically target certain classes of microorganisms such as thermophiles or alkaliphiles in faecal matter. The discovery of isolates that utilise a variety of substrates is an exciting indicator of the various research avenues that can be explored within the microbiology of lesser researched faecal materials. Consideration of storage and seasonal effects as well as the stability of faecal matter over time need to be more extensively explored in future research.

6. Appendices

Appendix A

Table 17: Hydrolytic activities (µg x g^{-1} x h^{-1}) of faecal and soil samples over four seasons (2011-2012)

Sample	Zebra shaker	Zebra static	Zebra soil control shaker	Zebra soil control static	Giraffe shaker	Giraffe static	Giraffe soil control shaker	Giraffe soil control static	Impala shaker	Impala static	Impala soil control shaker	Impala soil control static
March 2011	862.	541	150	71	186	57	245	129	706	607	172	55
August 2011	1228	467	120	99	1095	181	69	1	905	530	121	26
June 2011	1487	636	205	16	1148	167	245	65	764	348	335	88
January 2012	847	623	41	29	279	4	78	4	364	158	120	24

Plate counts of faecal and soil samples over summer and winter seasons

Winter 2011

Table 18: $\log_{10}$ (cfu/g) Plate counts of hydrolase-producing microorganisms in June 2011

Sample	PC agar	Starch agar	Casein agar	Tween agar	CMC agar
Giraffe	7.64	7.55	7.41	6.66	7.52
Giraffe soil control	5.80	5.40	<4	5.46	5.60
Impala	7.97	7.23	6.52	5.60	6.96
Impala soil control	6.36	5.06	5.49	5.09	5.25
Zebra	7.94	7.40	6.91	7.90	7.75
Zebra soil control	5.38	5.08	<4	5.29	<4

<4: microbial numbers not determined due to plate counts lower than required for weighted means estimation

Summer 2012

Table 19: log10 (cfu/g) Plate counts of hydrolase-producing microorganisms in January 2012

Sample	PC agar	Starch agar (positives)	Casein agar (positives)	Tween agar (positives)	CMC agar (positives)
Giraffe	8.53	8.51	8.50	7.78	8.39
Giraffe soil control	6.50	6.49	6.30	6.20	6.16
Impala	8.29	8.25	8.25	6.53	8.01
Impala soil control	6.27	6.10	6.08	<4	6.16
Zebra	8.30	8.13	7.55	8.16	7.63
Zebra soil control	6.68	5.92	5.09	<4	6.29

<4: microbial numbers not determined due to plate counts lower than required for weighted means estimation

Hydrolytic activities of faecal and soil control samples, over 4 weeks of storage at 4 °C and 30 °C in June 2011 and January 2012.

