Production and Characterization of Polyhydroxyalkanoates (PHA) from Corn Silage using *Thermus thermophilus* HB8

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PREFACE

The work described in this thesis was performed by the author under the supervision of Prof N. Deenadayalu and Prof K. Permaul at Durban University of Technology, Durban, South Africa, from 2016 – 2019. The study presents original work by the author and has not been submitted in any form at another university. Where use is made of the work of others, it has been clearly stated in the text.

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ABSTRACT

In this study, a biodegradable copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) was produced from an agricultural by-product namely corn silage through a fermentation process using *Thermus thermophilus* HB8. Two types of corn silage pre-treatment processes viz. deionized water treatment (unhydrolysed) and acid hydrolysis were carried out at different loadings of corn silage (6%, 12%, 24% and 48% m/v), at 70°C for 50 h.

Both pre-treatments were able to produce biopolymer where 6%, 12%, 24% and 48% unhydrolyse pre-treatment yielded 12%, 20.44%, 28.42% and 18.65% PHA, respectively; 6%, 12%, 24% and 48% acidic pre-treatment yielded 42.23%, 49.53%, 56,41% and 61.32% PHA, respectively. The extracted polymer was characterized by Fourier transform infrared spectroscopy (FT-IR) and ultraviolet-visible spectroscopy (UV-Vis) to study the characteristic bands; gas chromatography was used to identify the PHA monomers of the extracted methyl esters; scanning electron microscopy (SEM) was used to study the morphology of the bioplastic products; tensile testing was used to study the tensile properties of the bioplastics.

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Abbreviations

PHA	Polyhydroxyalkanoates
PP	Polypropylene
PET	Polyethylene terephthalate
PVC	Polyvinylchloride
PE	Polyethylene
PS	Polystyrene
PA	Polyamide
LMW	Low molecular-weight
NSP	Non-starch polysaccharides
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PAGE	Polyacrylamide gel electrophoresis (native)
VFAs	Volatile fatty acids
CDW	Cell dry weight
DCW	Dry cell weight
OD	Optical density
ADF	Acid-detergent fibre
ADL	Acid-detergent lignin
NDF	Neutral-detergent fibre
NDFD	Neutral-detergent fiber digestibility
RTIL	Room temperature ionic liquids
AW	Agricultural waste
SRT	Sludge retention time
LPW	Leguminous processing water
FPW	Fruit processing water
BOD	Biochemical oxygen demand
COD	Chemical oxygen demand
SFL	Sludge fermentation liquid
AFR	Anaerobic fermentation reactor

PHAR	PHA production bio-reactor
FAN	Free amino nitrogen
TDW	Total dry weight
OMW	Olive mill wastewater
DO	Dissolved oxygen
ACP	Acyl carried protein
CS	Corn silage

Polyhydroxyalkanoates (PHAs)

3HO	3-hydroxyoctanoate
3HD	3-hydroxydecanoate
3HV	3-hydroxyvalerate
3HB	3-hydroxybutyrate
3HN	3-hydroxynonanoate
3HUD	3-hydroxyundecanoate
3HDD	3-hydroxydodecanoate
Poly(3HB-co-3HV)	poly(3- hydroxybutyrate-co-3-hydroxyvalerate)

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CHAPTER **1**

INTRODUCTION

1.1. Background

Dependence on petroleum-derived plastics has significantly increased globally, resulting in the depletion of crude oil and growing apprehension about the environmental impact of fossil fuel derived materials has attracted much interest in biologically synthesised polymers. The change to renewable energy supplies and greater efficiency in the utilization of energy is more urgent, because of the threat to global warming caused largely by fossil fuel burning. Increased emission of greenhouse gases (GHG) and carbon dioxide (CO_2) due to the energy-driven consumption of fossil fuels, disrupt the global carbon cycle, leading to planetary warming. Utilization of renewable energy could be the potential answer for these issues and, in particular, the use of lignocellulosic biomass. Biomass can play a vital role in climate stabilization and may be utilized as an alternate and manageable energy and chemical supply for the future.

A large amount of research has been conducted on lignocellulosic biomass such as crop residues, agricultural wastes, agro-commercial by-products, and municipal solid waste (Hadar 2013). Agricultural biomass consists of rigid structures that can be deconstructed through effective pretreatment technologies to release sugars that can be converted by microorganisms into ethanol and other bio-based chemicals (Kai *et al*. 2003). Different pre-treatment methods have been studied including weak acid hydrolysis, strong acid hydrolysis, alkaline hydrolysis, liquid hot water, room temperature ionic liquids (RTILs), oxidation delignification (Harmsen *et al.* 2010). The different pretreatments have their own limitations for large-scale applications because of negative impacts on the environment. Some pretreatment strategies are not selective, hence creating undesirable products that inhibit fermentation and conversion of monosaccharides into ethanol and bio-based chemicals (Wang *et al.* 2011). Pretreatment of lignocellulose material by either physical, chemical or organic attack is a significant step for cellulose and hemicellulose bio-processing through hydrolysis for the production of basic sugars.

Lignocellulose biomass waste is an agricultural byproduct and it is always available as a renewable source (UNEP 2009). Potential products obtained from lignocellulosic biomass after pretreatment are shown in Figure 1.1.

