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Moyo, Phanankosi

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The use of dung beetle larvae gut microbial consortia in the production of biogas and bio-ethanol

Moyo Phanankosi





Research report submitted in fulfilment of the requirements of the degree MSc. (Biotechnology) at the University of Witwatersrand, Johannesburg.

Project Title

The use of dung beetle larvae gut microbial consortia in the production of biogas and bio-ethanol

By

Moyo Phanankosi

Student number: 396087

Supervisor

Dr. K. Rumbold

Co-supervisor

Dr. M. Ntwasa

Research report submitted in fulfilment of the requirements of the degree MSc. (Biotechnology) at the University of Witwatersrand, Johannesburg.

Abstract

The goal of this study was to develop a standard and reproducible method for the propagation of *Euoniticellus intermedius* (Coleoptera: Scarabaeida) larvae gut microbial consortia and subsequently use them in the production of bio-ethanol and biogas. Homogenized midgut and hindgut sections of the larvae were enriched in 4 different media (NB, TSB, M1A and M1B) under oxic conditions for 4 weeks with TSB giving the highest microbial counts with 3×10^7 CFU/ml for the hindgut consortium and 1.9×10^7 CFU/ml for the midgut consortium. M1B enriched microbes gave the lowest counts at 1.1×10^7 CFU/ml and 1.0×10^7 CFU/ml for hindgut and midgut consortium, respectively.

Midgut consortium enriched in M1B showed the greatest visible filter paper degradation along with the unenriched midgut consortium from the dung beetle larvae. However, HPLC analysis only detected D-glucose from filter paper degraded by hindgut microbes from TSB (0.34 g/l), midgut microbes from M1B (0.03 g/l) and hindgut microbes from the dung beetle (0.09 g/l).

Ethanol detected from the experimental runs could not be conclusively attributed to gut consortia metabolic activity. Methane production was detected from the cultures incubated anaerobically for 8 weeks. The hindgut consortium consistently gave the highest concentration of methane with an average of 0.034 moles/l of methane produced from medium AM1 (carbon sources were D-glucose and D-lactose).

Automated ribosomal RNA intergenic spacer analysis (ARISA) showed a clear distinction between the bacterial communities found in the midgut and hindgut of *E. intermedius* third instar larvae.

Declaration

I, Moyo Phanankosi, declare that this report is my own unaided work. It is being submitted to The University of Witwatersrand for the degree of MSc (Biotechnology). It has not been submitted for any degree or examination in this or any other university.

Moyo P. (Mr.)

Date ……/……/……

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Lastly a big thank you to my family for being there for me all the time, thank you and God bless you all.

Abbreviations

NB – Nutrient broth

TSB – Tryptone soy broth

DB – Dung beetle

NB M – Midgut consortium enriched in nutrient broth

NB H – Hindgut consortium enriched in nutrient broth

TSB M – Midgut consortium enriched in Tryptone soy broth

TSB H – Hindgut consortium enriched in Tryptone soy broth

DB M – Midgut consortium from dung beetle larvae that have not been enriched

DB H – Hindgut consortium from dung beetle larvae that have not been enriched

HPLC – High performance liquid chromatography

GC – Gas chromatography

ARISA – Automated ribosomal RNA intergenic spacer analysis

PCR – Polymerized chain reaction

rRNA – Ribosomal RNA

M1A – Aerobic basal media

M1B – Aerobic basal media

M1C – Aerobic basal media

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Chapter 1

Introduction

1.1 Fossil fuels

The use of fossil fuels has generally come to be accepted as unsustainable due to reduction of resources and the amassing of greenhouse gases in the environment that have already surpassed the threshold of 450 ppm CO_2 (Schenk *et al*., 2008; Chisti, 2007; Demirbas, 2008; Balat and Balat, 2009). An increasing gap between the energy needs of the industrialized nations and the failure to replace this need with the limited sources of energy like the fossil fuels in turn enhance the dangers of global warming and energy crisis (Mohan *et al.,* 2008). Balat and Balat (2009), state that known petroleum reserves are difficult to access and a global peak on oil production has been put at between 1996 and 2035 by different studies.

Motor vehicles are responsible for a significant proportion of urban air pollution in developing countries (Balat and Balat, 2009). They account for over 70% the world's carbon monoxide emissions and 19% carbon dioxide emissions (Goldemberg, 2008). Hansen (2004), states that there are over 700 million light duty vehicles on roads around the world and the numbers are predicted to rise to 1.3 billion by 2030 and to over 2 billion vehicles by 2050 with most of the increase coming in the developed world. This increase will affect the stability of ecological systems, global climate and global oil reserves (Balat and Balat, 2009).

Of late, there has been a great disquiet on the possible impacts of greenhouse gases on the global weather system (Zecca and Chiari, 2010). The world has witnessed the eight warmest years in history, a rise in sea levels, higher number of hurricanes being recorded than ever before and deadly infectious diseases spreading beyond the tropics. Khasnis and Nettleman

(2005), state that global warming will have serious implications in human health. Climate refugee migration is going to result in changes on disease patterns. Drought, induced by change in weather systems as a result of global warming, will leave human beings more vulnerable to diseases due to compromised immunity systems (Khasnis and Nettleman, 2005).

Clearly the world is in great need of alternative greener energy sources that practically can sustain the globes' demand for energy. The very existence of mankind on earth lies in the finding of an alternative, environmentally friendly fuel. It is then that the interest in the use of bio-fuels world wide has strongly grown in recent years. Renewable, low carbon foot print fuels are necessary for the environmental and economic sustainability (Chisti 2007; Schenk *et al*., 2008), and healthy living conditions for humans (Khasnis and Nettleman, 2005).

1.2 Bio-fuels

Bio-fuels are liquid, gaseous or solid fuels made using organic material, such as agricultural crops, municipal, agricultural and forest wastes (Balat and Balat, 2009; Demirbas, 2007). Bio-fuels can significantly reduce emissions by vehicles if they are widely used (Balat and Balat, 2009). Biomass energy technologies use waste or plant material to produce energy with lower level of greenhouse gas emissions than fossil fuel sources (Sheehan *et al.,* 1998). They has been an increase in the use of advanced systems and improved bio-energy transformations utilizing a range of bio-fuels by developed nations (Puhan *et al.,* 2005). Fig. 1 and Table 1 clearly show increased interest in bio-fuels (bio-ethanol) production.

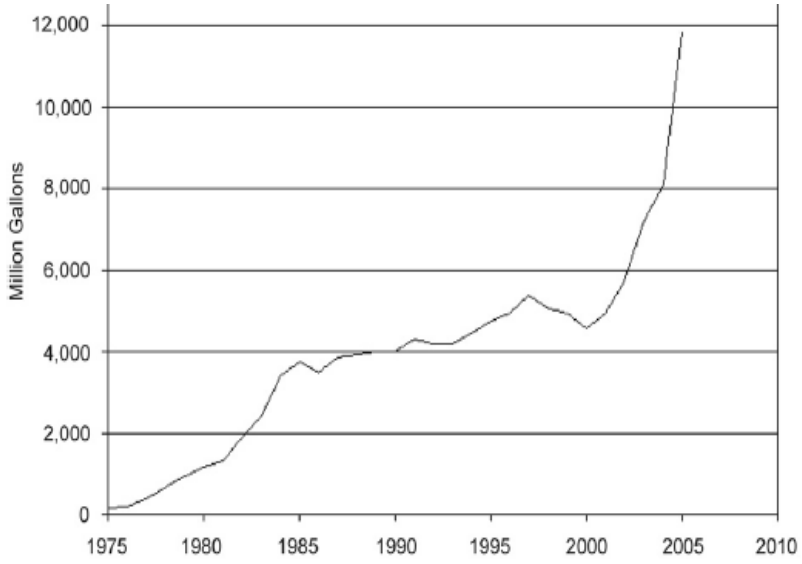


Fig 1: World ethanol production, 1975 – 2005

Adapted from Mathews, (2007)

Table 1: World ethanol production by Country, 2005

Country	Production	Production
	Million Liters	Million Gallons
United States	16,214	4,283
Brazil	16,017	4,244
China	3,800	1,004
India	1,700	449t
France	910	240t
Russia	750	198t
South Africa	390	103

Adapted from Mathews, (2007)

A variety of fuels can be produced from biomass resources including liquid fuels like ethanol, methanol and bio-diesel (Balat and Balat, 2009; Chisti, 2007; Demirbas, 2007; Jumbe *et al.*, 2009; Mshandete and Parawira, 2008), and gaseous fuels such as hydrogen and methane (Mshandete and Parawira, 2008; Demirbas, 2008; Tait *et al.*, 2009; Kaparaju *et al.*, 2009;

Coalla *et al.,* 2009; Lee *et al.,* 2009; Zhu *et al.,* 2007; Wang *et al.,* 2009; Kaparaju *et al.,* 2008; Schenk *et al*., 2008).

There are a numerous reasons why bio-fuels have been seen as potential replacements for fossil fuels by both developing and industrialised countries (Demirbas, 2007). These reasons range from energy and homeland security, environmental concerns, foreign exchange savings and socio-economic issues related to the rural sector (Demirbas, 2008). However, the prospect of oil exhaustion, the concerns of energy security and global warming are the main motives of bio-fuels promotion by public authorities in industrialized countries (Gnansounou *et al.,* 2009).

In general, bio-fuels have the following advantages (Puppan, 2002):

a.) they are made using readily available organic material,

b.) they have a low carbon foot print,

c.) there are many benefits for the environment, economy and consumers in using bio-fuels and

d.) they can be degraded biologically and do not persist in the environment for too long.

Vasudevan *et al.,* (2005), states that there are three ways in which biomass can be transformed to biofuels, namely thermochemical, chemical and biological. Biomass can be converted to bio-fuels via chemical (bio-methanol and biodiesel production), thermochemical (bio-crude oil, bio-syngas, bio-hydrogen production) and biochemical (bio-ethanol, biogas, bio-hydrogen and biodiesel production) methods (Demirbas, 2008).

Biomass seems to be an appealing feedstock for three main reasons. First, it is a renewable resource that can be sustainably developed in the future. Secondly, it appears to have good positive environmental properties resulting in no net release of carbon dioxide and very low

sulphur content. Thirdly, it appears to have significant economic potential provided that fossil fuel prices increase in the future (Cadenas and Cabezudo, 1998).

The abundance of biomass sources makes bio-fuels production more sustainable compared to other alternative green energy sources (Demirbas, 2008). The added advantage with biomass is its ability to produce bio-fuels in all three states of matter, which can be stored and used at places a distant from where they were made.

Industrialised and developing countries have set long term goals to substitute bio-fuels for fossil fuels. EU-Commission (2008), states that these goals are often in the range of 5-10% substitution to be reached within the next five years. However, today's production, in most countries, is under 2% and more effort has to be made to increase the percentage supplied by bio-fuels to reach these goals (Royal_Society, 2008). Scientific notion is now reaching an agreement that bio-fuels could account for 10% of all fuels by 2010, and for 20% by 2020, rising to 50% of a (reduced) consumption of transport fuels by 2050 (Mathews, 2007).

The use of bio-fuels has had its own fair share of problems. Among these problems has been the dilemma of using biomass resources like maize, rice and sugar cane to produce the so-called first generation bio-fuels (Zah and Ruddy, 2009; Balat and Balat, 2009; Stein, 2007). Though it may seem beneficial to use the readily available resources like maize and sugar cane, it raises many concerns about major environmental problems, food shortages and serious destruction of vital soil resources (Pimentel, 2008). The growth of biomass for bio-fuels production on arable land could also impact negatively on food security due to a reduction in agricultural land available for growth of food crops for human consumption. The draw back in producing bio-ethanol from sugar or starch is that the feedstock is expensive and is demanded for other applications as well (Enguidanos *et al.*, 2002). The high

feedstock price then translates to a high bio-fuel price which makes it uncompetitive to petroleum fuels.

Lignocellulosic biomass is potentially seen as being able to provide a great portion of the raw material needed for bio-fuel production (second generation bio-fuels, that is bio-fuels produced from lignocellulosic organic material where as first generation bio-fuels are produced from agricultural crops like maize, sugar and wheat) in the medium and long term due to its low cost and abundance (Gnansounou *et al.,* 2005). However, conversion technologies for producing bio-fuels from lignocellulose are under development and have not been demonstrated yet to be commercially viable (Balat and Balat, 2009)

1.2.1 Bio-ethanol

Bio-ethanol is produced by hydrolysis of organic material, followed by alcoholic fermentation in order to produce ethanol (Demirbas, 2008). Polysaccharides in plant materials can be transformed to monosaccharides by hydrolysis which is normally an enzyme catalysed process. Enzymatic hydrolysis of cellulose to glucose through treatment by enzymes is a major processing step in the ethanol plant (Kumar *et al.,* 2005). The glucose is subsequently fermented anaerobically and transformed to ethanol by the action of microorganisms, usually yeasts (Demirbas, 2008) like *Saccharomyces cerevisiae.*

Bio-ethanol can be blended with petroleum at different mixtures, for example E10 is a fuel mixture of 10% ethanol and 90% petroleum which can be used without having to adjust the car's engine (Demirbas, 2008). In fact, bio-ethanol has a higher octane number (Yoosin and Sorapipatana, 2007; Demirbas, 2008) or antiknock properties (Demirbas, 2008) compared to petroleum. Bio-ethanol also has broader flammability limits, higher flame speeds and a higher heat of vaporisation (Balat and Balat, 2009). These properties allow for a higher

compression ratio and shorter burn time which lead to theoretical advantages over petroleum fuels in an internal combustion engine (Balat, 2006).

However, bio-ethanol has the following disadvantages:

a.) it vaporizes more easily (Demirbas, 2008),

b.) has lower energy density,

c.) it is corrosive and

d.) has low vapour pressure and toxicity to ecosystems (Dufey, 2006).

Lignocellulosic materials serve as a less expensive and most available feedstock (Sassner *et al.,* 2008). Producing bio-ethanol form lignocelluloses, a non-food source for humans, is seen as a solution to the environmental and food-versus fuel concerns that are the major draw backs of producing bio-ethanol from food crops like sugar or corn (Seelke and Yacobucci, 2007).

1.2.2 Biogas

Biogas, a mixture of methane and carbon dioxide, is an environment friendly, clean, cheap and versatile fuel (Kapdi *et al*, 2005) produced by an anaerobic digestion process. The digestion process occurs in three sequential stages; in the first stage, hydrolytic microorganisms convert organic matter into a form that can be utilised by a second group of microorganisms, acetogens, in the second stage. Acetogens produce organic acids which are utilised by methanogens to produce methane in third and final stage of methane production (Parawira *et al.,* 2004; Kelleher *et al.,* 2000; Marty *et al.,* 2001; Demirbas, 2008).

There are a number of factors that impact on the relative measure of biogas produced during the anaerobic digestion process (Demirbas, 2008; Rastogi *et al.,* 2008). Amongst these factors are pH, temperature, hydraulic retention time, carbon/nitrogen ratio and microbial balance

(Demirbas 2008; Rastogi *et al*., 2008; Demirel and Yenigun, 2002; Chen *et al.,* 2008). Temperature is the most important parameter in the successful production of biogas (Demirbas, 2008; Rastogi *et al*., 2008; Demirel and Yenigun, 2002; Chen *et al.,* 2008).

1.2.2.1 Effect of temperature on biogas production

The most optimal temperature for the activity of anaerobic bacteria is 36.75 to 54.45 ºC with a temperature tolerance range from under 0 ºC to over 57.25 ºC (Demirbas, 2008). Temperature does not only affect the carbon and electron flow, but it also affects the composition of the methanogenic consortium (Chin *et al.,* 1999; Fey and Conrad, 2000). Chen *et al.,* (2008), states that temperature affects the growth rate of microorganisms and the concentration of free ammonium. A high temperature normally results in increased microbial metabolic activity. However, there is also an increase in the concentration of free ammonium as temperature increases (Chen *et al.,* 2008). Kroeker *et al.,* (1979), states that free ammonia is the main cause of anaerobic digestion process inhibition since it is freely membrane-permeable

1.2.2.2 Effect of pH on biogas production

In a review written by Chen *et al.,* (2008), pH is stated as one of the inhibitors of anaerobic digestion. This is due to the fact that pH affects the concentration of total ammonia nitrogen and growth of microorganisms (Kroeker *et al.,* 1979; Hashimoto, 1983, 1984; Hansen *et al.,* 1999). Increase in pH would result in shift to a higher free-ammonia-to-ionized-ammonia ratio (Chen *et al.,* 2008).