June 2011 (South African Winter) at 4 °C storage

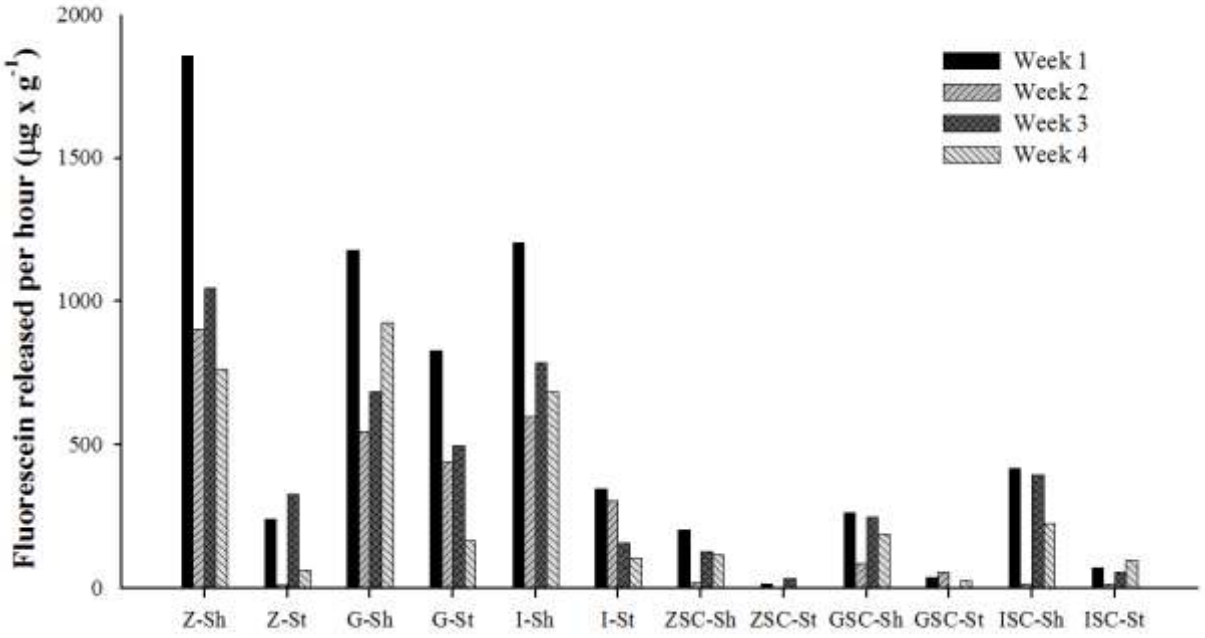


Figure 22: Changes in hydrolytic activities of faecal and soil samples stored at 4 °C in June 2011

Key: Z-Sh: Zebra shaker; Z-St: Zebra static; G-Sh: Giraffe shaker; G-St: Giraffe static; I-Sh: Impala shaker; I-St: Impala static; ZSC-Sh: Zebra soil control shaker; ZSC-St: Zebra soil control static; GSC-Sh: Giraffe soil control shaker; GSC-St: Giraffe soil control static; ISC-Sh: Impala soil control shaker; ISC-St: Impala soil control static

June 2011(South African winter) at 30°C storage

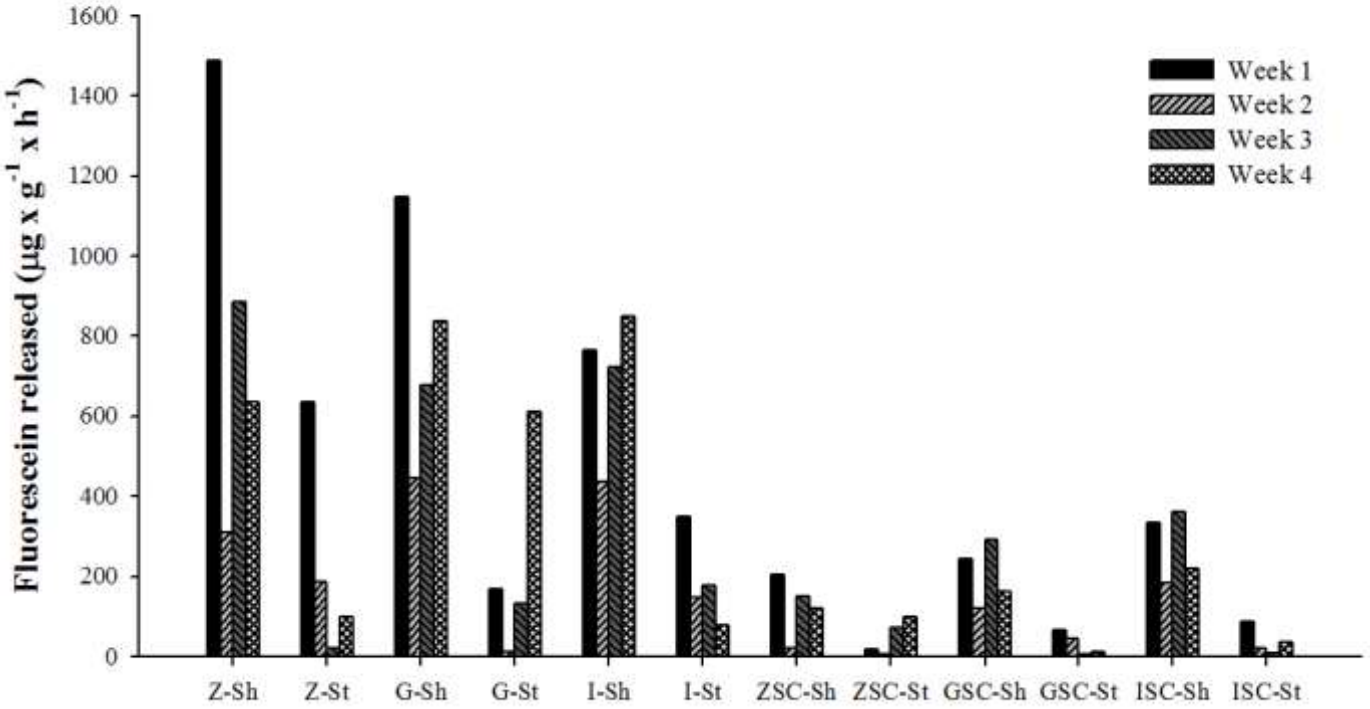


Figure 23: Changes in hydrolytic activities of faecal and soil samples stored at 30 °C in June 2011