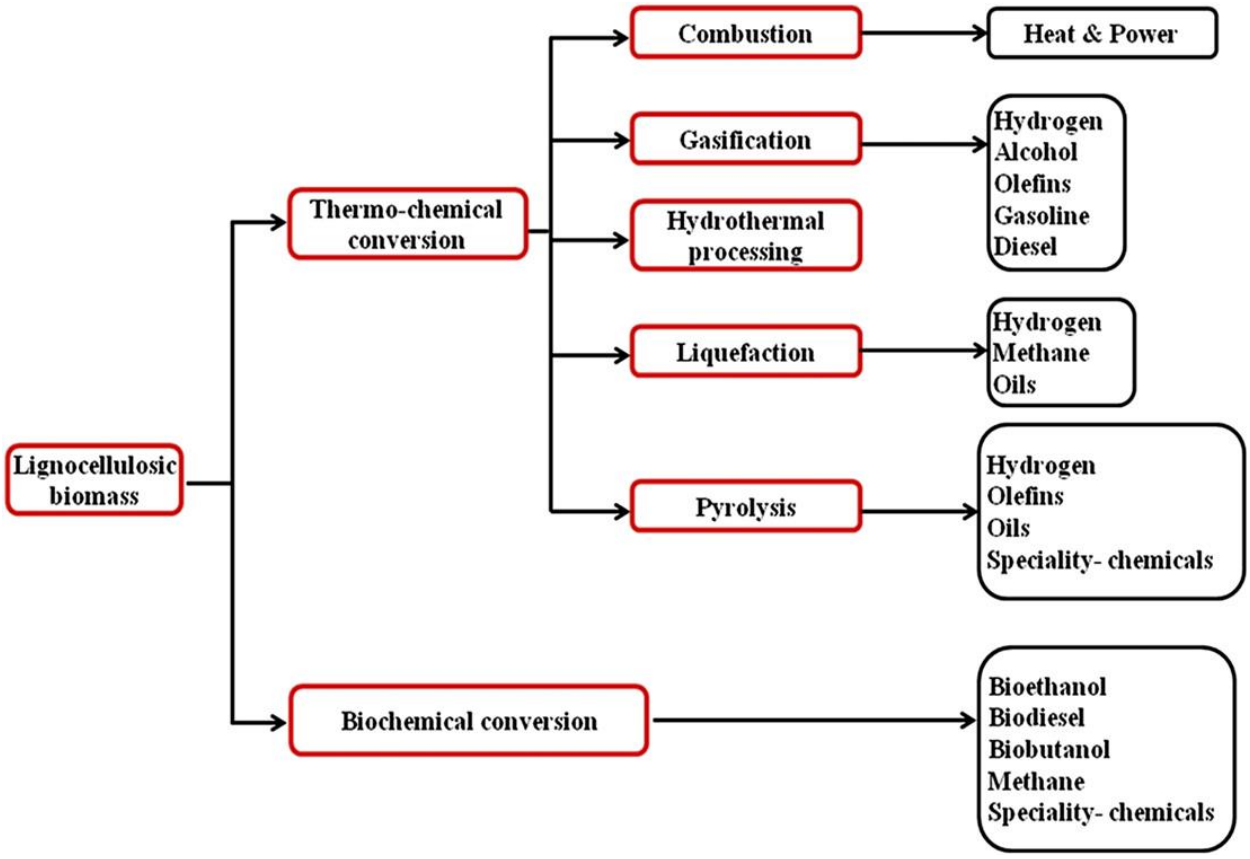


Figure 1.1 Possible products that are produced after different pre-treatments of lignocellulosic biomass (Menon and Rao 2012).

1.2. Biomass

Biomass is a biological-produced matter derived from plant material. It is carbon-based material composed of hydrogen, oxygen or nitrogen atoms. The carbon used to build biomass is assimilated from the climate as CO_2 by plants utilizing energy from the sun. Biomass is organic material that stores chemical energy from sun. Biomass is produced by means of green plants transforming sunlight into plant material via photosynthesis and it is converted into energy and fuel (Figure 1.2) (Hall *et al.* 1993).

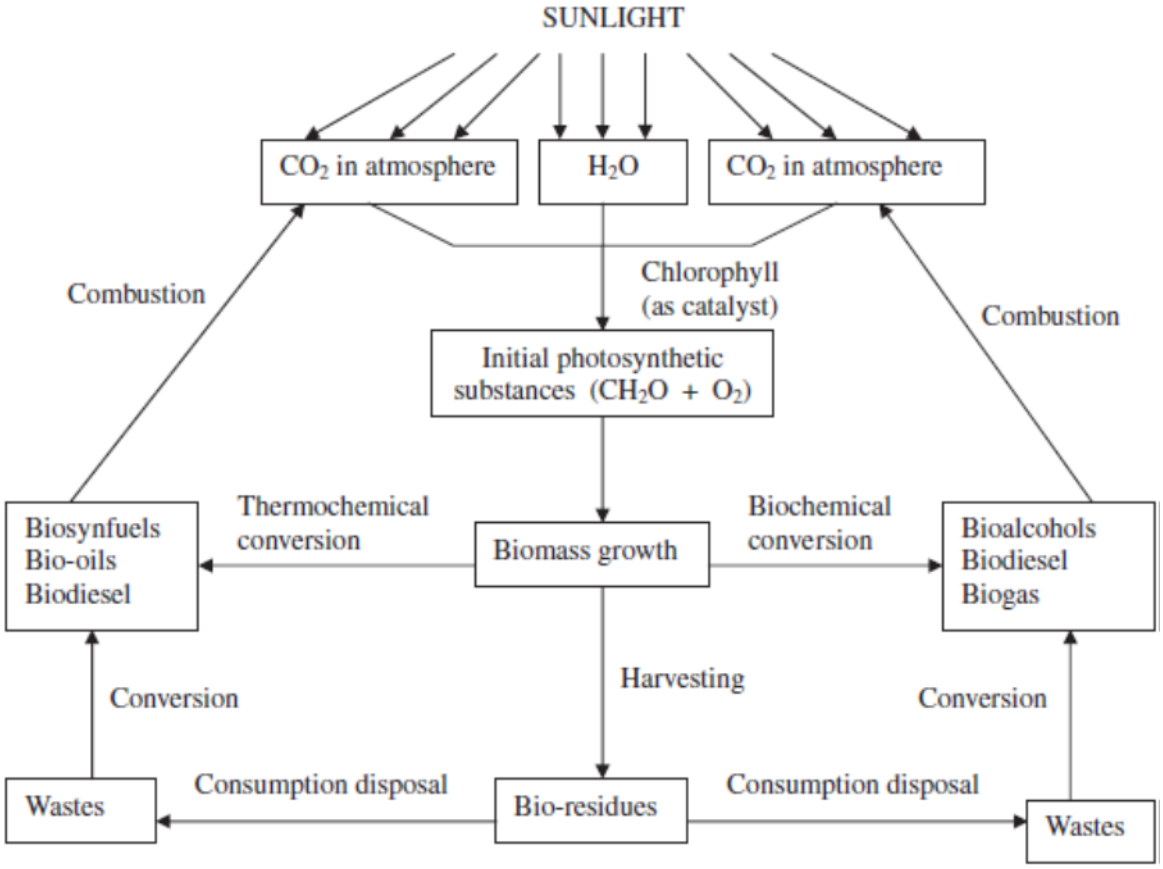


Figure 1.2 Main steps involved in biomass formation and its conversion into energy and biofuel. (Demirbas 2008).

Agricultural plant material that is not for human consumption is either utilized as animal feed or incinerated. The biomass utilized by animals discharges carbon into the environment as either CO_2 or methane (CH_4) through methane oxidation. These processes form part of the carbon cycle. Examples of agricultural plant materials that results into agricultural biomass (Figure 1.3).

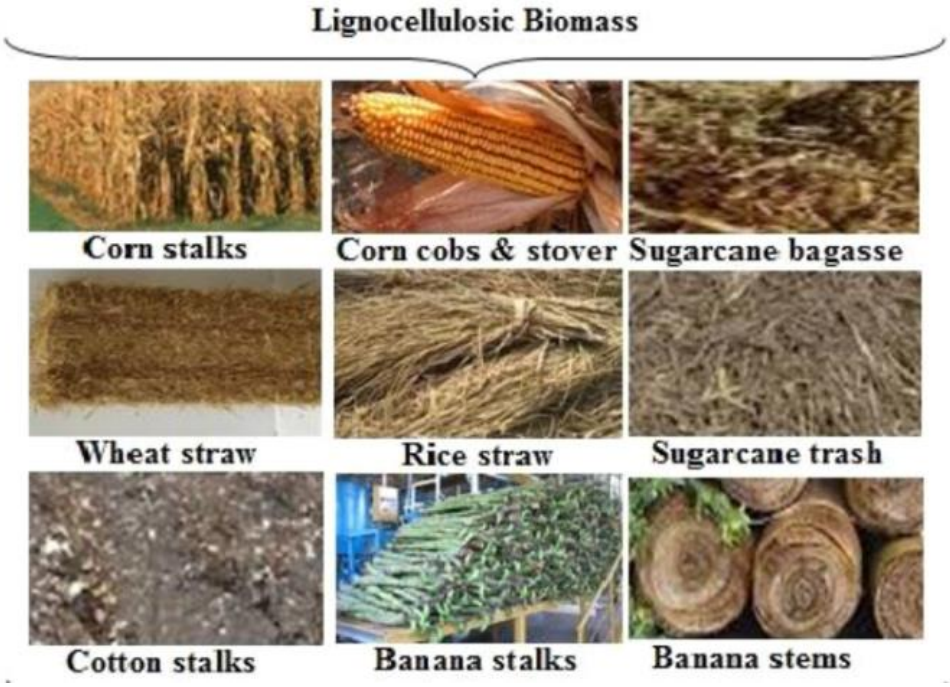


Figure 1.3 Typical examples of agricultural materials that result into agricultural biomass (Anwar *et al*. 2014).