1.2.2.3 Effect of volatile fatty acids on biogas production

Volatile fatty acids are key intermediate metabolites in the anaerobic digestion process (Wang *et al.,* 2009; Wang *et al.,* 1999). Acetic acid and butyric acid are the main fatty acids

from which methane is produced by methanogens (Wang *et al.,* 2009; Wang *et al.,* 1999). All higher volatile fatty acids have to be first transformed into acetic acid before they can be used for methane production (Wang *et al.,* 2009).

1.2.2.4 Effect of microbial imbalance on biogas production

The inability of a significant number of biogas digesters to operate efficiently has generated an interest to look into a more detailed role of microorganisms in biogas production (Rastogi *et al*, 2008). Microbial imbalances lead to reduced efficiency of a biogas plant (Rastogi *et al*, 2008). Acid forming and methane producing bacteria, which have a key role in methane production, have been reported to differ widely in terms of physiology, nutritional needs, growth kinetics and sensitivity to environmental conditions (Pohland and Ghosh, 1971). The inability to maintain the balance between these two groups of microorganisms is the main cause of anaerobic reactor failure (Demirel and Yenigun, 2002).

1.2.2.5 Methanogens

Methanogens are strict anaerobic microorganisms which are methane producing that belong to the kingdom *Archaebacteria* (Abbanat *et al.*, 1989; Zinder, 1998). There are found at the end of a chain of a group of anaerobic microorganisms that synergistically work together in transforming biomass to methane (Abbanat *et al.*, 1989; Zinder, 1998). The methanogens metabolize acetic, formic acid or hydrogen produced by acidogens to produce methane (Abbanat *et al.*, 1989; Zinder, 1998; Wang *et al.,* 2009). Methanogens are categorized into three genuses, namely Methanobacterials, Methanococcales and Methanomicrobiales (Jones *et al.*, 1987).

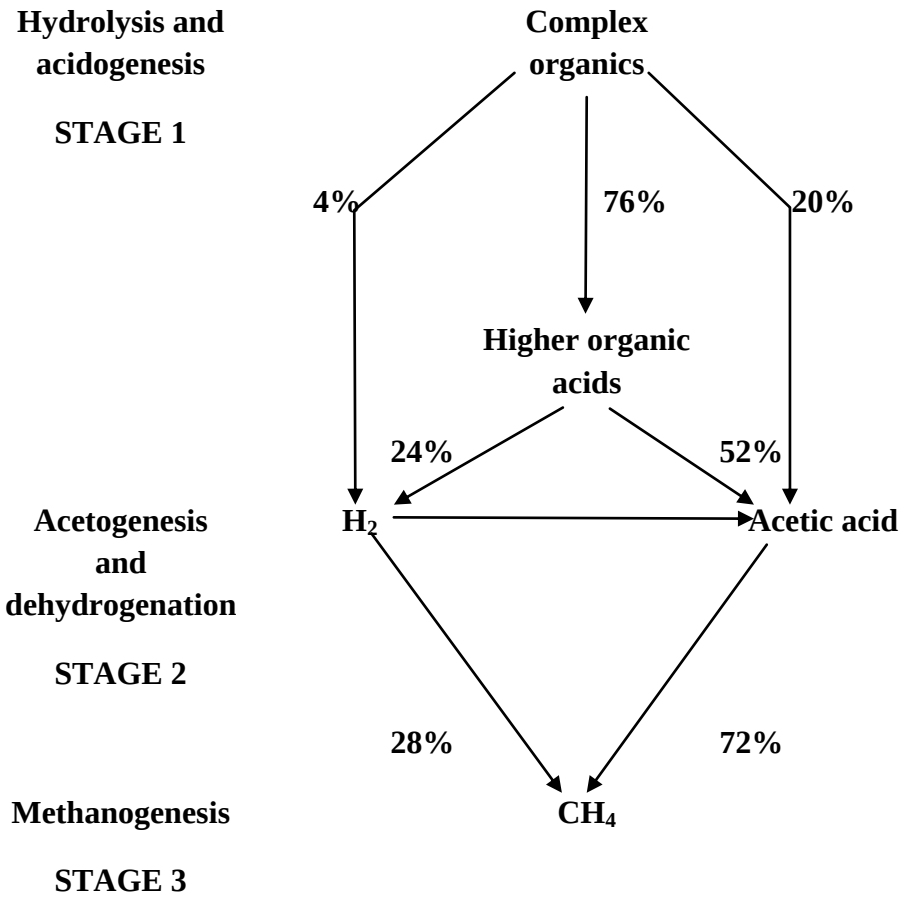


Fig 2: Methane production (McCarty, 1982).

There are two important pathways used in the dissimilation of organic materials during methane production; the reduction of carbon dioxide with hydrogen or formate as the electron donors and the conversion of acetate to methane and carbon dioxide (Abbanat *et al.,* 1989). There are many one carbon carrying coenzymes, only found in methanogens, which are involved in the carbon reduction pathway. These coenzymes are methanofuran, tetrahydromethanoprotein and coenzyme M (Abbanat *et al.,* 1989; Zinder, 1998).

Eikmanns and Thauer (1984), states that acetate is first activated before the cleavage of the carbon-carbon bond in electron and carbon transfer as it is transformed to methane. Increased levels of acetate kinase and phosphotransacetylase in *Methanobacterium formicicum* cells grown on acetate, suggest the combined process of the two enzymes may drive the activation of acetate to acetyl-CoA in this organism (Terlesky *et al.,* 1987).

1.3 Insect gut microbial consortia

Brune (2009), states that insects are the most thriving class of land animals, with respect to both species diversity and biomass. One important reason that has been attributed to this success has been their ability to feed on a variety of diets (Harada and Ishikawa, 1993) which includes fibre-rich foods (Brune, 2009). These feeding habits are closely related to endo-symbionts harboured by the insects with an estimated 10% of all insect species believed to contain microorganisms within their cells (Harada and Ishikawa, 1993). These intracellular symbionts are harboured by epithelial cells of the gut or some special cells present closely around the gut (Buchner, 1965; Ishikawa, 1989).

Research work by many scientists has shown that different insects have gut microbial consortia capable of digesting cellulose or hemicelluloses material into many different metabolites such as organic acids, ethanol and biogas (Egert *et al*., 2003; Egert *et al*., 2005; Lemke *et al*., 2003; Tholen *et al*., 1997; Tholen *et al*., 2007; Italo *et al*., 2005; Wagner and Brune, 1999; Bignell, 1994; Brennan *et al.,* 2004; Tokuda and Watanabe, 2007). These gut microbial consortia have been shown to play a vital role in the survival of these insects. Eutick *et al.,* (1978) and Fakatsu and Hosokawa (2002), have shown that loss of the gut consortia often results in abnormal development and reduced survival of the insect host.

Egert *et al.,* (2003) characterised the microbial community structure of bacteria and archaebacteria in the midgut and hindgut of the humus feeding larvae *Pachnoda ephippiata* (Coleoptera: Scarabaeida) using culture independent techniques. They showed that the bacterial consortium was dominated by phylogenetic groups with a fermentative metabolism. This was supported by high concentrations of lactate and acetate in both gut compartments (Lemke *et al*., 2003). *Actinobacteria* were dominant in the midgut while the hindgut was dominated by members of the *Cytophaga-Flavobacterium-Bacteroides* family (Lemke *et al*.,

2003). *Methanobactriaceae* related 16s rRNA genes were most frequent in the hindgut (Lemke *et al*., 2003).

Lemke *et al.,* (2003) showed the ability of the gut consortia in cellulose degradation only under oxic conditions as well as methane production exclusively by the hindgut microbial consortia. Analysis of the physicochemical gut conditions larvae showed the two gut compartments, midgut and hindgut, to have different pH values and redox potentials. The midgut was shown to have a more alkaline pH with a maximum value of 10.1 and 10.7 whilst the hindgut was slightly alkaline with a pH of 8.4 ± 0.1 (Lemke *et al.,* 2003). The reducing potential was shown to shift from oxidising conditions in the midgut of the first instars to reducing conditions in the second and third instar larvae. The hindgut was shown to be reduced at all the stages of growth (Lemke *et al.,* 2003).

Egert *et al.,* (2005), showed that *Melolontha melolontha* larvae also had a similar consortia pattern with that for *Pachnoda ephippiata* with the major difference being that *Methanobrevibacter* and not *Methanobacteriaceae* were the methanogens found in the hindgut. This may suggest that insects from the same order and subfamily may have significant similarities in their gut consortia.

Gut microbial consortia found in termites have been shown to play a role in the digestion of lignocellulose material ingested by termites (Brune, 1998; Tokuda and Watanabe, 2007; Tholen *et al.,* 1997). However, Brune (2009) and Tokuda and Watanabe, (2007) quickly point out that digestion of cellulose in termites is a combined effort of enzymes produced by both the host and the symbiotic consortia. Tokuda and Watanabe (2007) showed a significant decrease in cellulase activity within the hindgut of the termite *Nasutitermus takasagoensis* that had been treated with an antibiotic, implying a bacterial origin of the hindgut cellulases. In contrary, the midgut cellulase activity was not affected by antibiotic activity (Tokuda and

Watanabe, 2007). Their experimental results suggest the termites to be the source of the enzymes and not the bacteria as they would have been killed by the antibiotics.

In addition to their ability to digest cellulose, termites also have the ability to produce methane (Wagner and Brune, 1999; Brune, 2009; Brauman *et al.,* 1992; Shinzato *et al.,* 1992; Wheeler *et al.*, 1996; Bignell *et al.,* 1997; Sugimoto *et al.,* 1998). Wagner and Brune (1999) showed that termite gut emissions were more pronounced in the P3/4a compartment; whereas low rates were only observed in the P1 and P4b compartments (Fig. 3). This possible indicates the influence of the different microorganisms colonising the different gut compartments in methane production. As a result of difference in gut consortia, they could be a difference in concentrations of methane in different guts.

Brune (2009), states that the only insects known to produce methane are termites, cockroaches and scarab beetles. Methanogenesis occurs in the hindgut and is fueled by hydrogen and reduced one-carbon compounds that are formed during the fermentative breakdown of plant fiber and humus (Brune, 2009).

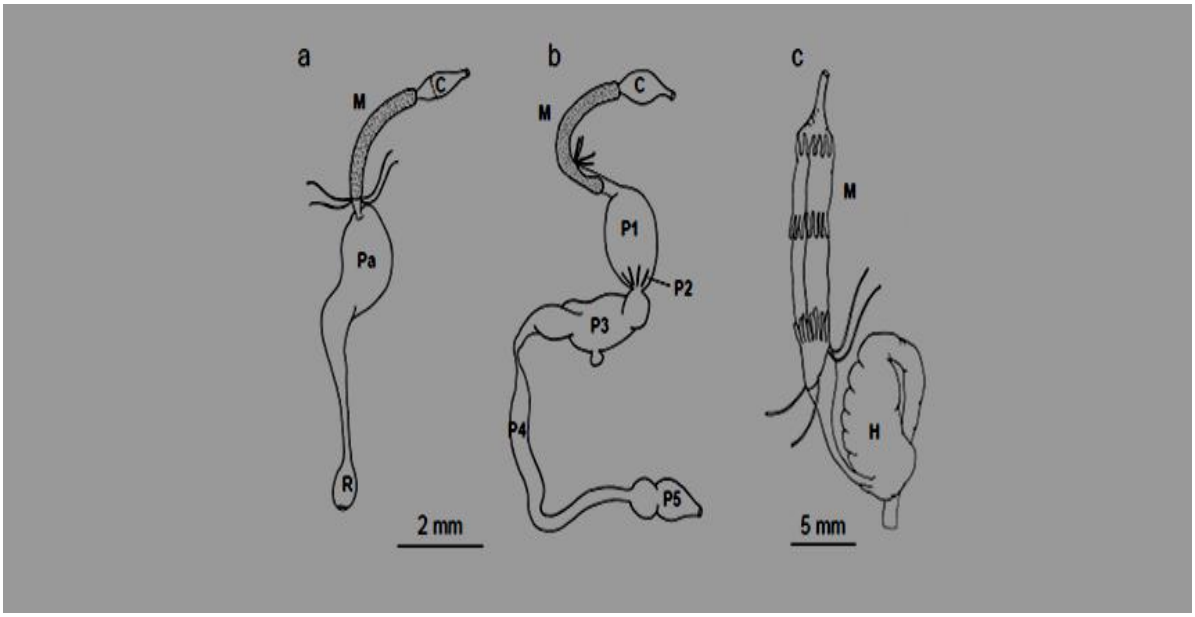


Fig 3: Structure of the intestinal tract of a lower termite (a), a higher termite (b), and a scarab beetle larva (c), crop (C) and the midgut (M) and hindgut (H) (Adapted from Brune, 2009).

Critically important to the production of methane is the supply of substrates used by methanogens; hydrogen, formate or acetic acid. It has been generally shown that gut consortia in different insects use different substrates. Lemke *et al.,* (2003), states that in cockroaches, hydrogen is transferred from the midgut to the hindgut where it is used by the methanogens as the electron donor in the process of methanogenesis. Similarly in termites, because of the proximity of the hydrogen producing and the hydrogen consuming compartments, hydrogen is used as the electron donor in methanogenesis (Wagner and Brune, 1999).

However, in scarab beetles like *Pachnoda epphippiata*, it has been shown that midgut fermentations are coupled to methanogenesis in the hindgut by formate transported via the hemolymph (Lemke *et al.,* 2003) and possible acetate. As part of this research project, it will be critical to try and point out which substrate is utilised efficiently by *E. Intermedius* gut consortia in methane production.

1.4 Justification

The world is quickly realising the need to find a new source of alternative energy as global warming becomes a big problem and world oil reserves become limited. Bio-fuels offer a reliable alternative and yet there are still not being extensively used. One of the major problems is their productive expense which translates to high product prices. One way of making them less expensive is by making the production process more efficient. Microbial starter cultures can play a vital role in improving the production of bio-fuels. Dung beetle larvae have gut microbial consortia that have the ability to degrade celluloid material to glucose and organic acids using them to produce bio-ethanol and biogas. The consortium is well balanced containing different microbes which work together synergistically in metabolising celluloid material to produce different metabolites particularly ethanol and biogas. The aim of this project is to isolate gut microbial consortia from dung beetle larvae and to propagate them to produce starter cultures that can be used in the production of biogas and bio-ethanol.

1.5 Project Aim

The main aim of this project is to evaluate the bio-ethanol and biogas production capabilities by the use of cellulose degrading microbial gut consortia of the dung beetle larvae of *E. intermedius* (Coleoptera: Scarabaeida).

1.6 Project objectives

1. To develop a standard and reproducible method for the isolation, propagation and characterisation of dung beetle larvae microbial gut consortia.
2. To evaluate dung beetle larvae microbial consortia in the production of bio-ethanol and biogas.

Chapter 2

Materials and Methods

2.1 Collection and maintenance of dung beetle *Euoniticellus intermedius* (Coleoptera: Scarabaeida)

Live adult female and male dung beetles were collected on a farm south of Johannesburg, South Africa between September and October 2009. Captured insects were placed into empty plastic (160mm x 130mm x 130mm) containers. The insects were bred in an insectry room in plastic (160mm x 130mm x 130mm) containers that were half filled with slightly moist soil.

Cow dung (fresh stock that was kept in a –20ºC freezer for a week) was placed on the soil and three breeding pairs of beetles (3 males and 3 females) were placed in the containers. Every 3 – 4 days, fresh cow dung was placed in the container. Once a week the containers were sieved and any broodballs found were removed. Surviving breeding pairs were placed in a new container with fresh soil and dung.