Key: Z-Sh: Zebra shaker; Z-St: Zebra static; G-Sh: Giraffe shaker; G-St: Giraffe static; I-Sh: Impala shaker; I-St: Impala static; ZSC-Sh: Zebra soil control shaker; ZSC-St: Zebra soil control static; GSC-Sh: Giraffe soil control shaker; GSC-St: Giraffe soil control static; ISC-Sh: Impala soil control shaker; ISC-St: Impala soil control static

January 2012 (South African summer)

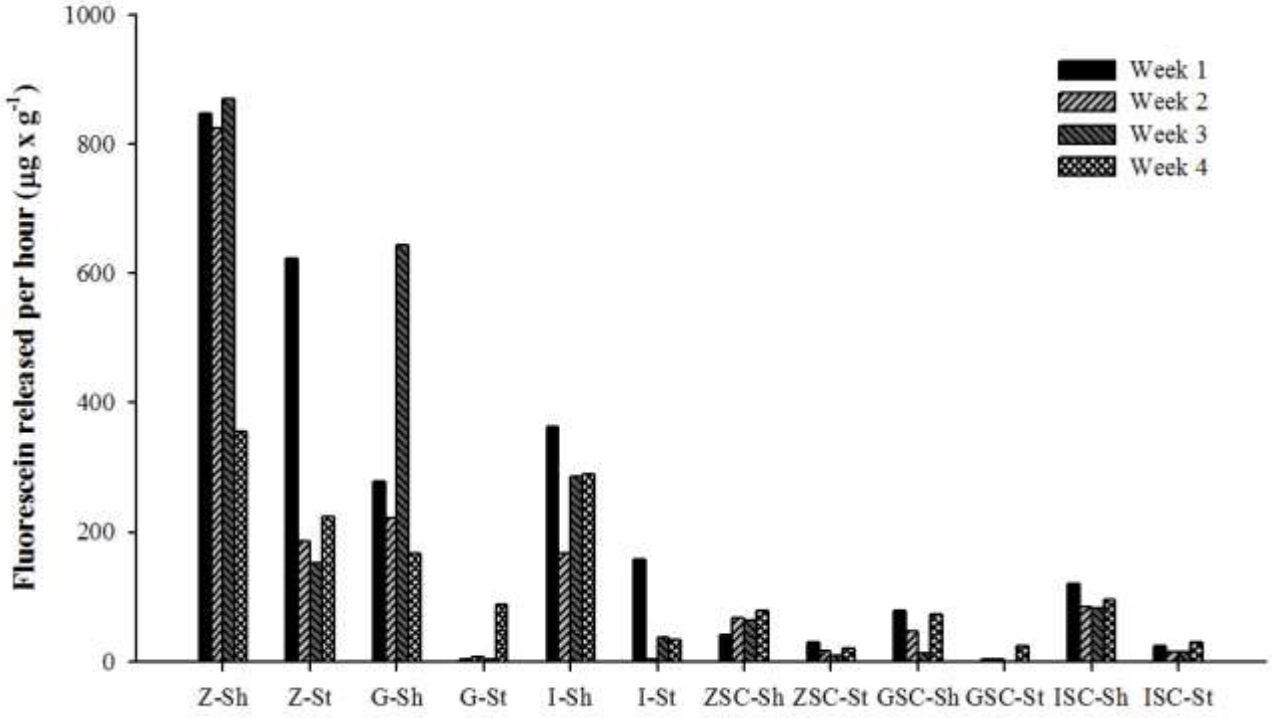


Figure 24: Changes in hydrolytic activities of faecal and soil samples stored at 4°C in January 2012