1.2.1. Lignocellulosic biomass

Plant material produced contain cell walls, primarily composed of three bio-based chemicals: cellulose; hemicellulose; and lignin (Figure 1.4). Together they are called lignocellulose with cellulose embedded in a cross-connected framework of lignin and hemicellulose that link the strands. Extractives (gum, phenol, and inorganic chemicals) and minerals (calcium, magnesium, potassium) are other chemicals contained by lignocellulose plant structures in the framework.

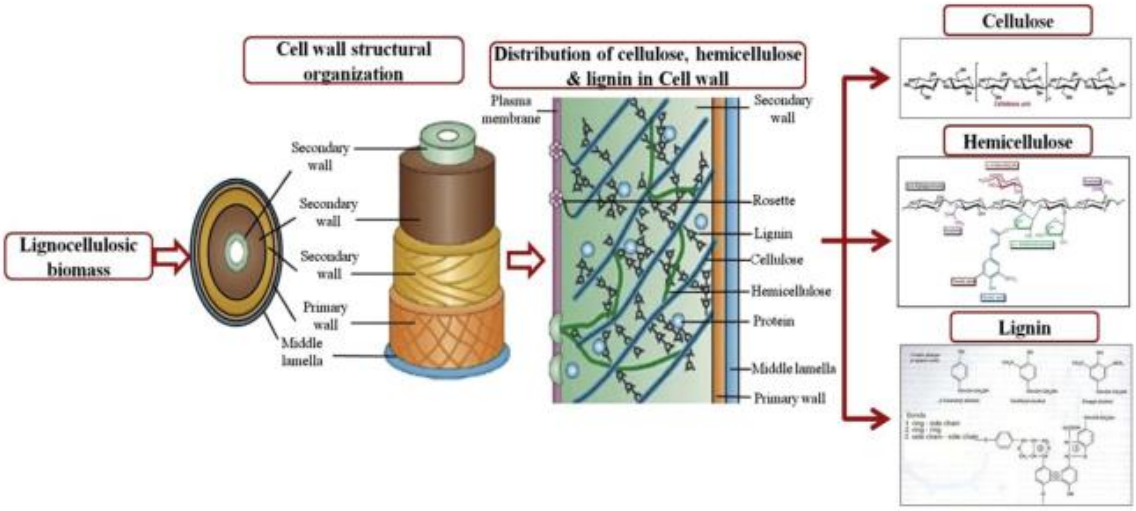


Figure 1.4 Identification of plant cell wall compounds in the lignocellulose biomass (Anwar *et al*. 2014).

Corn is one of the major agricultural crops in South Africa. Agricultural residues like corn stover and corn cobs are "waste" that remains after corn harvesting in agricultural industries and previous studies showed great potential for the conversion of corn residue to biofuel while delivering agro-economic benefits. Previous studies indicated that at maturity corn cobs contain structural polymers with 34% cellulose, followed by 32% hemicellulose and 6% lignin (Table1.1) (Mussatto and Teixeira 2010). Agricultural lignocellulose wastes contain different amounts of structural polymers (Table 1.1).

Table 1.1 Composition of agricultural lignocellulosic waste (Mussatto and Teixeira 2010)

Lignocellulose waste	Cellulose (wt %)	Hemicellulose (wt %)	Lignin (wt %)
Barley straw	33.8	21.9	13.8
Corn cobs	33.7	31.9	6.1
Corn stalks	35.0	16.8	7.0
Cotton stalks	58.5	14.4	21.5
Oat straw	39.4	27.1	17.5
Rice straw	36.2	19.0	9.9
Rye straw	37.6	30.5	19.0
Soya stalks	34.5	24.8	19.8
Sugarcane bagasse	40.0	27.0	10.0
Sunflower stalks	42.1	29.7	13.4
Wheat straw	32.9	24.0	8.9

1.3. Maize in South Africa

Maize residues are fibrous materials remaining after harvesting and is one of the most abundant renewable and eco-friendly feedstock. According to Southern African Grain (2010/2011) South Africa produced on average 10 360 000 tons of maize annually where the predominant maize-producing domain is Free State (4 051 500 tons), followed by North West Province (2 332 500 tons) and Mpumalanga (2 190 000 tons). About 59% is white maize and 49% is yellow maize (DAFF 2017). According to DAFF (2017), white maize is primarily utilized for human consumption and yellow maize is frequently utilized for animal feed production. Previous studies indicated that the major crop produced in South Africa is sugarcane followed by maize being the second major crop produced, hence the agricultural sector is an important industry to the economy both as an earner of foreign currency because of its multiplier effects and as an employer. Also, maize is utilized as a raw material for manufacturing products such as paints, textiles, paper, food and medicine (Mogala 2012). Maize is produced in all nine provinces in South Africa (Figure 1.5) hence, it's a major feed grain and staple food.

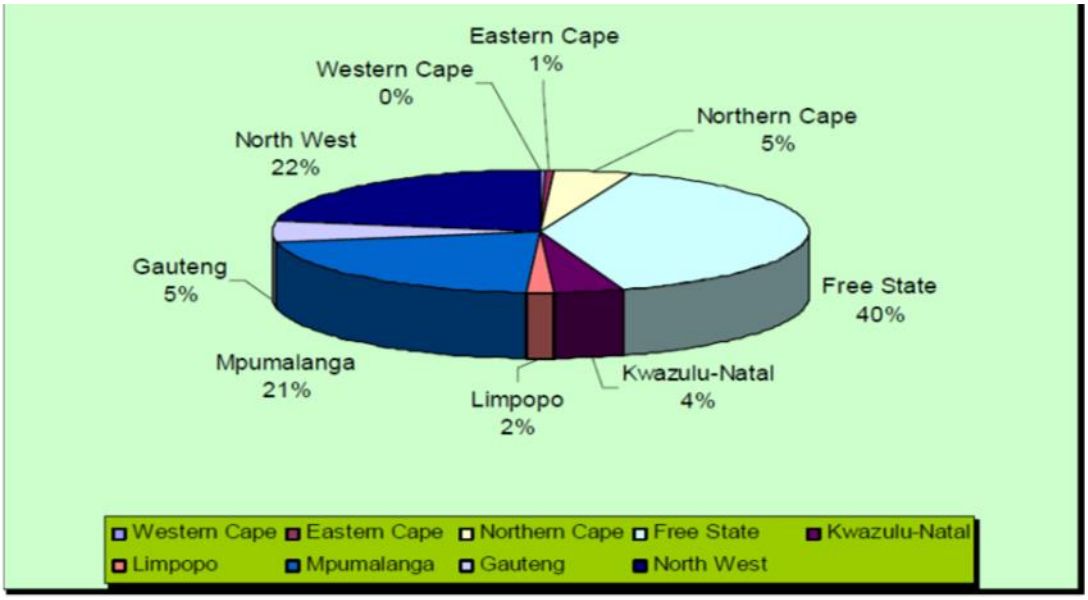


Figure 1.5 Maize production in nine provinces of South Africa (Mogala 2012).