Collected broodballs were placed in a large (400 x 300 x 200mm) plastic container. These were covered with compact soil. A wet sponge was placed on the soil to keep it moist. Once beetles began to emerge, small plastic dishes filled with the dung were used as traps to capture them. These captured beetles were then used as the new breeding pairs to produce brood balls.

2.2 Dissection of the dung beetle larvae

The dissection method of Lemke *et al.,* (2003) was used with some modifications. A preparation dish filled with wax was sterilized leaving it for 24 hours covered with 70% ethanol. The dish was then placed in a laminar flow cabinet 2 hours prior to dissection to allow the ethanol to evaporate completely. Steel pins, forceps and scissors were sterilized by autoclaving (Vertical steam sterilizer, HL 341, Taiwan) for 15 minutes at 121ºC. Insect Ringer solution (0.9 g NaCl (Merck), 0.02g $CaCl_2$ (Merck), 0.02g KCl (Merck) and 0.02g $NaHCO_3$ (Merck)) was prepared following a protocol described by Hayashi and Kamimura (2001). The insect Ringers solution was autoclaved for 15 minutes at 121ºC.

Third instar larvae were used for all the experiments and all the dissection was done under the dissection microscope in a laminar flow cabinet. The larvae were first anesthetized by exposure to a nitrogen, hydrogen and carbon dioxide (71/7/22 vol/vol respectively) (Afrox grade) gas mixture for 15 minutes as described by Lemke *et al.,* (2003). Larvae were then fixed with steel pins, with the larvae laid on its sides, in a preparation dish filled with sterile insect ringer solution

The cuticle was cut along the sidelines and the ventral integument, circular muscles and trachea carefully removed. The head was decapitated followed by a circular cut on the anus. The intestinal tract was then carefully removed from the body. The midgut and hindgut were then separated at the midgut hindgut muscular junction.

2.3 Microbial gut consortia identification

Borneman and Triplett (1997), state that DNA fingerprinting methods are much faster and much less expensive techniques for studying microbial communities. A community is characterized in a shorter period of time than would have been done using cloning techniques and also includes DNA from unculturable microorganisms (Steele *et al.,* 2005). Automated ribosomal RNA intergenic spacer analysis (ARISA) is one of the techniques used in fingerprinting. Steele *et al.,* (2005), states that ARISA amplifies the variable spacer regions between the highly conserved 16s RNA and 23s RNA regions. The DNA from amplification is then run on an automated sequencer that produces an electropherogram separating the amplicons according to their size. The intergenic spacer sequence and size varies amongst the different taxa (Leuders and Friedrich, 2003).

2.3.1 DNA extraction

DNA was extracted from the midgut and hindgut samples using the ZR bacterial DNA kit (Zymo research, USA). Extracted and purified DNA was separated on a 1% agarose gel stained with ethidium bromide and visualized using ultraviolet light.

2.3.2 ARISA - PCR amplification

PCR reactions were performed on the DNA using a eubacterial specific primer set ITSReub and FAM (carboxy-fluorescein) labelled ITSF specific for the 16 rDNA intergenic spacer region. PCR reactions were done using a GeneAmp PCR System 2400 (AppliedBiosystems, USA). The reaction mixture contained 0.5 µl of the purified genomic DNA extracted from soil, 10 nM of each primer and 5 µl of KapaTaq readymix (KapaBiosystems, South Africa) in a total volume of 10 µl. The PCR conditions consisted of an initial denaturing step of 3 min at 95 °C followed by 38 cycles of 95 °C, for 30 s, 56 °C for 30 s and 72 °C for 60 s. The

reaction was completed with a final extension at 72 °C for 5 min and then cooled and held at 4 °C. PCR for each sample was performed in triplicate and pooled to eliminate background noise from the ARISA profile and reduce the PCR variability occurring. PCR samples were separated on a 1% agarose gel, stained with Ethidium Bromide and visualized using ultraviolet light.

The PCR products of every sample were run on an ABI 3010xl Genetic analyser to obtain an electropherogram of the different fragment lengths and fluorescent intensity. ARISA samples were run with ROX 1.1 size standard which varied from 20 to 900 bp. GeneMapper 4.1 software converted fluorescence data to an electropherogram and the peaks which represented fragments of different sizes are termed operational taxonomic units (OTU). Fragments less than 1% of total fluorescence was considered background

2.4 Aerobic cultivation of gut consortia

2.4.1 Medium preparation

Four different types of media were used in the aerobic cultivation of gut consortia. Nutrient broth (16g/l, Merck), Tryptone soy broth (30g/l, Merck), basal media M1A, modified from MM5 described by Lemke *et al* (2003), and M1B were used in the enrichment of the gut microbes. Composition of M1A was as follows, 1g/l NaCl, 0.5g/l KCl, 0.1 g/l $MgCl_2 \cdot 6H_2O$, 0.015g/l $CaCl_2 \cdot 2H_2O$, 0.3g/l NH_4Cl, 0.2g/l KH_2PO_4, 0.15g/l Na_2SO_4, 0.5g/l yeast extract, 0.225 g/l D-glucose and 0.855g/l D-lactose. 1ml of trace element solution SL11 (1.5 g/l $FeCl_2 \cdot 4H_2O$, 100 mg/l $CoCl_2 \cdot 6H_2O$, 100 mg/l $MnCl_2 \cdot 4H_2O$, 70.0 mg/l $ZnCl_2$, 36.0 mg/l $Na_2MoO_4 \cdot 2H_2O$, 24.0 mg/l $NiCl_2 \cdot 6H_2O$, 6.0 mg/l H_3BO_3, 2.0 mg/l $CuCl_2 \cdot 2H_2O$, 10.0 mg/l HCl (25%)) and Selenium tungstate solution (0.5 g/l NaOH, 4.0 mg/l $Na_2WO_4 \cdot 2H_2O$, 3.0 mg/l $NaSeO_3 \cdot 5H_2O$) were added to a liter of the prepared solution. M1B was similar to M1A

with the only difference that 0.405 g/l cellulose microcrystalline (Merck) was added and not the D-glucose and D-lactose as the carbon source.

Nutrient broth (16g/l, Merck) and Tryptone soy broth (30g/l, Merck) were prepared following manufactures instructions and then autoclaved at 121ºC for 15 minutes. M1A and M1B were prepared as described in the above paragraph with medium being corrected to pH 7 using Na_2HPO_4 (3.56g/l Na_2HPO_4). Prepared medium were then autoclaved at 121ºC for 15 minutes.

2.4.1.1 Medium inoculation and culturing

Euoniticellus intermedius larvae were dissected following the protocol described in section 2.2 of this report. The midgut and hindgut were separately homogenized in 8ml sterile phosphate buffered saline solution (8g/l NaCl, 0.2g/l KCl, 1.44g/l Na_2HPO_4 and 0.24g/l KH_2PO_4). 0.5ml of homogenate was then inoculated into culture tubes containing 4.5ml of the 4 different medium in triplicate for each gut section for each medium. Control media were set up by incubating the media without inoculation. The tubes were then incubated in the dark at 30ºC in a rotary shaker (Shaking incubator, 5082U, Labcon) at 100 rotations per minute for 4 weeks.

2.4.1.2 Plate counts and pH analysis

After 4 weeks of incubation (enrichment of microbes), plate counts were done for the inoculated culture medium from 2.2.1.1 using the pour plate method using solid Tryptone soy broth at 30g/l with Agar bacteriological (Merck) at 12g/l. From each culture bottle 1 ml was serially diluted in sterile saline solution (8.5g/l sodium chloride) and only the 10^{-4} dilution plated. The plates were then incubated for 24 hours at 30ºC with the plate counts read after the 24 hour period using a colony counter (3329, Dark field Quebec, USA). The pH (pH meter – D-82362, Inolab pH level 1) of all tubes was determined after pour plating.

2.4.2 Cellulose degradation

Basal media M1C was prepared using a similar recipe to that for M1A with the only difference being that Whatman filter paper strips (5.5cm x 1cm) were used as carbon source and not D-glucose and D-lactose. A pair of Whatman filter paper strips was added per tube containing 10 ml of the salts solution. The media was then autoclaved at 121ºC for 15 minutes. The tubes were inoculated with a loop of inoculum from plates after the plate counts from 2.2.1.2. Midgut and hindguts were inoculated in separate culture tubes in duplicate for each gut section. Control experiment was an uninoculated M1C.

Four M1C culture tubes (containing 9ml medium) were inoculated with each 1ml of the homogenized gut sections as described in protocol 2.2.1.1 of this report. Two tubes were inoculated with midgut homogenate (DB M) while the other two were inoculated with hindgut homogenate (DB H). All culture tubes were then incubated in the dark at 30ºC in an orbital incubator at 100 rotations per minute for 4 weeks.

2.4.2.1 Glucose, acetic acid and ethanol production analysis

After 4 weeks of incubation, liquid samples of 1ml were taken from the tubes from 2.4.2 and centrifuged (Mini spin, Epperndorf AG, Germany) at 10,000 rpm for 15 min, and the supernatant was passed through a 0.45µm membrane filter for the analysis of glucose, acetic acid and ethanol using high performance liquid chromatography (HPLC). The principle behind the function of HPLC is similar to that of gas chromatography as described in section 2.5.5 of this report. The difference is that in HPLC mobile phase is a liquid and not an inert gas as is the case with gas chromatography. HPLC used was the Agilent Technologies 1200 series, using a Biorad Fermentation Monitoring column (Particle size 9µm, 150 x 7.8mm), fitted with a refractive index column detector (Temperature at 40ºC). Mobile phase was 0.001M sulphuric acid and HPLC grade water with a flow rate of 0.8ml/minute.

2.5 Anaerobic cultivation of gut consortia

2.5.1 Medium preparation

The most critical step in preparing anaerobic media is oxygen removal by boiling the media and doing all the steps that follow in an oxygen free environment, for example under a nitrogen or carbon dioxide blanket (Zinder, 1998). Reducing agents like l-cysteine, sodium sulfide and palladium catalyst are added to remove oxygen. Sodium sulfide reacts with oxygen producing sodium sulfate, thereby removing the oxygen from the medium. Palladium catalyst catalysis the reaction between hydrogen and oxygen producing water and by so doing reduces the medium. Sodium sulfide allows less growth of contaminants in mixed culture systems studies than cysteine, which can be used as an energy source by some fermentative heterotrophs. However, some anaerobes find sodium sulfide toxic (Zinder, 1998). Resazurin

is used as an oxidation-reduction indicator. The redox indicator, resazurin, changes from purple to pink to clear when reduced.

Basal media AM5 described by Lemke *et al.*, (2003) with some modifications was used in the cultivation of the dung beetle larvae gut consortia. Composition of AM5 was as follows, 10mg/l resazurin, 1g/l NaCl, 0.5g/l KCl, 0.5g/l $MgCl_2 \cdot 6H_2O$, 0.1g/l $CaCl_2 \cdot 2H_2O$, 0.3g/l NH_4Cl, 0.2g/l KH_2PO_4, and 0.5g/l yeast extract.

After complete dissolution of the salts and yeast extract, the medium was then split into four conical flasks in equal volumes. Four different media were prepared, namely AM1, AM2, AM3 and AM4. The carbon sources for the different medium were, for AM1 0.225g/l D-glucose and 0.855g/l D-lactose, for AM2 0.405g/l cellulose microcrystalline (Merck), for AM3 0.225g/l D-glucose, 0.855g/l D-lactose and 0.17g/l sodium formate and for AM4 0.225g/l D-glucose, 0.855g/l D-lactose and 0.2g/1 sodium acetate.

2.5.2 Reducing solution preparation

1. 0.2 N NaOH.....200.0 ml
2. $Na_2S \cdot 9H2O$ .....2.5 g
3. L-Cysteine · HCl.....2.5 g

The sodium hydroxide solution was brought to a boil and bubbled with carbon dioxide (99.999%, Afrox) for 15 minutes. The solution was then allowed to cool and sodium sulfide and cysteine were added.

2.5.3 Hungate tube perpetration

Each Hungate tube (15ml size tubes) was first dusted with carbon dioxide (99.999%, Afrox) for 3 minutes so as to remove oxygen from the sealed tubes. Two sterile needles were used both plugged with cotton wool. One needle was connected to the gas supply line, to bring in carbon dioxide, whilst the other allowed the air to come out the tube during gassing. After autoclaving, the tubes containing the reduced media were further dusted for 3 minutes removing any traces of oxygen.

2.5.4 Reduced medium preparation

The medium, AM1, AM2, AM3 AND AM4, were brought to a boil while gassing with carbon dioxide. The solution was kept boiling for several minutes until the indicator color turned from blue to reddish-pink at which stage the reducing solution from 2.5.2 was added (40ml/l) during continuous gassing and boiling. The pink color disappears, indicating reduction. All media were corrected to pH 7 using 1 M $NaHCO_3$. A palladium catalyst was then added to the reduced media which was left overnight in an anaerobic chamber (Forma anaerobic systems, 1025/1029 USA). The reduced media were added to dusted Hungate tubes (4.5 ml/ tube), from 2.3.2, anaerobically under nitrogen, hydrogen and carbon dioxide (71/7/22% vol/vol, respectively) gas mixture in an anaerobic chamber (Forma anaerobic systems, 1025/1029 USA). The tubes were then autoclaved for 15 minutes at 121ºC. Anaerobically homogenized (Prepared by homogenizing midgut and hindgut sections separately in 8ml anaerobic sterile phosphate buffered saline solution in an anaerobic chamber) gut sections were then inoculated into the reduced medium (0.5ml of homogenate to 4.5ml reduced medium) working in the anaerobic camber. The inoculated tubes were then incubated in the dark at 30ºC in an orbital incubator at 100 rotations per minute for 8weeks.

2.5.5 Methane analysis

Methane gas analysis was done using a gas chromatography. As for all other chromatographic techniques, a mobile and a stationary phase are required. The mobile phase is comprised of an inert gas e.g. helium, argon and nitrogen. The stationary phase consists of a packed column where the packing or solid support itself acts as stationary phase, or is coated with the liquid stationary phase. More commonly used in many instruments are capillary columns, where the stationery phase coats the walls of the small-diameter tube directly.

The main reason why different compounds can be separated this way is the interaction of the compound with the stationary phase. The stronger the interaction is, the longer the compound remains attached to the stationary phase, and the more time it takes to go through the column. Different detector types are used in gas chromatography. These include flame ionization detector, thermal conductivity detector and electron capture detector. Gas chromatography is well suited for more volatile compounds.

After 8weeks of incubation of samples from 2.5.4, all samples were analyzed for methane and hydrogen production using a gas chromatography (Clarus 500, Perkin Elmer USA) equipped with a thermal conductivity detector (temperature set at 200ºC), using argon as the carrier gas at flow rate of 30ml/minute. 50 µl of gas sample was injected from each Hungate tube into the gas chromatography.

Chapter 3

Results and discussion

3.1 *Euoniticellus intermedius* culturing



Fig 4: Adult female ***Euoniticellus intermedius.***



Fig 5: ***Euoniticellus intermedius*** egg in brood ball.



Fig 6: 5 day larvae in brood ball.