Key: Z-Sh: Zebra shaker; Z-St: Zebra static; G-Sh: Giraffe shaker; G-St: Giraffe static; I-Sh: Impala shaker; I-St: Impala static; ZSC-Sh: Zebra soil control shaker; ZSC-St: Zebra soil control static; GSC-Sh: Giraffe soil control shaker; GSC-St: Giraffe soil control static; ISC-Sh: Impala soil control shaker; ISC-St: Impala soil control static

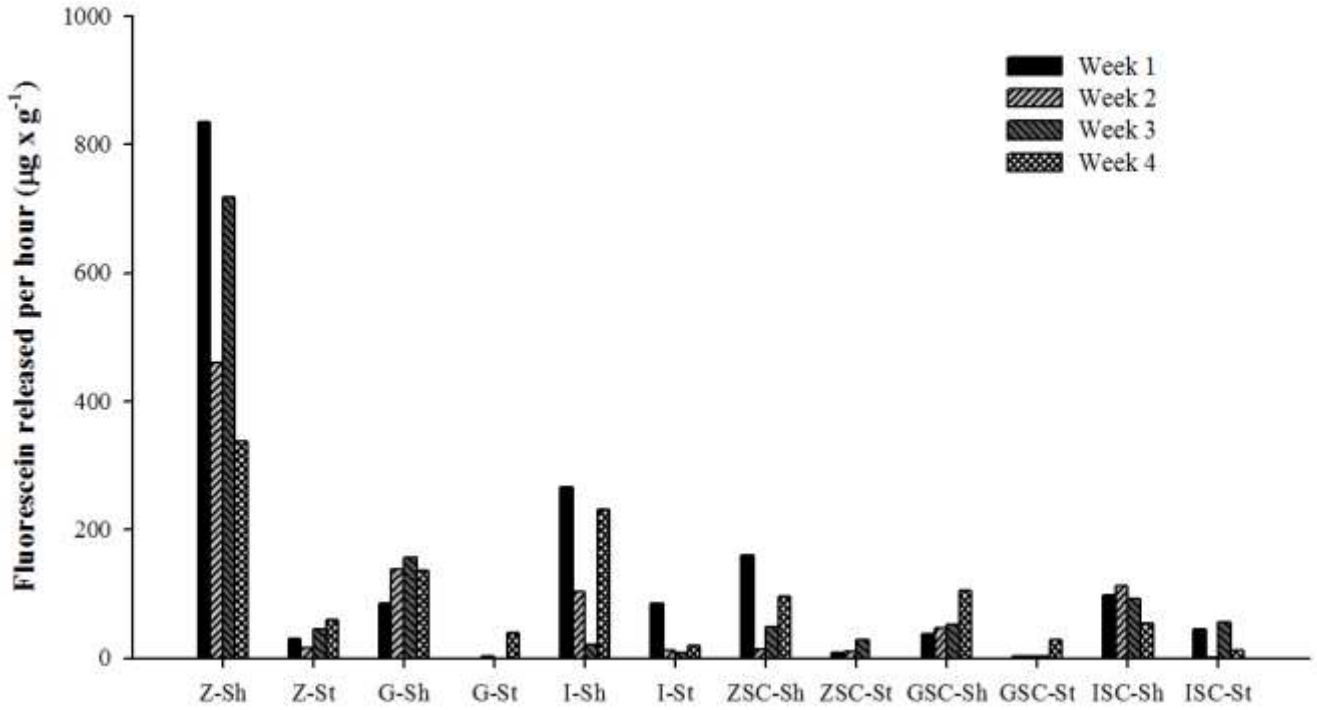


Figure 25: Changes in hydrolytic activities of faecal and soil samples stored at 30°C in January 2012

Key: Z-Sh: Zebra shaker; Z-St: Zebra static; G-Sh: Giraffe shaker; G-St: Giraffe static; I-Sh: Impala shaker; I-St: Impala static; ZSC-Sh: Zebra soil control shaker; ZSC-St: Zebra soil control static; GSC-Sh: Giraffe soil control shaker; GSC-St: Giraffe soil control static; ISC-Sh: Impala soil control shaker; ISC-St: Impala soil control static