Humans consume maize as a stable food and its nutritional value makes it a good source of energy. Industries such as livestock industries, millers and some snack industries depend on maize planting and production. Both white and yellow maize are utilized by livestock as feed for cattle as corn silage while yellow maize contributes extensively to the production of dairy products and production of red and white meat (DAFF 2008). Humans consume about 50% of the produced maize in South Africa, while animal feed industry utilize 40% and about 10% is utilized for seed industry (Mogala 2012). In the past six years, maize production has experienced significant variations in all the maize producing provinces due to climate change (Table 1.2) (DAFF 2015/2016)

Table 1.2 Maize production by provinces from 2009/10 to 2015/16 production season (tons)

Season	2008/09	2009/10	2010/11	2011/12	2012/13	2013/14	2014/15
PROVINCE							
Western Cape	50 000	18 000	14 000	30 000	33 000	33 000	45 000
Eastern Cape	92 000	80 000	68 000	92 000	107 000	111 000	70 000
Northern Cape	634 000	609 000	538 000	606 000	601 000	664 000	712 000
Free State	4 527 000	5 076 000	4 052 000	4730 000	5334 000	6247 000	2264 000
KwaZulu-Natal	521 000	524 000	450 000	516 000	519 000	559 000	454 000
Limpopo	247 000	210 000	173 000	226 000	300 000	307 000	248 000
Mpumalanga	2 870 000	2 745 000	2 190 000	2504 000	2666 000	2783 000	2108 000
Gauteng	534 000	685 000	543 000	552 000	617 000	648 000	441 000
North-West	2 575 000	2 868 000	2 332 000	2574 000	2226 000	2898 000	914 000

1.4. Corn silage

Corn silage (CS) is primarily made from annual crops like corn as feed source by chopping corn residues. The agricultural industry utilizes corn silage as a cost-effective source of nutritious roughage in dairy cattle (Van de Vyver *et al*. 2013). Preservation of corn residue nutrients after harvesting is conducted through ensiling method. This fermenting process encourages animal growth by promoting tasty and digestible feed while promoting optimal feed intake. Nutritionally, corn silage contains about 50% corn grain which makes it a high energy content feed (Martin *et al.* 2008).

Agricultural forage plays an important part in animal production system (Van de Vyver *et al*. 2013). Two physiological changes take place late in the life cycle of a corn plant. Firstly, the plant incorporates as much digestible starch as possible. Secondly, the cell wall components increases in the stover part of the plant (Kezar 2001). Subsequently the cell wall material is the single biggest forage component. The forage cell walls digestibility is a primary determinant of animal productivity and growth. Each plant cell is surrounded by a complex matrix of polymers which is the plant cell wall. Primarily walls provide physical sturdiness significant for plant growth in terrestrial climate and serves many different critical functions, including being a barrier for pathogens and insects attacks. Cell walls shape the structural framework of the plant architecture that offers mechanical support for plant organs (Varner and Lin 1989;

Jung 2012). Also, cell walls take part in ion exchange, cell recognition, water balance and defense from biotic stress (Moore and Jung 2001). The cells of the corn plant would be still alive when a corn plant is chopped and put in a storage facility. The plant cells and the microorganism respiration in the silage yield carbon dioxide and warmth. This is referred to as aerobic respiration, because oxygen is utilized. As the content of CO_2 increase and the oxygen content decrease, anaerobic fermentation commences. In anaerobic fermentations, bacteria utilize soluble carbohydrates within the cells to produce mostly lactic acid.

The structural plant cell wall in ruminant nutrition is known as "fibre". It is divided chemically into neutral detergent fibre (NDF) and an acid detergent fibre (ADF), both being inversely associated with energy density (Kawas *et al.* 1991). Fibre is measured as NDF as part of the detergent fibre analysis system (Van Soest 1967). As the grain portion of corn silage is mostly digestible, stover typically comprises half of the silage dry matter (DM) and carries high concentrations of fibre which is of restricted digestibility. Both physical and chemical natures of fibre are significant in describing it efficiency in producing a necessary ruminal environment (Martin *et al.* 2008). The NDF, ADF and starch content and starch digestion are primary factors used in setting the price of corn silage for dairy cattle.

1.5. Environmental impact of petroleum-based products

Global dependence on fossil fuels, combined with diminishing petroleum resources causes environmental apprehensions hence increased focus to develop strategies for the bio-synthesized compounds production (Sawant et al. 2015b). Previous studies have shown that greenhouse gases (GHG) emission such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), arises from fossil fuel incineration thus polluting the earth's climate (Cherubini 2010). Petroleum-based plastics are persistence in the environment, this concerns increases the cost of solid waste disposal due to their non-degradable nature, as well as, possible hazards from incineration of waste causes synthetic plastics a waste management issue (Ojumu et al. 2004). Combustion of plastic waste forms hydrogen cyanide from acrylonitrile-based plastics and poses health hazards (Khanna and Srivastava 2005). Recycling is challenged in sorting the altered plastics and from the changes that the recycling strategies caused to the properties of the polymer of which the plastics are composed (Bugnicourt *et al.* 2014). In this modern life,

every activity has been influenced by utilizing plastics and many rely completely on plastic products due to their advantageous properties. Plastics comprises of resourceful qualities of strength, lightness, sturdiness because of thermal and mechanical properties and they resist degradation. They have become a vital commodity to improve the quality and comfort of human existence. In almost all industries, plastics are essential and have substituted glass and paper in packaging (Khanna and Srivastava 2005). Additionally, they are easy and economical to manufacture and process. They are moulded into any form of shape and are therefore promptly replacing glass, metals and wood in numerous application (Nawrath *et al.* 1994). Polyethylene and polypropylene are traditional synthetic polymers derived from non-renewable raw materials (Jain and Tiwari 2015).

The vast use of petroleum-derived thermoplastics polypropylene (PP), polyethylene terephthalate (PET), polyvinylchloride (PVC), polyamide (PA) and polystyrene (PS) as packaging materials due to their great availability at relatively low cost and excellent mechanical performance which include: tensile and tear strength; good barriers to oxygen, carbon dioxide, anhydride and aroma compounds, and heat stability. Their use has been constrained due to the fact that they are non-recyclable and/or biodegradable hence they are posing serious environmental issues (Siracusa *et al.* 2008). The challenge for the new polymer is to retain the physico-chemical characteristics of the conventional plastic, however benefit from biocompatibility and biodegradability (Lee 1996; Amass *et al.* 1998; Müller *et al.* 2001).

1.6. New applications of corn silage

It is of importance for research to study diversified utilization of maize corn silage utilization for value-added chemicals and materials production. Corn silage (CS) is utilized by microorganisms able to produce high yields of thermostable xylanases and traits desired for industrial applications (Robledo *et al.* 2016). Maize silage is implemented on hydrothermal carbonization yielding nano-and micro carbon particles with distinctive properties consisting of a high energy value, an excessive thermal and chemical balance, and a moderately large surface area and adsorption capacity (Mumme *et al.* 2011).

1.7. Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates are natural aliphatic polyesters, synthesized via a fermentation process of sugars (glucose, sucrose) or lipids (vegetable oils, even glycerine from the production of bio-diesel) by microorganisms, and are stored as an intra-cellular carbon and energy reserve, when the cells develop in stressful conditions. A hydroxyalkanoate monomer is shown between the brackets under (Figure 1.7) and the R group indicates the residual group. R group consists of diverse carbon atoms (C_4-C_{16}) (Tan *et al.* 2014). PHA consists of 3-5 carbon atoms is classified as short-chain length PHA (scl-PHA), 6-14 carbon atom is classified as medium-chain length PHA (mcl-PHA) and 15 or more carbon atoms is classified as long-chain length PHA (lcl-PHA).

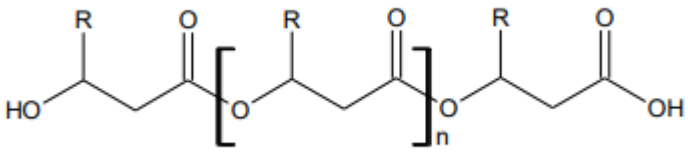


Figure 1.7 The general structure of PHA showing the monomer group between the brackets (Jiang 2010).