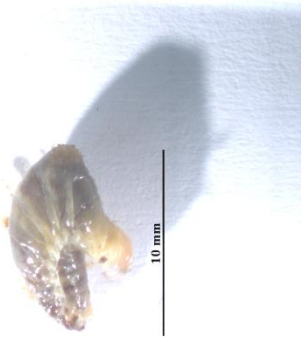


Fig 7: 17 day larvae ***Euoniticellus Intermedius.***

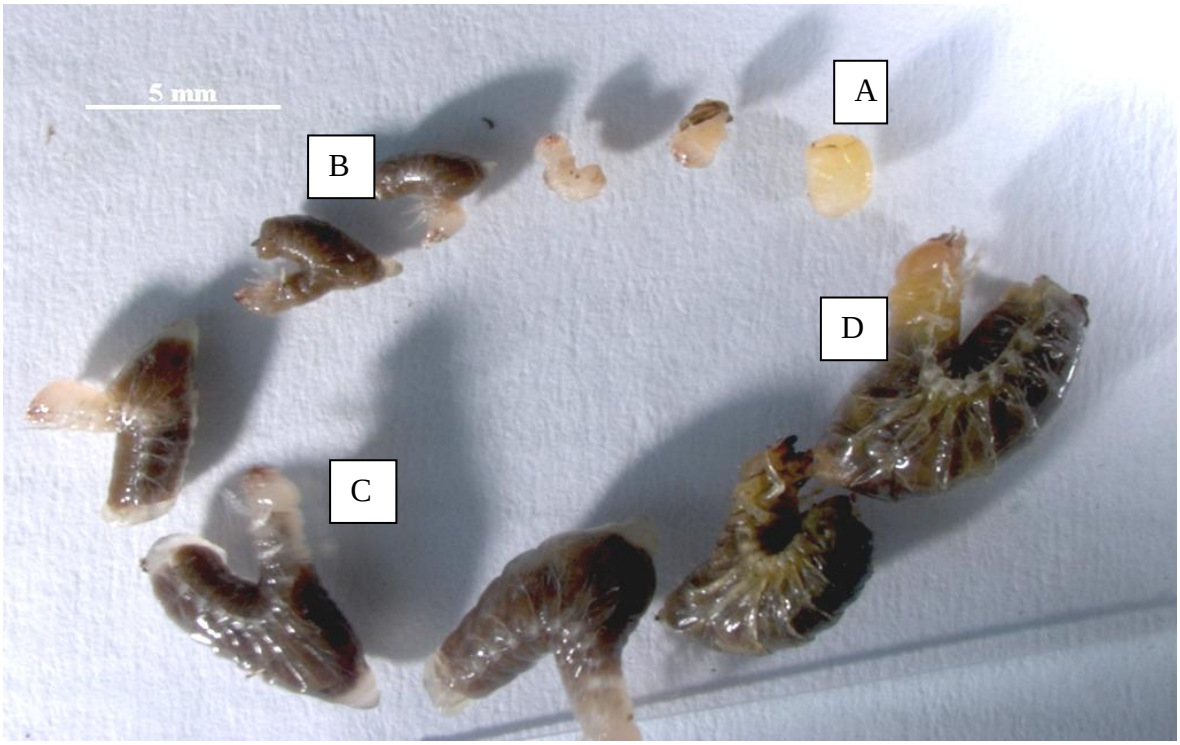


Fig 8: Life cycle of larvae from egg to first instar, second instar and finally third instar. A is a 1 day old egg, B is a first instar, C is a second instar and D is a third instar. Third instar larvae intestinal tract was used all through for the experiments in this project.

Euoniticellus intermedius adults (Fig. 4) collected reproduced in a normal life cycle. The females tunnelled into the soil within the containers where they buried brood balls each containing a single egg inside as seen in Fig. 5. It took a period of 5 – 6 days for the egg to develop into an embryo. Embryo then matured into a first instar within a week, the first instar then developed into second and third instar within a 2 week period. Third instar finally matured into a pupa which eventually developed into an adult dung beetle that hatch out of the broodball. The developmental stages time period shown by this particular culture indicated that we were working with a normal and healthy culture of *Euoniticellus intermedius* dung beetle larvae as the time lines of the life cycle are similar to those reported by Tyndale-Biscoe (1990).

3.2 *Euoniticellus intermedius* Larvae dissection

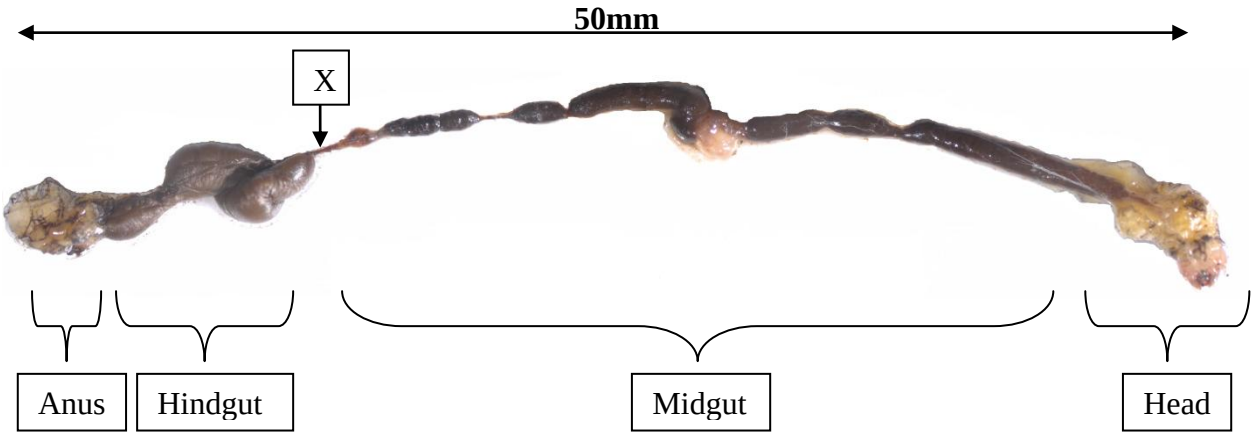


Fig 9: Photographic image of ***Euoniticellus intermedius*** larvae intestinal tract. Point X indicates where midgut was separated from hindgut. All pictures shown (Fig. 4 – Fig. 9) were captured using AxioVision 4.8.0.0. Hindgut is clearly seen as a chamber whilst midgut is long and relatively undeveloped. There is no clear difference between midgut and foregut. For this project the midgut included the foregut.

The intestinal tract of *Euoniticellus intermedius* showed two clear distinct gut systems, a long cylindrical midgut and bulbous hindgut (Fig. 9). This type of intestinal tract resembles that of humus feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae) reported by Lemke *et al.*, (2003). Similarity in physical structure of intestinal tracts of the insects pointed out that the intestinal tract system of the culture we used was normal considering that the insects belong to the same order and subfamily. However, to our knowledge there is no literature available that directly shows the intestinal tract of *Euoniticellus intermedius* and, therefore, we cannot fully conclude that the structure of the gut system was normal or abnormal. The accurate identification of the gut systems was vital as we specifically looked into culturing the different microbes in the midgut and hindgut separately. An Entomologist, who has worked extensively with the *Euoniticellus intermedius* larvae, was consulted and confirmed the midgut and hindgut sections as we had identified them.

3.3 DNA extraction and ARISA - PCR amplification

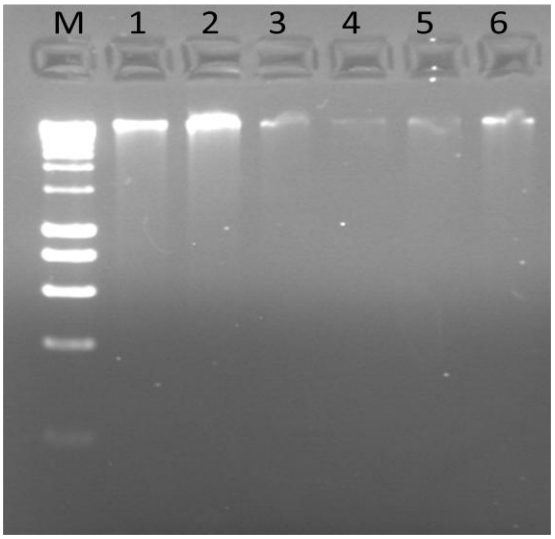


Fig 10: Agarose gel electrophoreses total extracted DNA from samples 1 – 6. Sample 1 is midgut and its contents, 2 is hindgut and its contents, 3 is midgut contents only, 4 is hindgut contents only, 5 is midgut empty and 6 is hindgut empty.

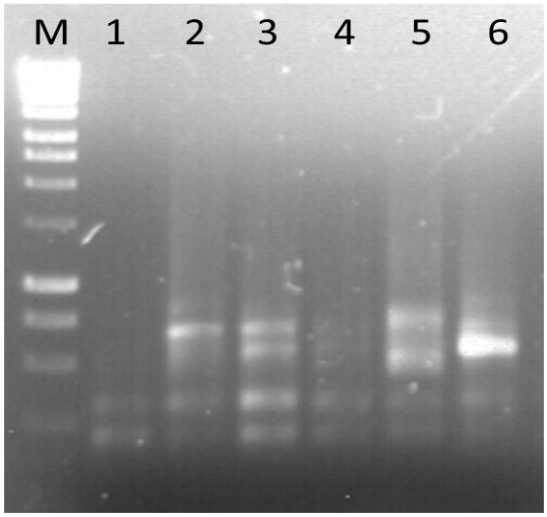


Fig 11: Agarose gel electrophoreses of bacterial ARISA PCR amplification of total extracted DNA. Sample 1 is midgut and its contents, 2 is hindgut and its contents, 3 is midgut contents only, 4 is hindgut contents only, 5 is midgut empty and 6 is hindgut empty.

Table 2: **The Shannon diversity and number of OTUs observed with ARISA in samples 1-6.**

Sample	1	2	3	4	5	6
Diversity index	2.502142	3.141821	2.275637	2.845046	2.854476	3.051267
Number of OTUs	13	23	13	17	17	23

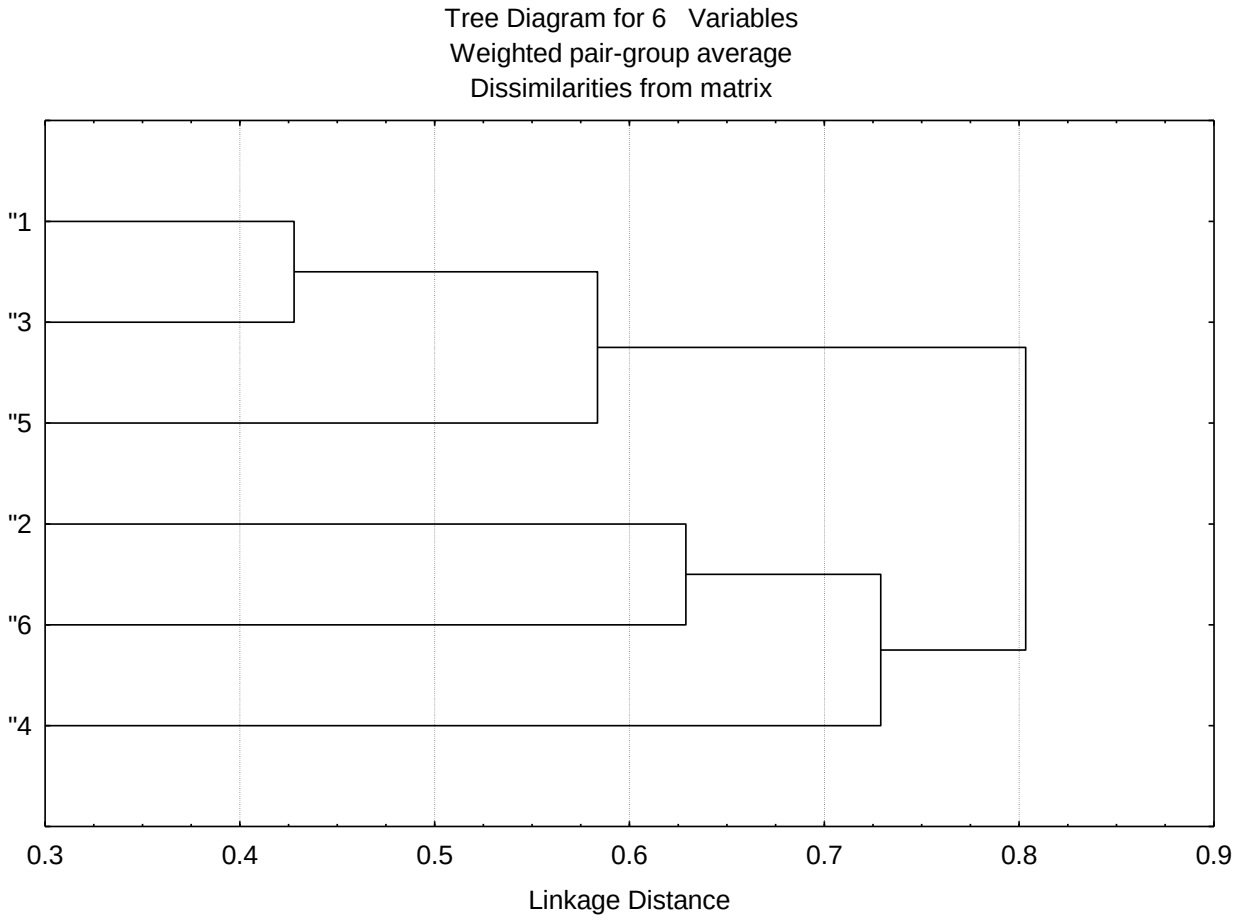


Figure 12: Tree diagrams of the Cluster analysis of the Whittaker similarity analysis for the bacteria using weighted pair-group average.

Automated ribosomal RNA intergenic spacer analysis (ARISA) is molecular fingerprinting method applied in the study of microbial communities (Steele *et al.,* 2005: Ranjard *et al.,* 2001: Fisher and Triplett, 1999). Ranjard *et al.,* (2001), states that the principle behind the method is based on the measurement of the intergenic spacer (IGS) length between small (16S) and large (23S) subunit genes in the *rrn* operon. ARISA has been used in the characterisation of bacterial and fungal soil communities (Ranjard *et al.,* 2001), monitor the structure of microbial communities in ethanol bioreactors (Steele *et al.,* 2005), comparing fungal communities from four salt mash plants (Torzilli *et al.,* 2006) and in the study of fresh water bacterial communities (Fisher and Triplett, 1999).

The different ARISA-PCR bands shown on Fig. 11 show that there are different bacterial communities found in samples 1-6. This was further confirmed by the differences in the Shannon diversity index and the difference in the number of operational taxonomic units (OTU) as seen in Table 2. Tree diagrams of the Cluster analysis of the Whittaker similarity analysis for the bacteria using weighted pair-group average from Fig. 12 clearly shows that there is a distinction between the samples 1, 3 and 5 from samples 2, 4 and 6. Samples 1, 3 and 5 were all coming from the midgut while 2, 4 and 6 all came from the hindgut, therefore, this suggest that there is a difference in the community of bacteria found within the midgut and those in the hindgut. These results are similar to those reported by Egert *et al.,* (2003) and Egert *et al.,* (2005) who have also showed that there is a difference in the communities of bacteria found between the midgut and hindgut of *Pachnoda ephippiata* and *Melolontha melolontha* larvae (Coleoptera: Scarabaeidae), respectively.

3.4 Aerobic culturing results

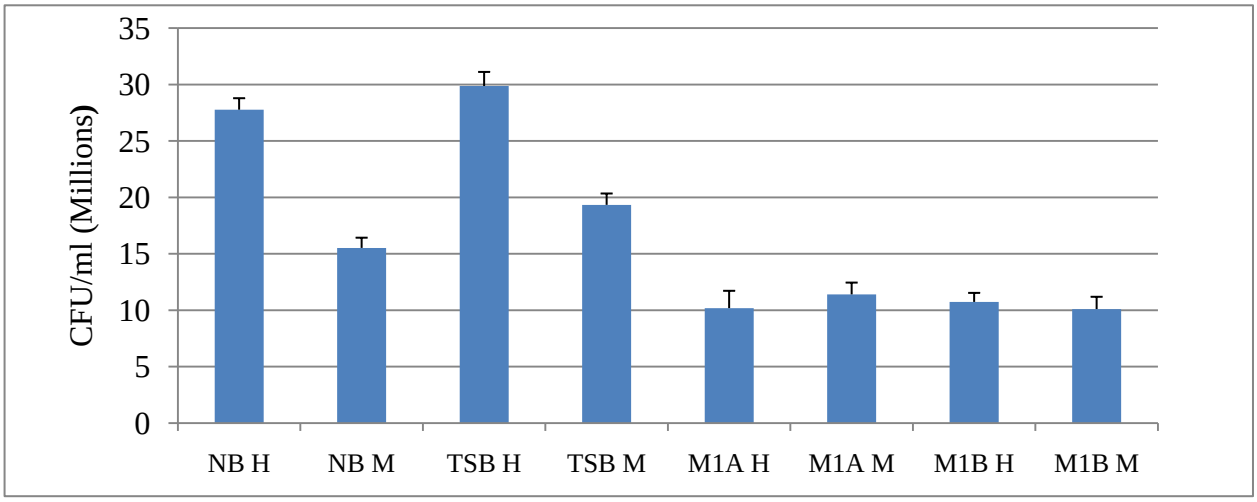


Fig 13: Plate counts of *Euoniticellus intermedius* larvae after enrichment from Nutrient broth (NB), Tryptone soy broth (TSB), Basal medium M1A and M1B. H is hindgut while M is midgut consortia. All control experiments gave 0 CFU/ml after incubation. Incubation period was 4 weeks at 30ºC in the dark.