Plate count data

January 2012 log_{10} (cfu/g) plate count changes with storage over four weeks

Week 1

Sample	PC agar	Starch agar (positives)	Casein agar (positives)	Tween agar (positives)	CMC agar (positives)
Zebra 4°C	8.63	7.86	8.58	8.48	8.59
Giraffe 4°C	8.02	7.9	7.28	7.36	7.2
Impala 4°C	8.27	6.5	8.22	7.98	8.22
Zebra soil control 4°C	6.26	0	5.08	6.16	6.09
Giraffe soil control 4°C	6.69	6.39	6.49	6.35	6.67
Impala soil control 4°C	6.66	<4	5.08	6.27	5.91
Zebra 30°C	8.11	7.18	7.49	7.41	7.85
Giraffe 30°C	8.8	8.74	8.79	8.28	7.65
Impala 30°C	8.07	7.14	7.71	8	7.13
Zebra soil control 30°C	6.94	5.08	<4	5.3	<4
Giraffe soil control 30°C	6.23	5.4	<4	5.47	5.61
Impala soil control 30°C	7.12	5.04	5.47	5.06	5.23

*<4: microbial numbers not determined due to plate counts lower than required for weighted means estimation

Week 2

Sample	PC agar	Starch agar (positives)	Casein agar (positives)	Tween agar (positives)	CMC agar (positives)
Zebra 4°C	8.26	7.58	7.88	7.88	7.4
Giraffe 4°C	7.61	7.2	7.43	7.2	7.26
Impala 4°C	7.64	7.3	7.53	7.66	7.08
Zebra soil control 4°C	5.79	<4	5.57	5.04	<4
Giraffe soil control 4°C	5.24	<4	5.21	<4	<4
Impala soil control 4°C	5.83	5.41	5.48	5.31	<4
Zebra 30°C	7.94	7.45	7.77	7.81	7.29
Giraffe 30°C	7.19	7.4	<4	7.14	7.17
Impala 30°C	7.23	7.23	4.08	<4	<4
Zebra soil control 30°C	5.74	5.24	5.54	5.17	<4
Giraffe soil control 30°C	5.09	<4	<4	4.98	<4
Impala soil control 30°C	5.83	5.43	5.6	5.04	<4

*<4: microbial numbers not determined due to plate counts lower than required for weighted means estimation

Week 3

Sample	PC agar	Starch agar (positives)	Casein agar (positives)	Tween agar (positives)	CMC agar (positives)
Zebra 4°C	7.93	7.52	7.53	7.59	7.36
Giraffe 4°C	7.37	7.28	7.24	7.18	7.23
Impala 4°C	7.32	7.39	7.17	7.15	<4
Zebra soil control 4°C	5.3	<4	5.06	5.17	4.98
Giraffe soil control 4°C	5.38	4.49	<4	<4	5.38
Impala soil control 4°C	5.69	5.48	5.46	4.96	<4
Zebra 30°C	7.56	<4	<4	7.24	7.12
Giraffe 30°C	7.27	7.18	<4	7.16	<4
Impala 30°C	7.18	7.12	<4	<4	<4
Zebra soil control 30°C	5.71	5.44	5.45	4.86	5
Giraffe soil control 30°C	5.59	5.52	<4	<4	<4
Impala soil control 30°C	5.67	5.22	5.14	4.98	5.22

*<4: microbial numbers not determined due to plate counts lower than required for weighted means estimation

Week 4

Sample	PC agar	Starch agar (positives)	Casein agar (positives)	Tween agar (positives)	CMC agar (positives)
Zebra 4°C	7.36	7.17	7.22	7.1	<4
Giraffe 4°C	7.66	7.32	7.11	7.24	<4
Impala 4°C	7.39	7.33	7.34	7.05	<4
Zebra soil control 4°C	5.58	5.32	5.49	4.98	5.3
Giraffe soil control 4°C	5.43	5.39	<4	<4	<4
Impala soil control 4°C	5.6	<4	4.66	5	<4
Zebra 30°C	7.61	<4	<4	7.26	<4
Giraffe 30°C	7.28	7.24	<4	7.09	<4
Impala 30°C	7.2	7.12	<4	6.58	7.08
Zebra soil control 30°C	5.43	4.98	4.94	<4	5.06
Giraffe soil control 30°C	<4	<4	<4	<4	<4
Impala soil control 30°C	5.15	5.08	5.12	<4	<4

*<4: microbial numbers not determined due to plate counts lower than required for weighted means estimation