1.7.1 PHA production

Microbial or plant route are processes used for the production of biosynthetic polymers although previous studies have indicated that microbes are the major source for PHA production (Keshavarz and Roy 2010). Over 300 species of Gram-negative and Gram-positive bacteria (for example *Bacillus* sp., *Methylobacterium* sp. and *Pseudomonas* sp.) biosynthesises PHAs through metabolic ability and store them as granules in the cytoplasm as carbon and energy source (Reddy *et al*. 2003; Suriyamongkol *et al.* 2007). Stressful environments such as nitrogen limitation with excess carbon source promotes bacterial biosynthesis of PHA (Lee 1996; Ojumu *et al.* 2004). *Alcaligenes latus*, Azotobacter *vinelandii* UWD, a mutant *Azotobacter vinelandii* and *Alcaligenes eutrophus* are some bacteria with storage capability of PHAs under non-limiting conditions (Keshavarz and Roy 2010). The major point of

consideration is the appropriateness of the bacteria for its ability to produce PHAs from cheap resources. Biosynthetic pathways PHAs are associated with the bacterium's central metabolic pathways (Figure 1.7).

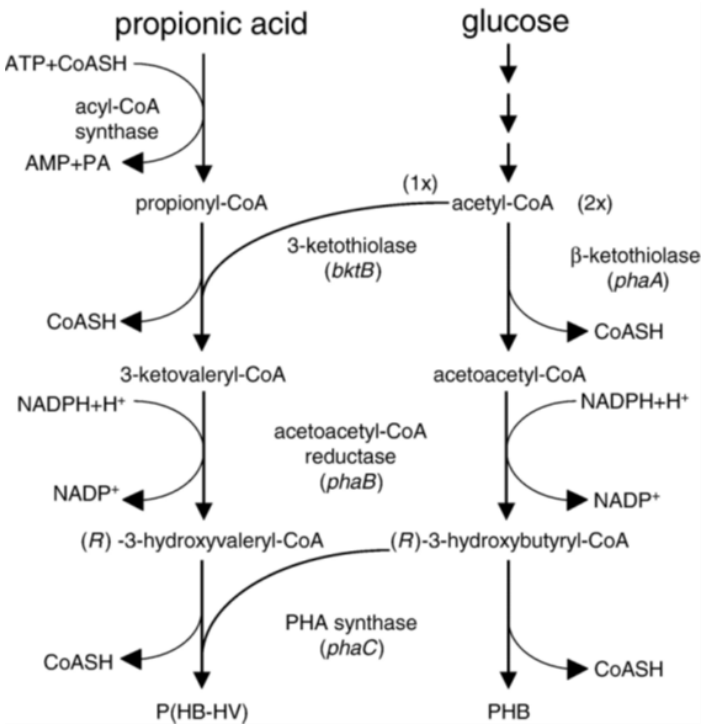


Figure 1.7 Poly(3HB) and poly(3HB-co-3HV) biosynthetic production pathways in *Ralstonia eutropha* (Suriyamongkol *et al*. 2007).

1.7.2 Advantages and disadvantages of PHAs

Biological polymers are regarded as greener because of their biodegradable abilities and showing better transparency (Scott 2000). They are derived by utilizing renewable resources hence CO_2 encumbrance in the environment is neutral because of their eco-friendly nature. PHAs extracted from bacterial cells are the versatile fully biodegradable polymers with notable similarities to the well-known synthetic polymers i.e. low density polyethylene and polypropylene in addition their disposal as bio-waste made them more and more appealing inside the pursuit of sustainable development of biodegradable plastic (Steinbüchel and Lütke-Eversloh 2003; Yu and Stahl 2008). Brittleness is one of the drawbacks causing them to be of compostable nature under specific conditions. Some possess a strong hydrophilic character and

under wet conditions they show unsatisfactory mechanical properties (Varsha and Savitha 2011). The high manufacturing price of biopolymer and the supply of low-price petrochemical equivalence make polyhydroxyalkanoates economically unattractive, a more effectual fermentation process, higher recovery / purification and the usage of inexpensive substrates can also appreciably reduce the manufacturing cost (Verlinden *et al.* 2007; Verlinden *et al.* 2011).

1.7.3 Application of PHAs

Utilization and application of PHAs have grown drastically over the past decade both in specialisation and variety. Industrially, PHA latex are utilized to make water-resistance surface cover for paper or cardboards opposing the combination of cardboard or paper with aluminium used currently which is non-degradable (Lauzier *et al.* 1993). They are utilized to make films, foils and diaphragms (Babel *et al*. 990). In the medical field, PHAs are finding substantial applications where they are used in tissue engineering, slow release of drugs and hormones. PHAs are used widely as bone plates, surgical sutures and osteosynthetic materials. Swabs and dressing materials for surgery are specifically made by PHA fibres. This is promoted due to PHAs being produced through fermentation causing them to have no left-overs of undesirable metal caused by catalysis utilized in chemical synthesis (Philip *et al.* 2007). In the agricultural sector, PHAs are utilized as mulch film and urea fertilizer coatings used in rice fields for insecticides and herbicides. They are used in bacterial inoculants for enhancement of nitrogen fixation in plants (Philip *et al.* 2007).

1.8. Bacterial microorganisms

Chemical or biological techniques are procedures utilized for the synthesis PHAs (Kemnitzer *et al*. 1993; He *et al.* 1999). Biosynthesis technique has proven to be the paramount technique yielding higher molecular weight compounds as compare to chemical techniques. Biosynthesis of PHAs involves fermentation process, microorganisms grown in an aqueous solution containing sustainable resources which consist of starch, glucose, sucrose, fatty acids, or nutrients in waste water under optimum conditions. PHAs are taken into consideration as environmentally and ecologically friendly particularly when petroleum, a non-ecological

resource deplete quickly (Chen 2010). Studies have proven that bacteria containing PHA storage materials are able to endure during starvation period when compared to bacteria without PHAs, these finding make PHA a storage compounds for carbon and energy sources. This energy-reserve material is capable of slowing down the cell autolysis and finally slowing its mortality (Macrae and Wilkinson 1958). Numerous bacterial strains among Gram positive (Williamson and Wilkinson 1958; Findlay and White 1983), Gram negative bacteria (Forsyth *et al*. 1958), archaebacteria (Doi 1990), photosynthetic bacteria (Hashimoto *et al.* 1993; Hassan *et al.* 1996; Hassan *et al.* 1997; Hassan *et al.* 1998) and cyanobacteria (Jensen and Sicko 1971; Jau *et al.* 2005) showed accumulation of polyhydroxybutyrate (PHB) both in aerobic and anaerobic conditions. Even though more than 300 different microorganisms are able to synthesizing PHAs but for vast scale production, only a few produce sufficient yield (*Alcaligenes latus, Azotobacter vinelandii*, *Cupriavidus necator* (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*, *Pseudomonas oleovorans*, *Protomonas extorquens, Paracoccus denitrificans*, and recombinant *E. coli*) (Chanprateep 2010). Under optimum growth conditions, recombinant *E. coli* has been engineered to produce PHA because of its capability to lyse easily and it was engineered for PHB-manufacturing initially and subsequently a variety of co-polymers are being manufactured (Keshavarz and Roy 2010). The *phaA* gene encodes β-ketothiolase, the first enzyme for the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA. This is followed by the reduction step of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA, catalyzed by the acetoacetyl-CoA reductase. The enzyme is encoded by the *phaB* gene and is NADPH-dependent. Polymerization of (R)-3-hydroxybutyryl-CoA monomers catalyzed with the support of PHA synthase is the last reaction, encoded by the *phaC* gene. PHA synthase in *Ralstonia eutropha*, formerly known as *Alcaligenes eutrophus*, reacts with C3–C5 chain length substrates and prefers C4-substrates. Consequently, PHAs acquired with the aid of this pathway comprise short-chain-length monomers.