Microbial gut consortium enriched in Tryptone soy broth gave the highest number of plate counts showing that it was most suited for growing the gut consortium as seen on Fig. 13. On two media (NB and TSB) hindgut plate counts were consistently higher than those of the midgut. This was consistent with results given by other researchers (Lemke *et al.,* 2003; Egert *et al.,* 2005). Lemke *et al.,* (2003) report cell counts for hindgut to be 3 – 6 fold those of the midgut whilst Egert *et al.,* (2005) report cell counts for midgut to be 5 times lower than of the hindgut.

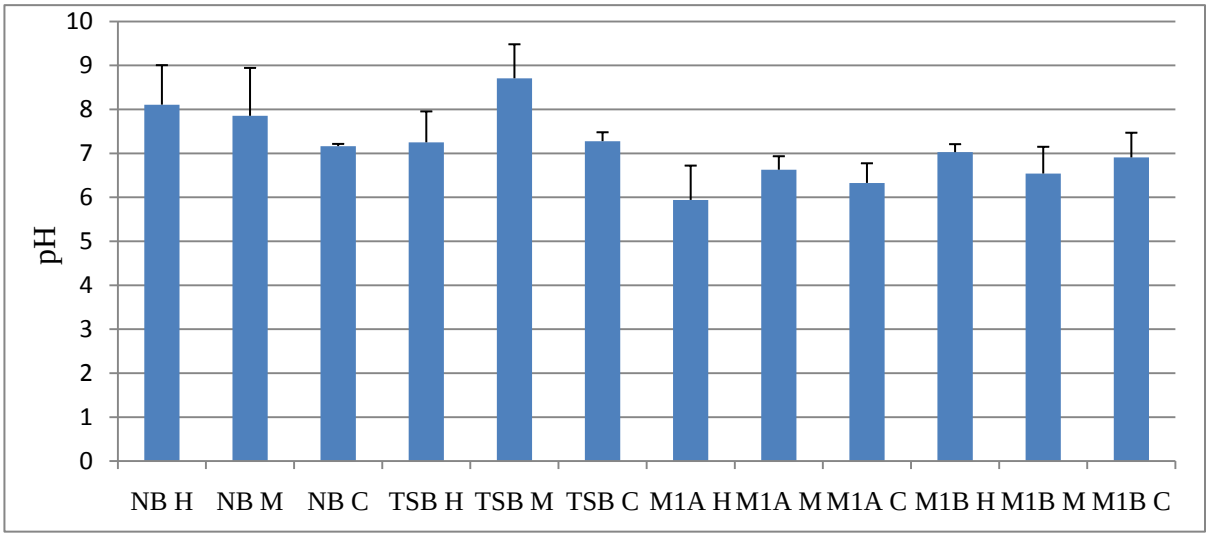


Fig 14: Average pH analysis of the liquid media after enrichment from Nutrient broth (NB), Tryptone soy broth (TSB), Basal medium M1A and M1B. H is hindgut while M is midgut consortia. C is the controls. Incubation period was 4 weeks at 30ºC in the dark.

Midgut consortia cultured in NB gave the highest average pH value of 9.5 with the lowest average pH value being recorded from M1A hindgut.

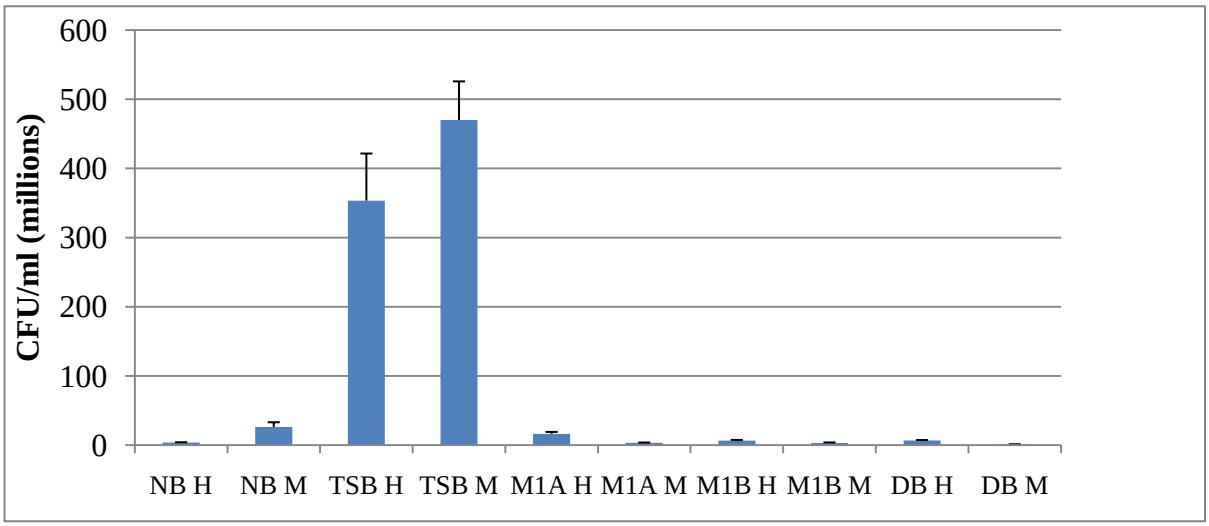


Fig 15: Plate counts of *Euoniticellus intermedius* larvae after cellulose filter paper degradation in M1C. Incubation period was 4 weeks at 30ºC in the dark.

Fig. 15 shows the number of microbial counts after degradation of the filter papers and it is very interesting to note that DBM which had the most visible filter paper degradation had the lowest number of microorganisms at 9.1 x 10^4 CFU/ml compared to TSB M with 4.7 x 10^8 CFU/ml.

3.5 Cellulose degradation

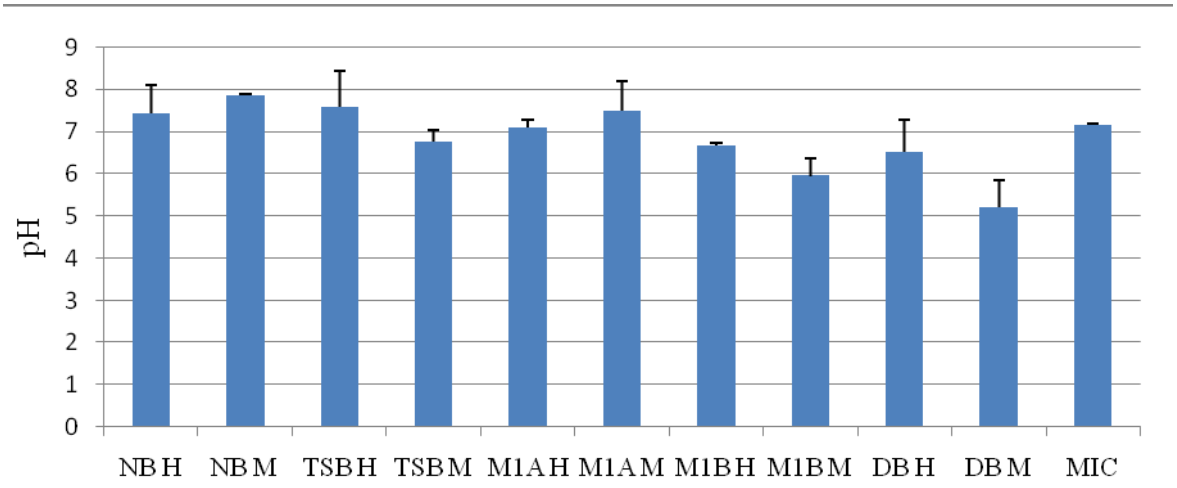


Fig 16: Average pH measurement of the liquid medium after cellulose filter paper degradation in medium M1C by microbial consortia initial enriched in Nutrient broth (NB), Tryptone soy broth (TSB), basal medium M1A and M1B. DB is microbial consortia coming directly from the gut larvae that had not been enriched and inoculated into M1C straight from homogenization. Incubation period was 4 weeks at 30ºC in the dark aerobically.

An average pH value drop from 7.14 (MIC control pH value) to an average pH value of 5.2 for DB M shown in Fig. 16 was the greatest drop recorded for all the samples. This drop could be attributed to the high concentration of acetic acid of 1.1 g/l shown in Fig. 17. There were, however, some unidentified peaks on the chromatogram (Fig. A9) that possible may be responsible for the drop in average pH. Lemke *et al.*, (2003) do report production of other organic acids like formate, lactate and succinate by microbial consortium in the larvae of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Production of these organic acids by microbial consortium in the larvae of *Euoniticellus intermedius* (Coleoptera: Scarabaeidae) could possible also explain the decline in pH after filter paper degradation.

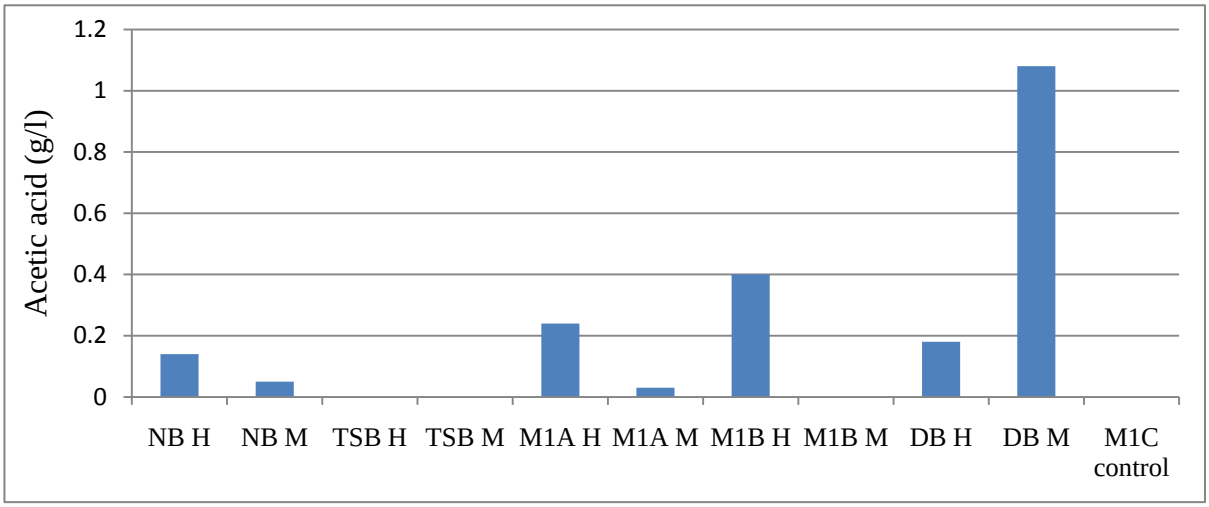


Fig 17: HPLC results for acetic acid production by gut consortia of *Euoniticellus intermedius* larvae after 4 weeks of incubation.

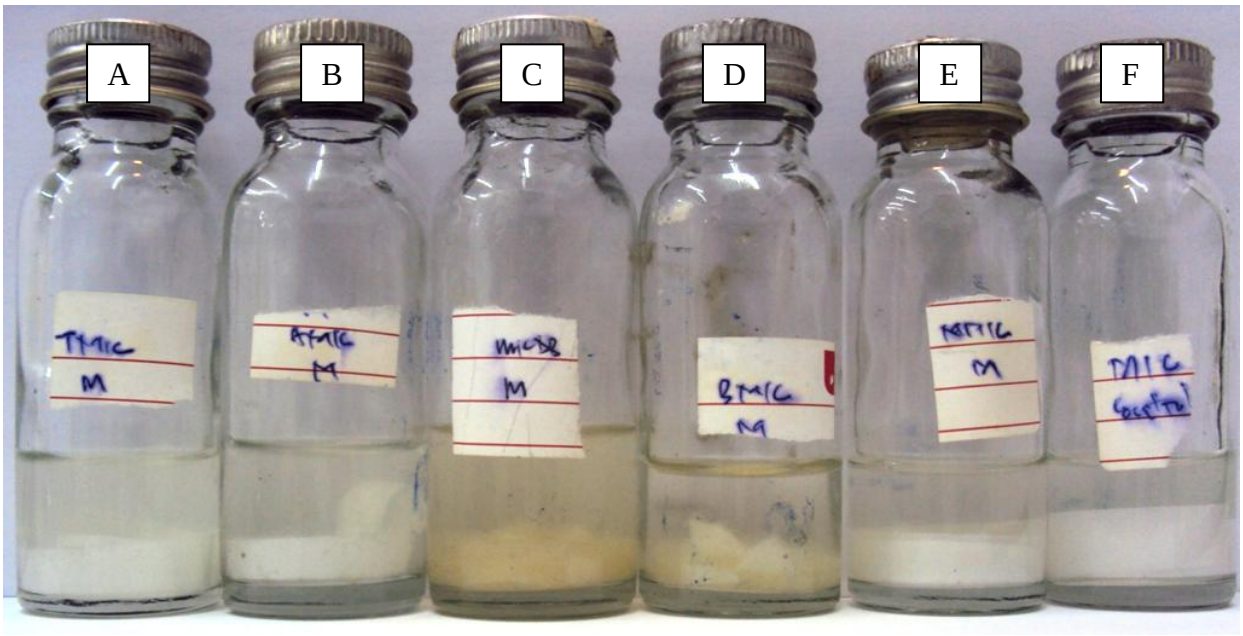


Fig 18: Photographic images of filter paper degradation by *Euoniticellus intermedius* midgut consortia. A is the midgut consortia enriched in TSB (TSB M), B is midgut consortia enriched in M1A (M1A M), C is midgut consortia originally from the larvae which had not been enriched (DB M), D is midgut consortia enriched in M1B (M1B M), E is midgut consortia enriched in NB (NB M) and F is the control (non inoculated culture). Control filter paper is still completely intact. Incubation was done for 4 weeks at 30ºC in the dark.