Miscellaneous carbon sources ranging from inexpensive, complex waste effluents to fatty acids, alkanes, plant oils and in addition to simple carbohydrates are capable of producing PHAs by microorganisms. Agricultural and food processing industries discharge a great amount of waste materials annually, which constitutes a great renewable feedstock for PHA manufacturing. Utilization of these waste substances as carbon source for PHA production not only reduces the substrate expense, but also saves the cost of waste disposal (Chee *et al.* 2010b).

1.9. Objectives of the study

In this work corn silage will be used as a biomass for valorization. The objectives of this study was:

I. To produce PHAs under nitrogen starvation using *T. thermophilus* HB8 fermentation of glucose.

II. To recover PHA using sodium hypochlorite in the extraction process.

III. To identify and characterize PHA using GC and GC-MS.

IV. To incorporate corn silage into the growth medium as a renewable carbon source.

V. To characterize the PHA bio-plastic and assess its production and biodegradability

CHAPTER **2**

LITERATURE REVIEW

2.1. Sustainable production of PHA

Efforts have been made in recent decades to discover ways to achieve low-cost production of PHA (Munir and Jamil 2015). PHAs, a family of bio-polyesters with various structures, are the only bio-plastics entirely produced by microorganisms (Chen 2010). The foremost PHA cost in the manufacturing process is the substrate type (Yamane 1993). With the intention to reduce PHA production cost in the future, emphasis has shifted to the utilization of valuable, renewable material as substrates for PHA manufacturing (Ashby *et al.* 2011; Palmeri *et al.* 2012).

Waste materials in great quantities are discarded each year from the agricultural sector, industrial waste water and food processing industries. These waste materials signify a prospective renewable feedstock for PHA manufacturing (Chee *et al.* 2010b; Munir and Jamil 2015). Using these waste materials as carbon sources for PHA production not only lessens the substrates cost, but also reduces the cost of waste discarded (Yu and Stahl 2008). Production of biodegradable plastics with renewable resources is a subject of interest, however there need to be further investigation into the utilization of renewable resources for production of PHA.

The biomass-derived PHAs are bioplastics with a green agenda. They are entirely acknowledged as biosynthetic and biodegradable with nil toxic waste, and completely recyclable into organic waste (Chanprateep 2010). They are intracellularly produced as storage compounds for carbon and energy by an extensive range of microorganisms (Steinbüchel *et al.* 1992; James *et al.* 1999). They have a broad spectrum of properties, comprising of materials that emulate electrometric properties and possess thermoplastic properties. Also, studies have shown that biocompatibility is one of the main features that have generated global importance of this polymer (Braunegg *et al.* 2015). Their degradation in the environment is effective because numerous microorganisms in soil are able to utilize PHA depolymerases, enzymes that hydrolyze the ester bonds of a polymer into water-soluble monomers and oligomers. PHA

degrades into H_2O and CO_2 through microorganism's metabolism (Jendrossek and Handrick 2002; Lim *et al*. 2005; Sridewi *et al*. 2006). The first PHA to be known was P(3HB), discovered as intracellular granules in the Gram-positive bacterium *Bacillus megaterium* by a French microbiologist Maurice Lemoigne (Lemoigne 1926*)*. To contest petroleum derived polymers, research is involved in making PHA production economically viable since their production is unattractive due to high cost of substrate and recovery process. Production costs of PHA in fed-batch cultures using beet molasses was found to be one-third compared to glucose substrate using *Azotobacter vinelandii* UWD, a substantial reduction in raw material using beet molasses (Page 1992).

2.2. Production of PHAs from "waste"

Carbon-rich industrial waste can be utilized as an appropriate feedstock and can contribute in making PHAs economically competitive while avoiding conflict with nutritional or animal feed (Koller *et al*., 2013).

Albuquerque *et al.* (2007) developed a three stage process for the production of PHA from agricultural sugar cane molasses: (1) molasses acidogenic fermentation; (2) PHA-accumulation culture selection; and (3) PHA batch accumulation utilizing enriched sludge and fermented molasses. The effect of pH (5-7) on the organic profile and productivity was assessed in the fermentation step with acetic acid and propionic acid produced at higher pH, while lower pH produced valeric and butyric acids. PHA accumulation was evaluated with two selected cultures either acetate or fermented molasses, where acetate-selected culture was studied for organic acids distribution effect on polymer composition and yield. A storage yield was found to be between 0.37 to 0.50 Cmmol HA/Cmmol VFA. Varying types of organic acids used and polymer composition showed a direct relationship. Low 0.1 Nmmol/l ammonia concentration in the fermented molasses selected culture promoted PHA yield of 0.62 Cmmol HA/Cmmol VFA. Low organic loading with great ammonia concentration combination selected a culture with a constant storage ability and with a polymer storage yield of 0.59 Cmmol HA/Cmmol VFA which is close to 0.62 Cmmol HA/Cmmol VFA attained from acetate-selected culture. In the study conducted by Chua *et al.* (2003), activated sludge treated municipal wastewater was studied for the production of PHA biodegradable plastics, where pH, concentration of acetate

in effluent and sludge retention time (SRT) operational factors affected the investigation. It was noted that PHA yield of 30% sludge dry weight was achieved through sludge acclimatized with municipal wastewater supplemented with acetate while sludge acclimatized with municipal water only yielded 20% of sludge dry weight. Higher PHA production was achieved through usage of activated sludge with an SRT of 3 days than with an SRT of 10 days. Also, sludge acclimatized under pH 7 and pH 8 environments in sequencing batch reactors (SBRs) displayed similar PHA-accumulation capability. The significance of PHA-accumulation behavior of activated sludge was greatly influenced by pH, in PHA production batch experiments, when pH was kept at 6 or 7, a low PHA yield was achieved while a great degree of PHA yield was achieved when pH was controlled at 8 or 9.

Bengtsson *et al.* (2008) described manufacturing of PHA in activated sludge through pure culture fermentation where PHA production process at a laboratory scale was examined using paper mill wastewater. In this study, three stage processes were looked at which consisted of conversion of wastewater organic matter to VFA through acidogenic fermentation, enrichment of PHA producing organism by an activated sludge system operative under feast/famine settings and PHA accumulation in batch experiments. About 74% of soluble chemical oxygen demand (COD) was available as VFA after fermentation of wastewater and 31-47mol% of hydroxybutyrate and 53-69 mol% of hydroxyvalerate was achieved as a resulting PHA after batch accumulation. The highest PHA content obtained was 48% of sludge dry weight and the three stage process displayed a possibility to yield 0.11 kg of PHA per kg of influent COD treated.

Tripathi *et al.* (2012) evaluated different carbon sources based upon the cost and availability for PHA production by the soil bacterium *Pseudomonas aeruginosa.* Cane molasses yielded a maximum cell biomass and PHB concentration of 6.63 ± 0.05 g/L and 4.14 ± 0.12 g/L with PHB content of 62.44% after cultivation for 60.0 h. The second-best carbon source was fructose with PHB yield of 50.92% while glycol was the worst carbon source with a PHB yeild of 12.41%. Temperature, pH and agitation speed were studied on PHA production and the strong nitrogen source utilized was urea for bio-plastic synthesis over other inorganic nitrogen sources. Under optimized cultural and physical conditions, batch cultivation kinetics revealed maximum cell mass and PHA concentration of 7.32 ± 0.2 and 5.60 ± 0.3 g/L, respectively after

cultivation for 54.0 h. Total sugars (4%) and urea (0.8%) increased the yield of the process to 0.70 with productivity of 0.11 g/L/h. Produced PHA was further characterized with FT-IR and it showed PHB content.

Haas *et al.* (2008) studied the importance of using cheap carbon substrates for the manufacturing of poly(3-hydroxybutyrate). Saccharified waste potato starch proved to be utilized as an alternative carbon source for high cell density PHB accumulation using *Ralstonia eutropha* NCIMB 11599 with limited phosphate. A 179 g/L biomass, 94 g/L PHB, $Y_{biomass/starch}$ = 0.46 g/g, $Y_{PHB/starch}$ = 0.22 g/g, and PHB productivity of 1.47 g/L was achieved. The maltose residue accumulated in the fed-batch rector with no inhibition of PHA accumulation. It was detected that utilization of saccharified starch was effectively similar to that with glucose.

Elain *et al.* (2016) studied two types of processing waters for the production of PHA, namely, one rich in vegetable proteins and the other rich in reducing sugars i.e., a mixture of saccharose and stachyose in leguminous processing water (LPW) and a mixture of glucose and fructose in fruit processing water (FPW). A distinctive marine bacterial species *Halomonas* i4786 was used in a two stage fermentation process where these processing waters were used as growth media for PHA production. Nile blue staining was used as a preliminary test to determine if the two media efficiently promoted bacterial growth and PHA accumulation in the preliminary shake flasks. PHA production of 1.6 g/L and 1.8 g/L were achieved within 72 h of cultivation, in LPW and FPW respectively. Differential scanning calorimetry and steric exclusion chromatography characterization of the polymer specified that the two substrates steered the biosynthesis of polymers with different chain length, crystallinity and distribution.

Ribera *et al.* (2001) examined *P. putida* development in olive oil wastewater called alpechín, which is harmful for some microorganisms. The plasmid pSK2665, harbouring *Alcaligenes eutrophus* genes required for synthesis of poly (3-hydroxybutyric acid), permitted the *Pseudomonas* strain to grow to high concentrations in the wastewaters, aggregating biodegradable thermoplastic. It was seen that *Pseudomonas putida* KT2442 harbouring the plasmid pSK2665 increased PHA synthesis compare to parent strain after 72 hours. This demonstrated that *Pseudomonas* was able to grow in a residue (alpechín) and generate the PHA

polymer. Alpechín served as an economical substrate for polymers. It was reasoned that in future the accumulation could be enhanced by the ceaseless culture of *P. putida* in alpechín to accomplish results for industrial yield.

Jia *et al.* (2014) demonstrated the generation of polyhydroxyalkanoates from an overabundant sludge fermentation liquid (SFL) at laboratory and pilot scales in a bioreactor. A PHA-accumulating bacterial consortium (S-150) was identified from activated sludge-utilizing simulated SFL (S-SFL) containing a high concentration volatile fatty acids and nitrogen. The maximal PHA content represented 59.18% in S-SFL and dropped to 23.47% in real SFL of the dry cell weight (DCW) at laboratory scale. The pilot-scale incorporated framework involved an anaerobic fermentation reactor (AFR), a ceramic membrane system and a PHA reaction bio-reactor (PHAR). The PHA content pilot scale SFL reached 59.47% DCW with the maximal PHA yield coefficient (YP/S) of 0.17 g PHA/g COD. Results demonstrated that VFA-containing SFL was reasonable for PHA accumulation. The unfavorable effect of excess nitrogen and non-VFAs in SFL were dispensed with mechanical scale domestication, which may have brought about community structure optimization and substrate specific capacity improvement of S-150.

The production of polyhydroxyalkanoates by *Bacillus tequilensis* biocatalyst utilizing spent wash effluents as substrate introduced by Amulya *et al*. (2014) demonstrated a conceivably greener PHA production process and decrease in PHA creation costs. In this investigation, spent wash was utilized as a substrate for biohydrogen (H_2) generation and the subsequent acidogenic effluents were utilized as substrates for PHA creation. Maximum H_2 generation of 39.8 L and greatest PHA production of 40% dry cell weight was accomplished. High substrate removal related with decrease in acidification (53% to 15%) demonstrated that the VFA generated were adequately used for PHA production. It was demonstrated that PHA composition displayed copolymer [P(3HB-co-3HV)] with varying contents of hydroxybutyrate and hydroxyvalerate. Obtained results demonstrated that the utilization of spent wash effluents as substrate can extensively diminish the production cost of PHA with concurrent waste valorization. PHA synthesis with *B. tequilensis* and spent wash effluents was reported for the first time and positive outcomes were accomplished.

The investigation by Sawant *et al.* (2014) aimed to convert corn stover to polyhydroxyalkanoates. Corn stover was hydrolyzed to sugars by an on-site prepared cellulose cocktail from co-culture of *Trichoderma reesei* and *Aspergillus niger*. PHA producer, *Paracoccus sp.* LL1, was isolated from Lonar Lake, India and could amass PHA up to 72.4% of its dry cell weight. PHA production yielded 9.71 g/L from corn stover hydrolysate containing 40 g/L sugar blend. The PHA synthase gene (*phaC*) sequence of the isolate demonstrated 79% identity with the phaC gene of *Paracoccus seriniphilus* (E71) strain from the NCBI database. It was indicated that the nature of PHA found was poly (3-hydroxybutyrate) and was affirmed by FT-IR.

This investigation conducted by Koutinas *et al.* (2007) assessed a wheat-based biorefining methodology for creation of polyhydroxybutyrate by *Cupriavidus necator* (previously named *Ralstonia eutropha* however as of now assigned *Wautersia eutropha*). Wheat was bio-converted into two feedstock streams, parasitic concentrate (FE) and wheat hydrolysate (WH) that were wealthy in glucose and nitrogen, respectively. WH and FE were blended in appropriate proportions to give media varying strengths of glucose (5 – 26 g/L) and free amino nitrogen (FAN) (0.1 – 1.2 g/L) for batch shake flask fermentation. Increasing FAN strength showed higher microbial growth but less PHB growth. Utilization of different carbon sources i.e. amino acids, carbohydrates, and peptides brought about high accumulation yields, up to 1.07 g cells (g/glucose) as identified with glucose. Explicit growth rates up to 0.16 h^{-1} were observed. Three WH with comparative glucose (200 – 220 g/L) and varying FAN (0.3 – 1.48 g/L) concentrations were assessed in fed-batch shake flask fermentation for *C. necator* development and PHB accumulation. WH3 medium with the maximum nitrogen concentration showed the highest microbial biomass concentration of 29.9 g/L, growth yield of (0.28 g residual microbial biomass (g/glucose) and PHB yielded 0.43 (g/glucose). While WH2 medium showed the highest PHB concentration of 51.1 g/L and PHB content of 0.7 g/g. Because of the high yield of homopolymer PHB, cost sparing can be accomplished by utilizing this novel feedstock obtained from wheat – based bio-refinery.