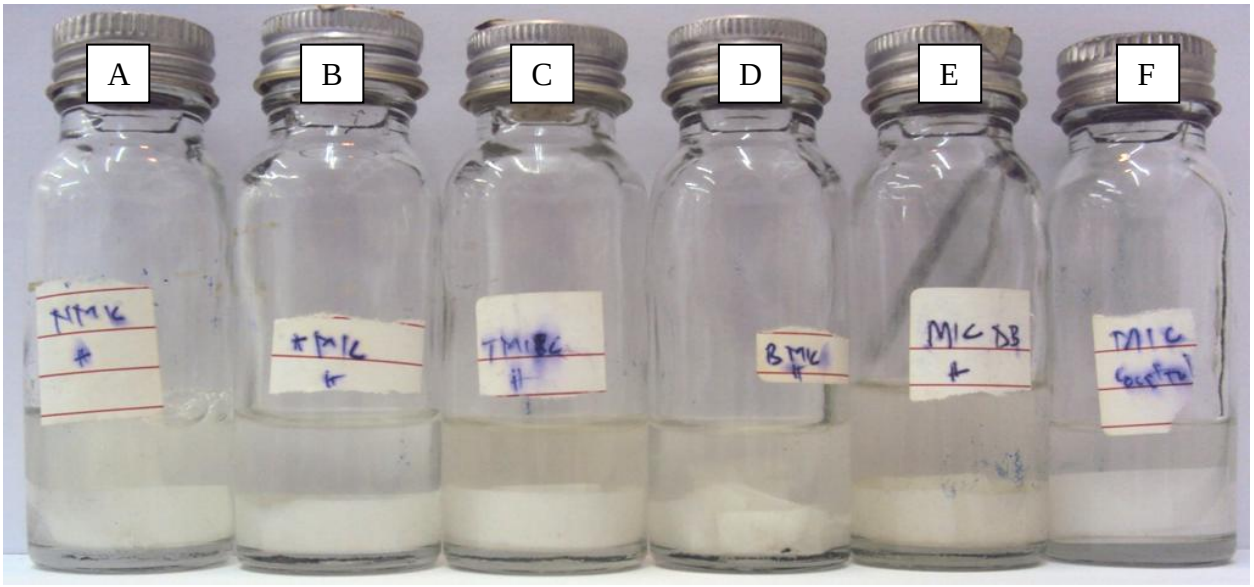


Fig 19: Photographic images of filter paper degradation by *Euoniticellus intermedius* hindgut consortia. A is the hindgut consortia enriched in NB (NB H), B is hindgut consortia enriched in M1A (M1A H), C is hindgut consortia enriched in TSB (TSH H), D is hindgut consortia enriched in M1B (M1B H), E is hindgut consortia originally from the larvae which has not been enriched (DB H) and F is the control (non inoculated culture). Control filter paper is still completely intact. Incubation was done for 4 weeks at 30ºC in the dark.

From Fig. 18, tubes C and D and Fig. 19, tube D, it is clearly visible that there was degradation of the filter paper by the midgut and hindgut microbial consortium under oxic conditions. Non-enriched midgut consortium, DB M, produced the greatest amount of degradation followed by midgut consortium enriched in basal medium M1B and lastly hindgut consortium enriched in M1B. Lemke *et al.*, (2003) report the complete dissolution of filter paper disks by the consortium under oxic conditions. Cellulose degradation amongst the larvae has long been debated as to whether the host produces the enzymes that degrade the cellulose or the symbionts do the degradation or alternatively play a supporting role. Results from Fig. 18 and 19 indicate that the symbionts in the midgut and hindgut of the *Euoniticellus intermedius* third instar larvae do degrade the cellulose and, therefore, could be degrading the cellulose feed for the host or possible play a supporting role with the host producing more cellulose degrading enzymes.

M1B H and M1B M show the greatest amount of filter paper degradation for all the enrichments, probably due to the fact that the consortium have been enriched on a cellulose carbon substrate basal medium M1B. This then meant that consortium does not go through a long lag phase in the basal medium M1C compared to the other enrichments.

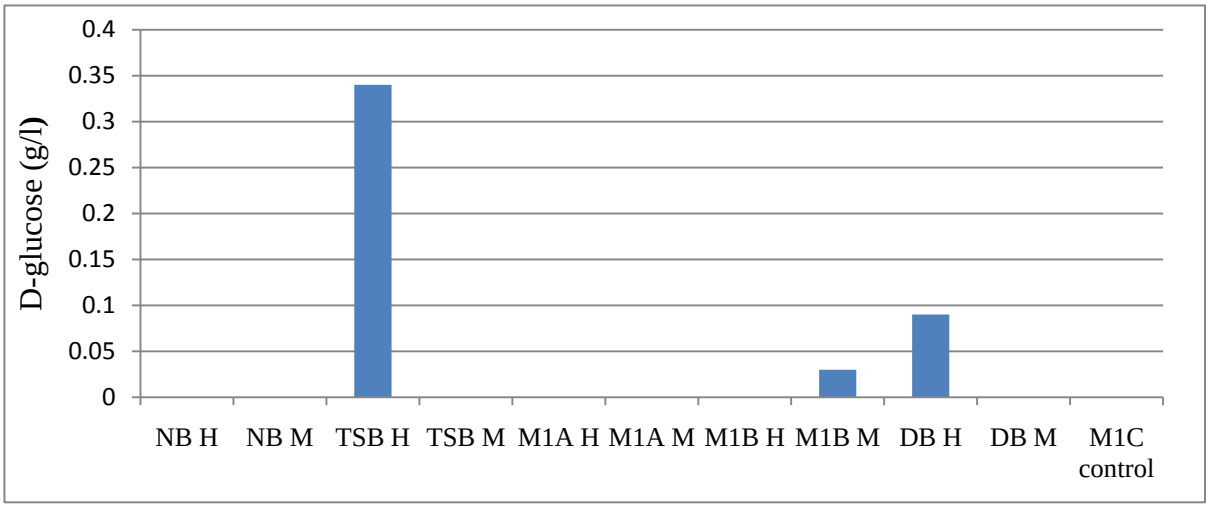


Fig 20: HPLC results for D-glucose production by gut consortia of *Euoniticellus intermedius* larvae after 4 weeks of incubation.

Visible filter paper degradation noted for DB M in Fig. 18 would have suggested a possible high concentration of glucose as the cellulose would have been degraded to its monomeric unit glucose. However, this proved not to be the case as seen on HPLC results for D-glucose on Fig. 20. Instead it was TSB H (hindgut consortium enriched in Tryptone soy broth) that produced the highest concentration of glucose with 0.34 g/l. Lack of glucose from DB M could be attributed to the fact that sampling for HPLC analysis was done after 4 weeks of incubation and possible by that time all the glucose produced would have been utilized by the microorganisms.

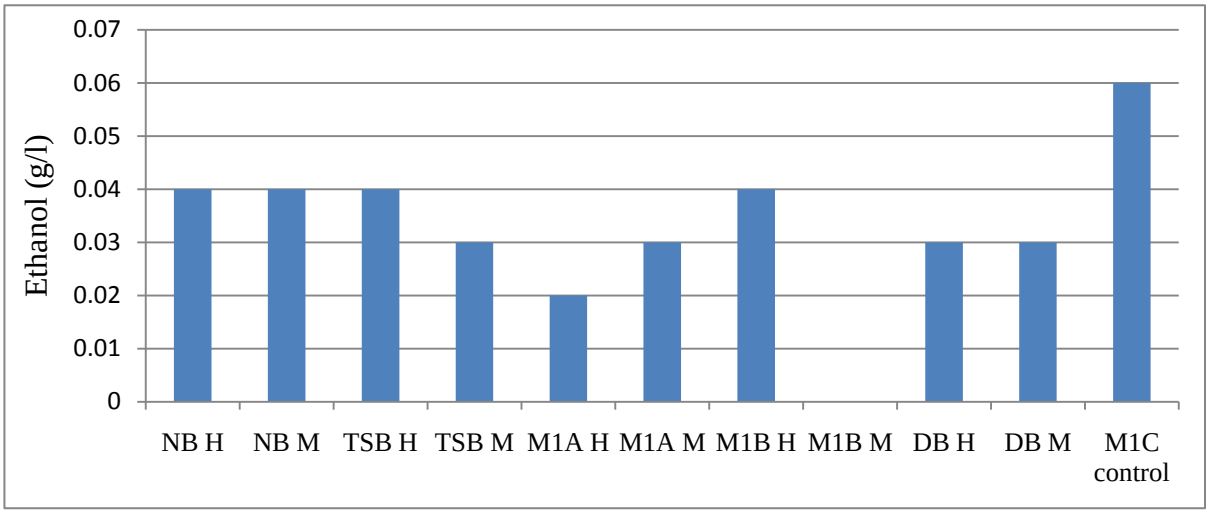


Fig 21: HPLC results for ethanol production by *Euoniticellus intermedius* larvae after 4 weeks of incubation. It was important to note that the control experiment had the highest amount of ethanol, therefore, ethanol peaks detected from sample runs cannot be conclusively attributed to gut microbial consortium.

Though research on related dung beetle larvae by Lemke *et al.,* (2003) showed the consortium to have an ability to produce ethanol, results from Fig. 21 cannot be interpreted to indicate that the midgut and hindgut consortium from *E. intermedius* larvae have the metabolic ability to produce ethanol as the control experiment had a higher ethanol concentration than anyone of the samples. Ethanol in the control could possible been due to contamination during medium preparation but, definitely not by microbial contamination as plate counts for control did not give any colonies at all.

3.6 Methane production

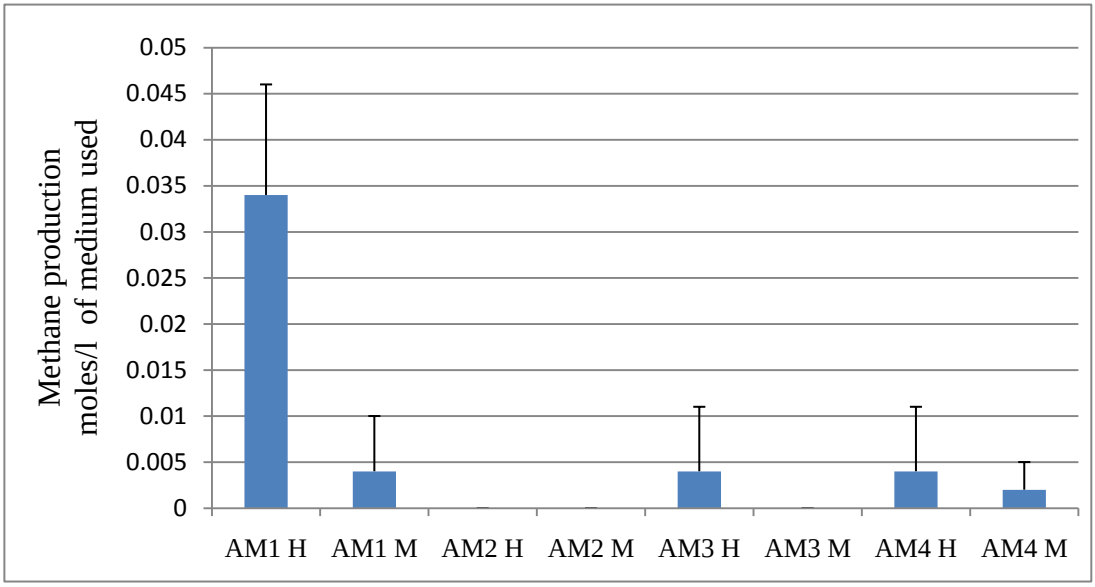


Fig 22: Gas chromatography results for methane production. Results are expressed in moles of methane produced per litre of media used. Carbon sources were 0.225g/l D-glucose and 0.855 g/l D-lactose for basal medium AM1. Carbon soruces for basal medium AM2 was 0.405 g/l microcrystalline cellulose, AM3 was 0.225 g/l D-glucose, 0.855 g/l D-lactose and 0.17 g/l sodium formate, AM4 was 0.225 g/l D-glucose, 0.855 g/l D-lactose and 0.21 g/l sodium acetate. No methane was picked out in the control experiments.

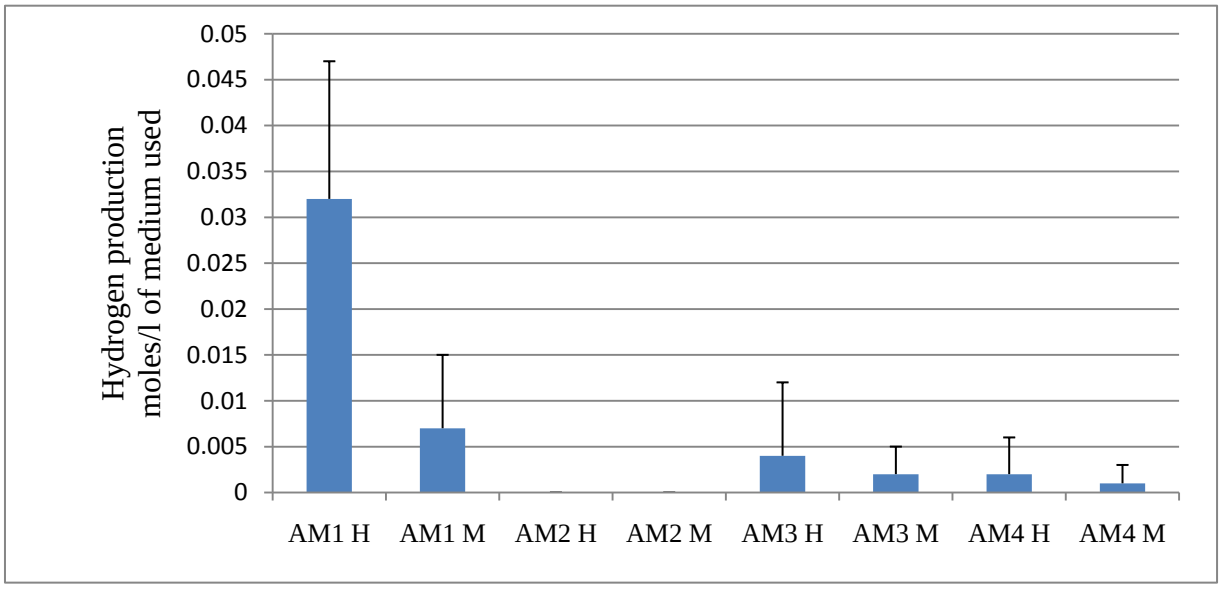


Fig 23: Gas chromatography results for hydrogen production. Results are expressed in moles of methane produced per litre of media used. Carbon sources were 0.225 g/l D-glucose and 0.855 g/l D-lactose for basal medium AM1. Carbon soruces for basal medium AM2 was

0.405 g/l microcrystalline cellulose, AM3 **was** 0.225 g/l D-glucose, 0.855 g/l D-lactose and 0.17 g/l sodium formate, AM4 was 0.225 g/l D-glucose, 0.855 g/l D-lactose and 0.21 g/l sodium acetate. No hydrogen was picked out in the control experiments.

In methane production, methanogens use two pathways to produce methane (Abbanat *et al.,* 1989). One pathway utilizes formate and hydrogen whilst the other utilizes acetic acid (Abbanat *et al.,* 1989). Therefore, in trying to monitor methane production it became necessary to look at which substrate is readily utilized by the methanogens to produce high concentrations of methane (between acetic acid and formate). From Fig. 22 it is seen that the highest concentration of methane was produced by microbial consortium grown in basal medium AM1 (carbon source was D-glucose and D-lactose), specifically the hindgut consortium with an average of 0.034 moles/l of the medium AM1 after 8 weeks of incubation. The high methane concentration production by hindgut consortium has been reported by Lemke *et al.,* (2003) and Egert *et al.,* (2005). They have, however, not reported the production of methane by midgut consortium. Methane detected in the midgut could be attributed to the action of transient methanogens (that is methanogens ingested inside dung by the larvae that will eventually colonize the hindgut not the midgut).

Failure of both methane and hydrogen production by consortia cultured in AM2 (carbon source was microcrystalline cellulose) possibly indicates failure of the cellulose degradation by the consortium under anaerobic conditions or it could be due to the fact that the microbes are still in the lag phase of growth.

Low methane production rates by consortia grown in AM3 and AM4 was rather surprising, as both formate (in AM3) and acetic acid (in AM4) are substrates directly utilized by the methanogens to produce methane. Therefore, the expectation would have been for both of them to produce a higher concentration of methane than AM1 midgut and hindgut consortia. Research by Lemke *et al.,* (2003) and Egert *et al.,* (2005) have shown that adding sodium

formate to isolated hindgut sections of the *Pachnoda ephippiata* and *Melolontha melolontha* larvae (Coleoptera: Scarabaeidae) increased methane output by up to 5 fold and 2 fold, respectively. What could possible explain the low methane production could be the possible that at the concentrations that both sodium acetate and sodium formate were used were inhibitory to the activity of the methanogens as they might have been possible too high.

Conclusion and Recommendations

Results from Fig. 13 clearly and conclusively show that we have successfully managed to develop a standard method for the propagation of *E. intermedius* larvae midgut and hindgut microbial consortia. However, the reproducibility of the method still remains to be conclusively shown. M1B proved to be the best medium for the propagation of *E. Intermedius* gut consortia as the few number of microorganisms that grew in it, compared to other media, degraded cellulose most extensively.

Ethanol results from Fig. 21 do not show the metabolic potential of the consortium in ethanol production due to the fact that the control experiment had the highest concentration of ethanol than other samples. However, the ability of the consortia to produce D-glucose is good, as the glucose produced could then be utilized by yeast like *S. cerevisiae* to produce ethanol. Results from Fig. 18 and Fig. 19 as well as those in Fig. 22 conclusively show the ability of the culture to degrade cellulose as well as produce methane, respectively.

It is recommended that more work be done in trying to optimise on cellulose degradation such that similar impressive results to those of DB M could be achieved in a shorter period of time with high glucose concentrations being detected. More experimental work needs to be done at different temperatures and pH of media to indentify what temperature and pH is most optimal for cellulose degradation using the gut consortia.

Screening for microorganisms involved in methane production should also be done. This is likely to yield impressive results like those that were achieved in the brewing and bakery industries when only pure *S. cerevisiae* was used compared to the old traditional system in which an unknown consortia was used. By identifying microorganisms in the consortia and removing microorganisms not within the methane production chain could improve on

concentration of methane produced. This because by removing microorganisms that are not involved in methane production but still compete with the three groups of microorganisms involved in methane production, more simple molecules are made available to acidogens which subsequently produce a higher concentration of organic acids. As a result, they will be an increase in the concentration of organic acids utilised by methanogens which in turn produce a higher concentration of methane.

Appendices

1.pH after 4 weeks of enrichment						
NB H	NB M	NB C	TSB H	TSB M	TSB C	
8.10667	7.85333	7.16333	7.25	8.70667	7.27667	Average
0.89857	1.08868	0.05033	0.70314	0.77358	0.20207	Standard deviation
8.49	6.82	7.17	6.73	8.15	7.51	
7.08	8.99	7.11	8.05	9.59	7.16	
8.75	7.75	7.21	6.97	8.38	7.16	
M1A H	M1A M	M1A C	M1B H	M1B M	M1B C	
5.94	6.62667	6.32667	7.02667	6.54	6.90667	Average
0.78045	0.30665	0.44636	0.18148	0.60828	0.56092	Standard deviation
5.45	6.47	6.84	6.82	6.94	6.52	
5.53	6.98	6.11	7.16	5.84	6.65	
6.84	6.43	6.03	7.1	6.84	7.55	

2. Plate counts after enrichment						
NB H	NB M	NB C	TSB H	TSB M	TSB C	
277.667	155.167	0	298.667	193.333	0	Average
10.1719	9.06458	0	12.4526	10.0731	0	Standard deviation
270	153	0	289	198	0	
264	144	0	294	204	0	
283	149	0	312	187	0	
290	170	0	300	187	0	
286	160	0	314	180	0	
273	155	0	283	204	0	
NB H	NB M	NB C	TSB H	TSB M	TSB C	
2.8E+07	1.6E+07	0	3E+07	1.9E+07	0	Average
1017186	906458	0	1245258	1007307	0	std deviation
M1A H	M1A M	M1A C	M1B H	M1B M	M1B C	
101.833	114	0	107.333	101	0	Average
15.3417	10.4499	0	7.99166	10.8628	0	Standard

						deviation
118	117	0	96	108	0	
96	98	0	104	96	0	
121	123	0	117	112	0	
104	120	0	104	110	0	
84	104	0	116	96	0	
88	122	0	107	84	0	
M1A H	M1A M	M1A C	M1B H	M1B M	M1B C	
1E+07	1.1E+07	0	1.1E+07	1E+07	0	
1534166	1044988	0	799166	1086278	0	std deviation

3. Plate counts after cellulose digestion					
NB H	NB M	TSB H	TSB M	M1A H	
37.3333	261	35.3333	47	162	Average
2.51661	67.9485	6.80686	5.56776	27	Standard deviation
35	270	33	42	162	
40	189	43	46	135	
37	324	30	53	189	
NB H	NB M	TSB H	TSB M	M1A H	
3733333	2.6E+07	3.5E+08	4.7E+08	1.6E+07	
251661	6794851	6.8E+07	5.6E+07	2700000	
M1A M	M1B H	M1B M	DB H	DB M	
31.6667	65.3333	29.6667	675	91	Average
4.50925	8.3865	7.37111	46.7654	22.2711	Standard deviation
36	75	24	648	115	
32	60	38	729	87	
27	61	27	648	71	
M1A M	M1B H	M1B M	DB H	DB M	
3166667	6533333	2966667	6750000	910000	
450925	838650	737111	467654	222711	

4. pH after cellulose digestion in M1C medium				
NB H	NB M	TSB H	TSB M	M1A H
7.42	7.845	7.57	6.75	7.095
0.66468	0.0495	0.84853	0.28284	0.19092
7.89	7.81	8.17	6.55	7.23
6.95	7.88	6.97	6.95	6.96
M1A M	M1B H	M1B M	DB H	DB M
7.475	6.665	5.945	6.5	5.195
0.70004	0.07778	0.41719	0.77782	0.64347
6.98	6.61	5.65	7.05	4.74
7.97	6.72	6.24	5.95	5.65

5. HPLC results						
NB H	NB M	TSB H	TSB M	M1A H	M1C control	
0	0	0.34	0	0	0	Glucose g/l
0.14	0.05	0	0	0.24	0	Acetic acid g/l
0.04	0.04	0.04	0.03	0.02	0.06	Ethanol g/l
M1A M	M1B H	M1B M	DB H	DB M	M1C control	
0	0	0.03	0.09	0	0	Glucose g/l
0.03	0.4	0	0.18	1.08	0	Acetic acid g/l
0.03	0.04	0	0.03	0.03	0.06	Ethanol g/l

6. Methane production moles /l medium used								
AM1 H	AM1 M	AM2 H	AM2 M	AM3 H	AM3 M	AM4 H	AM4 M	
0.034	0.004	0	0	0.004	0	0.004	0.002	average
0.012	0.006	0	0	0.007	0	0.007	0.003	std deviation

7. Hydrogen production moles/l of medium used								
AM1 H	AM1 M	AM2 H	AM2 M	AM3 H	AM3 M	AM4 H	AM4 M	
0.032	0.007	0	0	0.004	0.002	0.002	0.001	average
0.015	0.008	0	0	0.008	0.003	0.004	0.002	std deviation

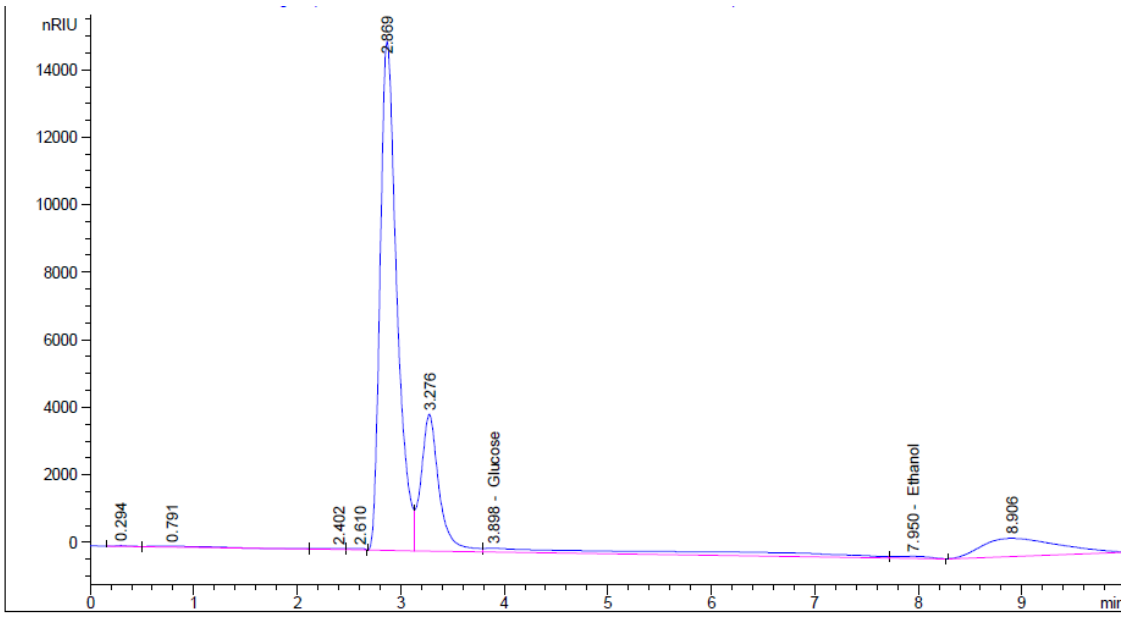


Fig A1: Chromatogram for TSB H showing two identified peaks at for D-glucose and ethanol.

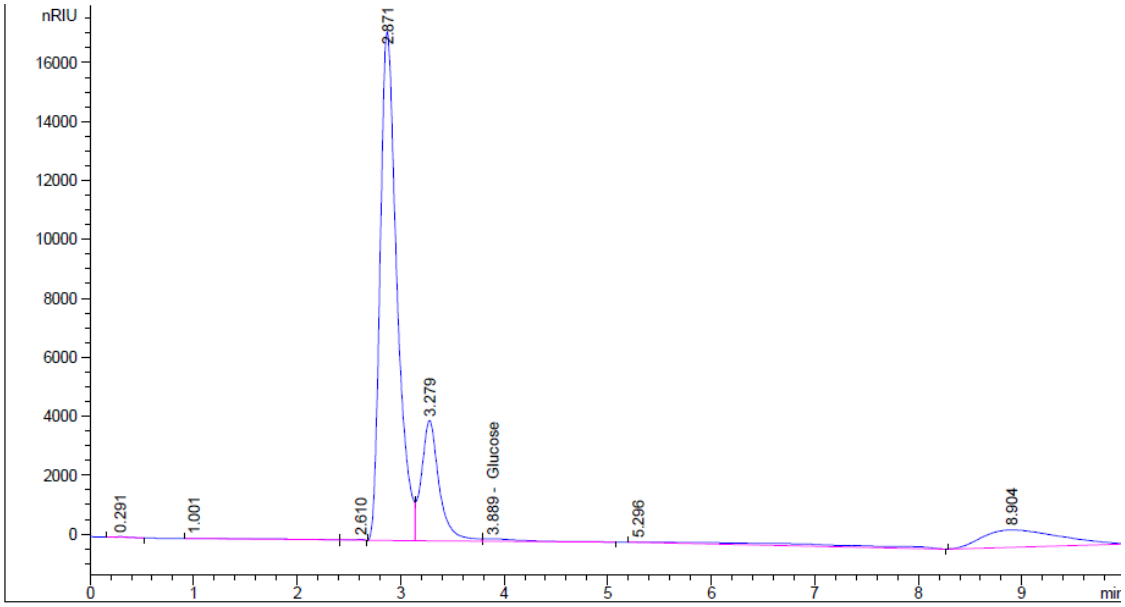


Fig A2: Chromatogram for M1B M showing one identified peak for D-glucose

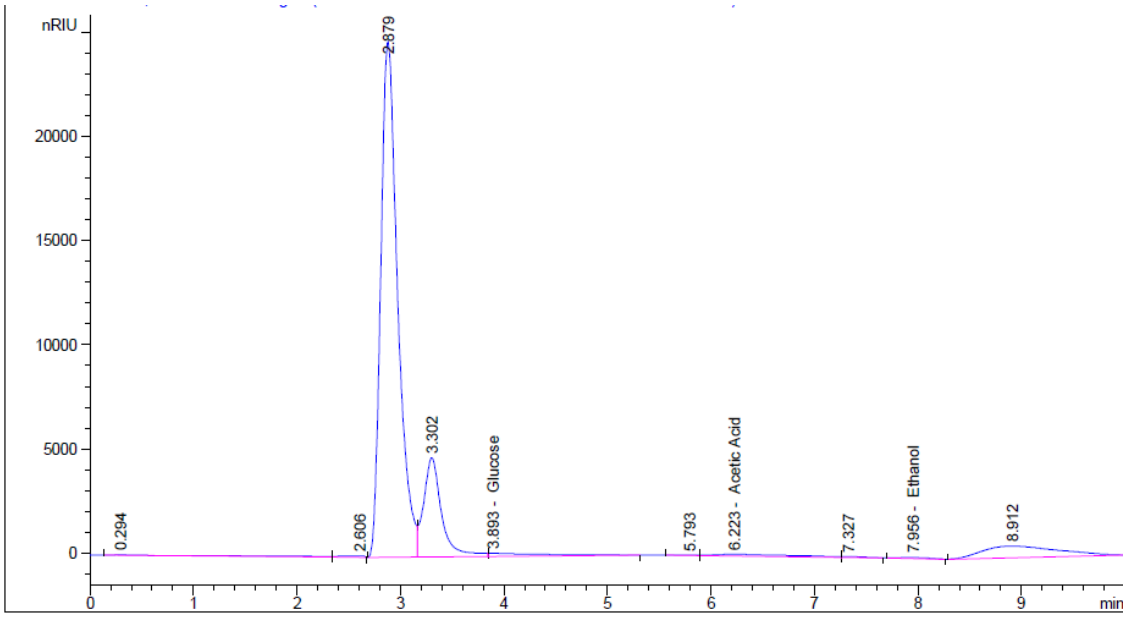


Fig A3: Chromatogram for DB H showing three identified peaks for D-glucose, acetic acid and ethanol.

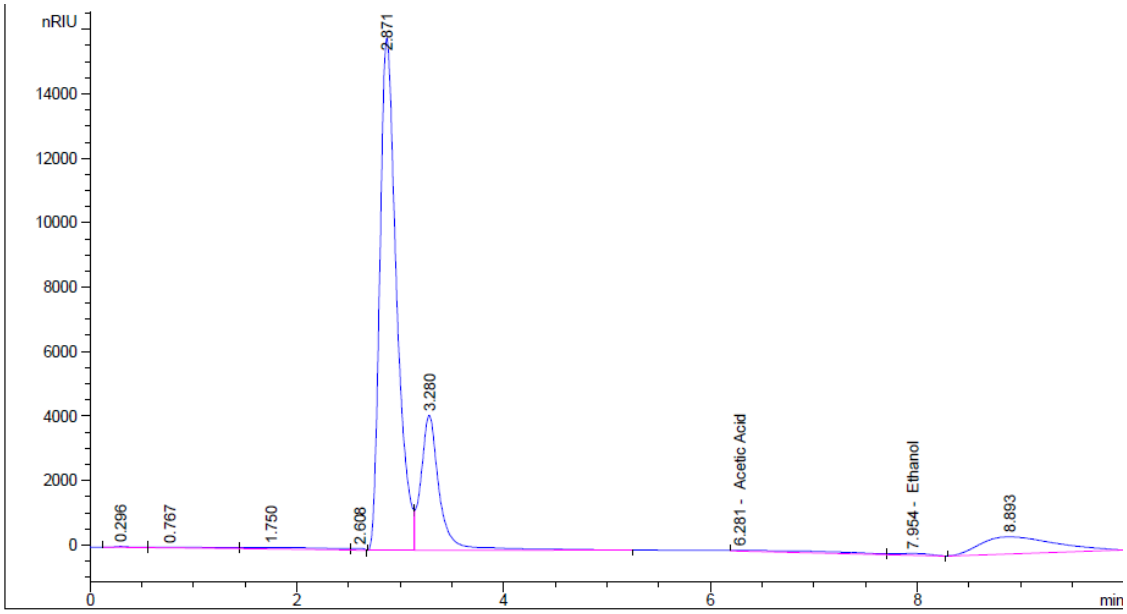


Fig A4: Chromatogram for NB M showing two identified peaks for acetic acid and ethanol.

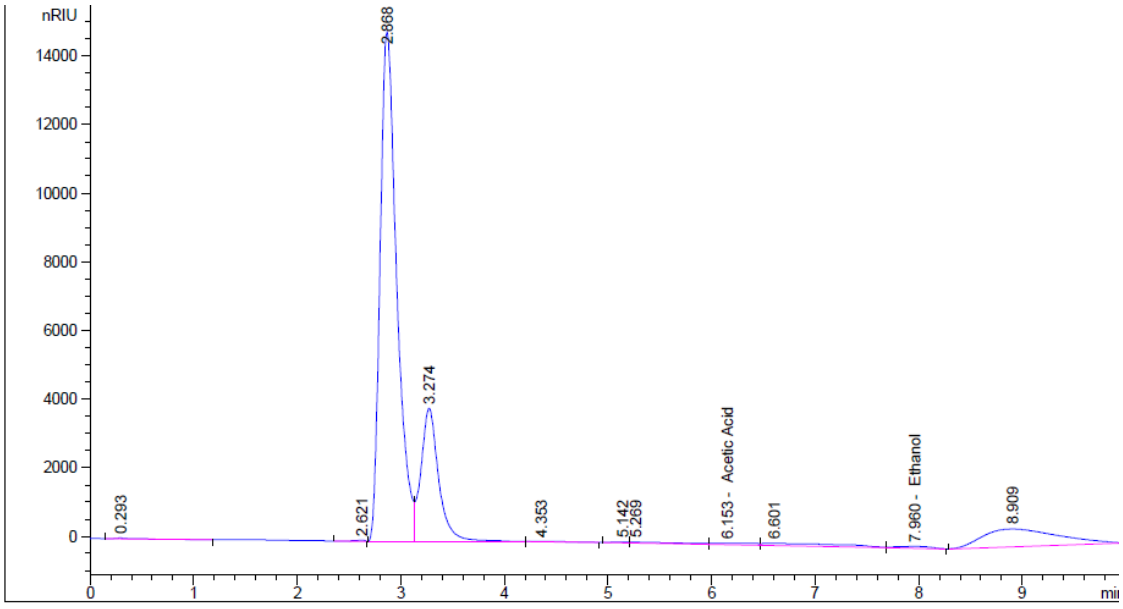


Fig A5: Chromatogram for NB H showing two identified peaks for acetic acid and ethanol

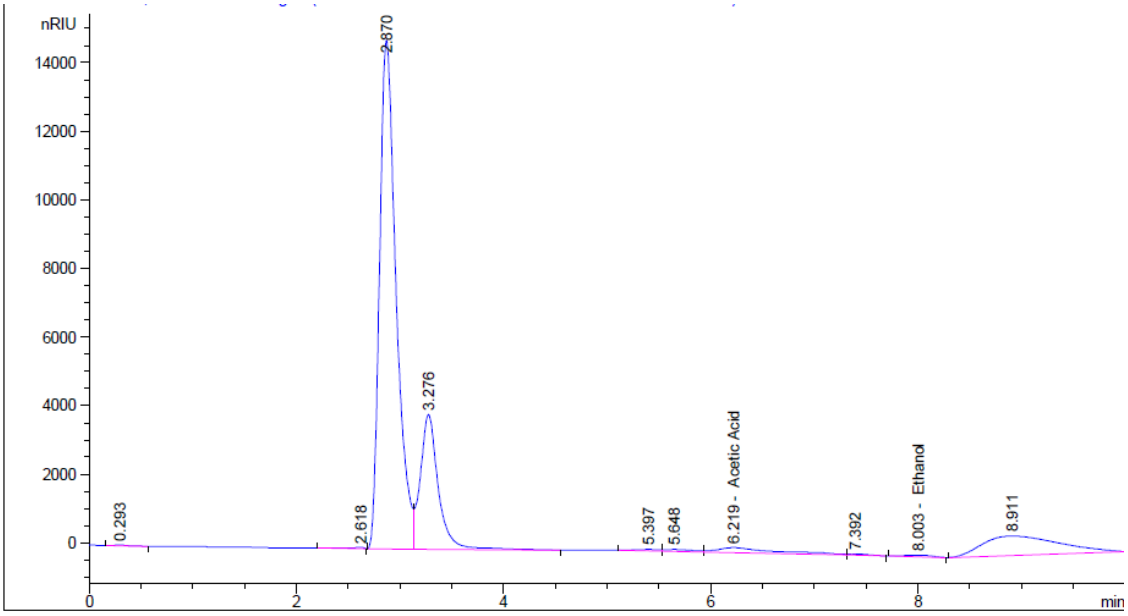


Fig A6: Chromatogram for M1A H showing two identified peaks for acetic acid and ethanol.

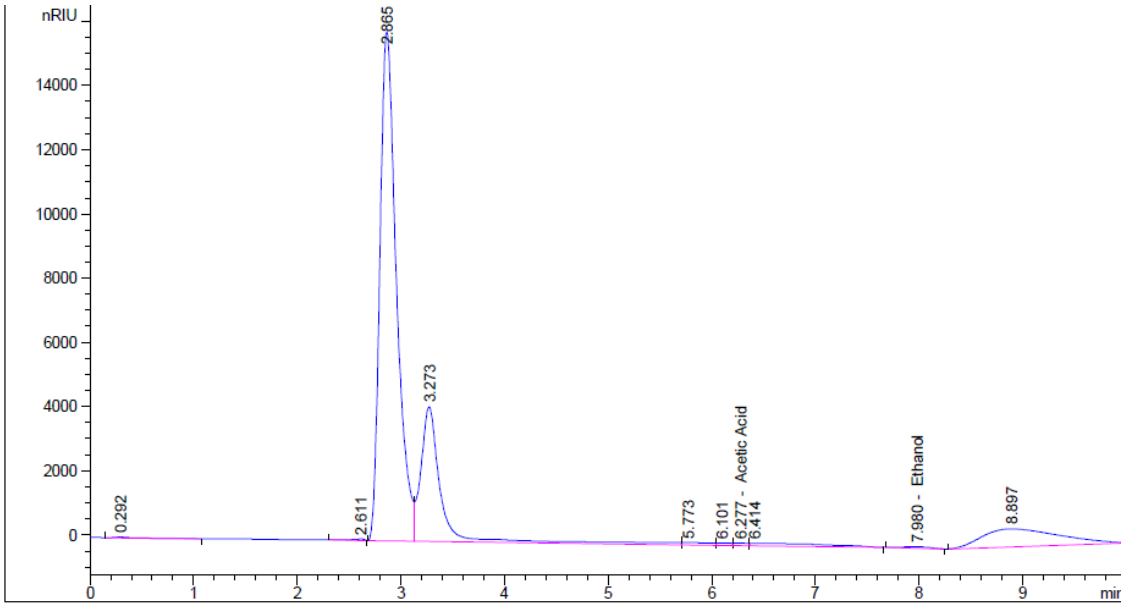


Fig A7: Chromatogram for M1A M showing two peaks for acetic acid and ethanol.

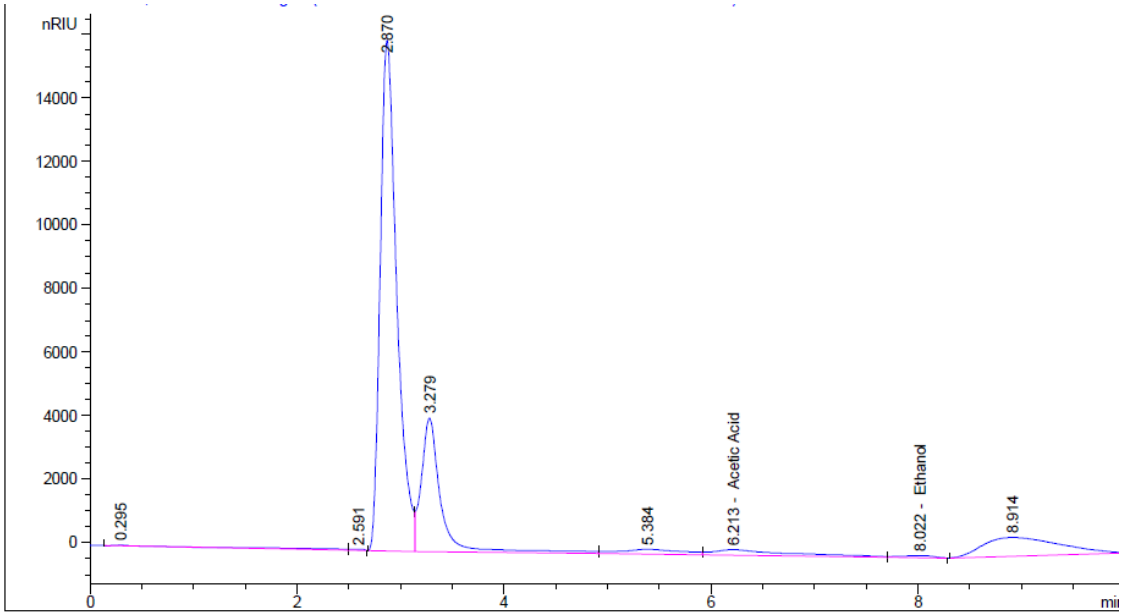


Fig A8: Chromatogram for MIB M showing two identified peaks for acetic acid and ethanol.

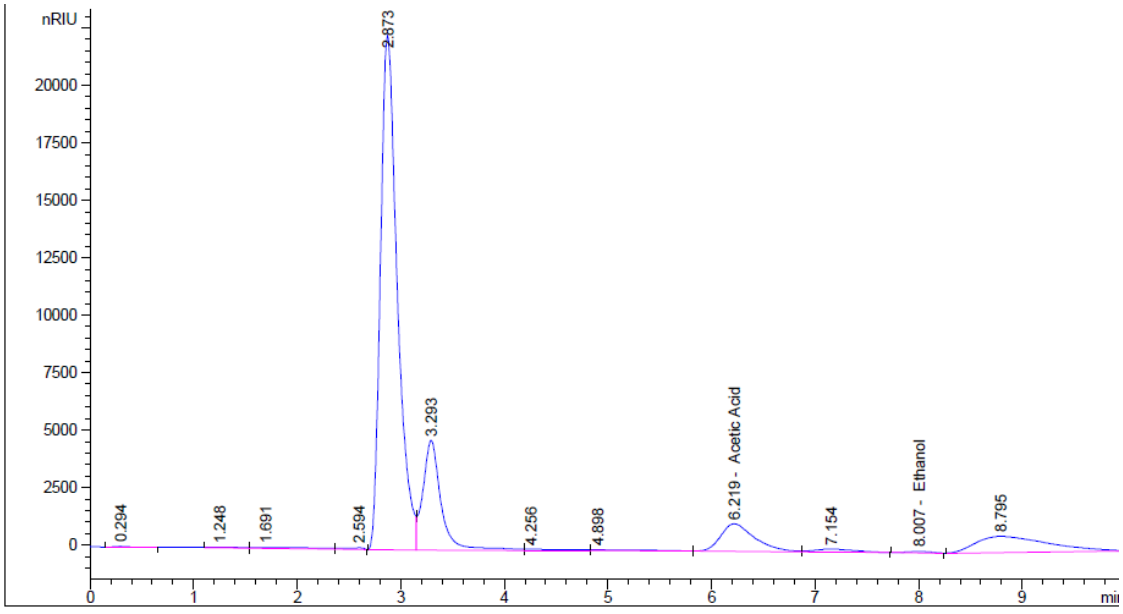


Fig A9: Chromatogram for DB M showing two identified peaks for acetic acid and ethanol

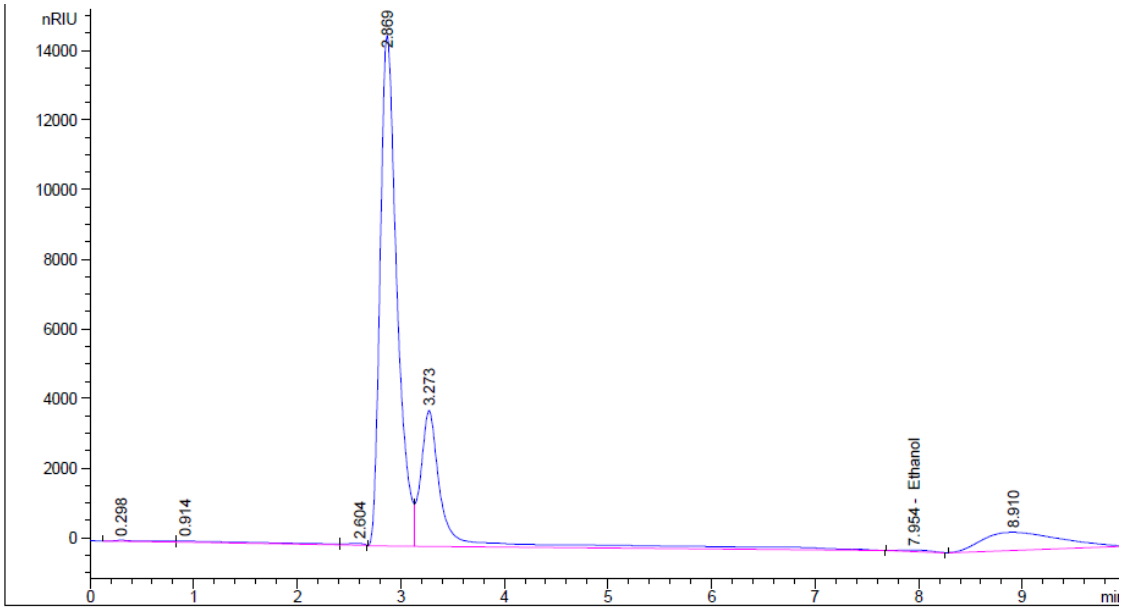


Fig A10: Chromatogram for TSB M showing a single identified peak for ethanol

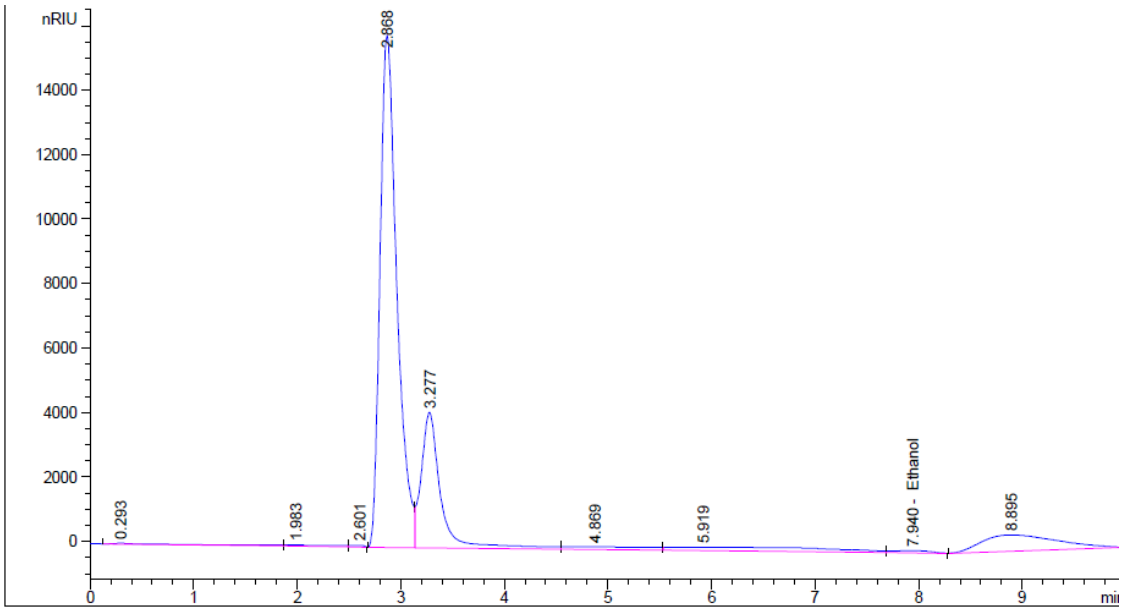


Fig A11: Chromatogram for M1C control showing a single peak for ethanol. No peaks were peaked out at all for glucose and acetic acid.

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