Xu *et al.* (2010) studied the restructuring of the present fermentation and recuperation practices utilized for the production of polyhydroxyalkanoates important for the commercialization of environmentally benign and cost-focused biodegradable plastics. Capability of a wheat-based bio-refinery for the production of PHB was displayed and it was seen that fed-batch bio-conversion utilizing *Wautersia eutropha* growing on wheat-determined media yielded 162.8 g/l of PHB. A high PHB to total dry weight (TDW) yielded 93% (w/w) and was acquired because of microbial autolysis toward the end of fermentation. Micrograph of bacterial cells were taken with a transmission electron microscope (TEM) and demonstrated the capability of bacterial autolysis intended to shorten downstream recovery and purification of PHB. It was noticed that the utilization of amino acids and peptides derived from wheat gluten hydrolysis brought about a high glucose to PHB conversion and yielded 0.47 g/g. Wheat utilized for the production of enzymes and PHB yielded 0.3 g PHB/g, which compared to 82.8% of the greatest theoretical conversion yield and the productivity accomplished was 0.9 g/l h. It was concluded that fermentations carried out on wheat-derived media and formulated media with different commercial sources of nutrients demonstrated that the proposed wheat-based bio-refinery technique improved PHB production.

Martinez *et al.* (2015) demonstrated the achievability of producing polyhydroxyalkanoates by feeding a pure culture of *Cupriavidus necator* with a pre-treated olive plant wastewater (OMW). The investigation was done in a 500 mL shaken flask scale where OMW was dephenolised and after that fermented for the production of the effluent, rich in volatile fatty acids. The last stream (OMW acid) was used as the source carbon for PHAs accumulation. Pre-grown cells were initially fed with different concentrations of OMW acid, in particular: 25, 50, 75 and 100% (v/v). When OMW acid strength was 75 and 100%, profound inhibitory impacts were observed. Laboratory-prepared solutions were thereafter conducted to simulate the OMW acid, essentially showing that polyphenols significantly contributed to the observed hindrance. Moreover, it was seen that the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate), containing 11% of hydroxyvalerate, was accumulated up to 55% of the cells dry weight when two consecutive accumulation batch processes were done with 25% of OMW acid and without including any exogenous carbon source. It was discovered that the outcomes obtained are promising in the point of view of proceeding with the production study at a bench-top bio-reactor scale and from that point examining the possibility of emerging a biotechnological PHAs production process as part of an incorporated OMW valorization process.

Kourmentza *et al.* (2015) explored the capacity of microbes confined from an enriched mixed culture to produce polyhydroxyalkanoates and researched the impact of nitrogen and dual nitrogen–oxygen restriction on PHA production, by utilizing both synthetic and olive plant wastewater (OMW). PHAs production was performed through batch experiments utilizing both the enriched culture and the segregated strains belonging to the genus of *Pseudomonas*. The aim was to compare PHAs production limits, yields and rates. Utilization of enriched cultures and engineered wastewater under nitrogen limited yielded a remarkable PHA production of 64.4% g PHAs/g of cell dry mass (CDM) but it was found that when OMW was utilized, PHAs accumulation significantly diminished to 8.8% g PHAs/g CDM. A similar pattern was followed by the isolated strains; however, it was seen that their capacity to synthesize PHAs was lower. Despite the fact that, double nitrogen– oxygen limitation generally decelerated PHAs biosynthesis, in certain strains PHAs creation was increased.

Gobi and Vadivelu (2014) studied the aerobic dynamic feeding (ADF) methodologies in sequencing batch reactor (SBR) for PHA accumulation in aerobic granules. Aerobic consuming granules could expel 90% of COD from palm oil mill effluent (POME). VFAs were the sole source of PHA accumulation in the POME and a 100% elimination of butyric and propionic acids was observed. Aerobic granules produced the highest amount of 0.6833 mg PHA/mg biomass. Obtained PHA was characterized further and portrayed to be poly(3-hydroxybutyrate-co-3-hydroxyvalerate) utilizing gas chromatography-flame ionization detector.

The study presented by Morgan-Sagastume *et al.* (2015) investigated at the industrial-scale which was operated for more than 22 months at the Brussels North Wastewater Treatment Plant (WWTP) in order to assess polyhydroxyalkanoate creation coordinated with municipal wastewater and activated sludge. Activated sludge was used for PHA accumulation potential (PAP) by applying feast–famine treatment selection while treating the promptly biodegradable COD from influent wastewater with average expulsions of 70% COD, 60% $COD_{soluble}$, 24% nitrogen, and 46% phosphorus were accomplished. The biomass PAP was assessed to be in abundance of 0.4 g PHA/g volatile suspended solids (VSS). A batch fermentation of full-scale WWTP sludge at 35℃, 42℃ and 55℃ temperatures produced 6 - 9.4 g COD_{VFA}/L consistent VFA arrangement, with optimal fermentation execution at 42℃. Centrate was utilized to accumulate PHA up to 0.39 g PHA/g VSS. In this investigation, it was noticed that the centrate

supplements were a challenge for the accumulation process however accumulating a biomass with 0.5 g PHA/gVSS was viewed as reasonably feasible inside the typical available carbon streams at municipal waste administration facilities.

According to Lee *et al.* (2008) management of wastewater by resource recuperation approach permits the change of wastewater into profitable resources. Fermented palm oil mill effluent (POME) biotransformation into biodegradable plastics polyhydroxyalkanoates was studied. The study was conducted through cultivation and enhancement of PHA-producing organisms in activated sludge and for ensuing production of PHA by the cultivated sludge. PHA-accumulating enriched organism by means of aerobic dynamic feeding process was viable and had fundamentally upgraded the PHA storage capacity of the sludge i.e. wt % PHA per sludge dry weight from 4 wt % seed sludge to 40-64 wt % sludge, cultivation for more than 50 days. Fluorescent hybridization assessed that the cultivated sludge involved 13 ± 4% *Gammaproteobacteria,* 35 ± 7% *Alphaproteobacteria,* and 42 ± 12% *Betaproteobacteria*. Effect of pH i.e. 4.5, 7, 8, and 9 on PHA production by the cultivated sludge was investigated, which resulted in PHA content of 64 wt % in 8 h at pH 7. This finding made the neutral state the most ideal for the production of PHA. PHA accumulated was made up of 77 mol% 3-hydroxybutyrate and 23 mol% 3-hydroxyvalerate.

Koller *et al.* (2005) investigated the opportunity of utilizing inexpensive complex nitrogen and phosphate-sources from agriculture, silage juice (SJ) and green grass juice (GGJ). Agricultural juices were added to cultivation medium to study their effect on growth of PHA-accumulating strain *Wautersia eutropha*. The impact of these green added substances was directly contrasted with cultivation on defined minimum mineral medium, as well as on the similar media enhanced with more costly complex additives substances which are casamino acids. Supplementation with most complex additives brought about shortening of lag-phase of bacterial development and higher end-concentrations of residual biomass contrasted with minimal mineral medium. high poly(3-hydroxybutyrate) volumetric productivity was achieved and further characterized by GC-FID. The impact of the cheap SJ on volumetric efficiency of 0.653 g/L h^{-1} was identical to the outcomes for the costly casamino acids that yielded 0.619 g/L h^{-1}, and consequently was a practical alternative. The similar results were viewed for the biomass concentration of 7.00 g/L versus 7.44 g/L. This work introduced financial evaluation

with its outcomes proposed that it is conceivable to make the sustainable process of microbial PHA production the more monetarily plausible.

Kahar *et al.* (2004) examined high accumulation of polyhydroxyalkanoates by *Ralstonia eutropha* H16 and its recombinant strain PHB-4/pJRDEE32d13 from renewable low-cost soybean oil. PHA formed by the wild type strain H16 was 118-126 g/L of dry cell weight and high poly[(R)- 3-hydroxybutyrate)] content per dry cell of 72-76% (w/w) was additionally obtained. A copolymer of 3HB with 5 mol% (R)- 3-hydroxyhexanoates, p(3HB-co-3HHx), was produced from soybean oil as a sole carbon source by recombinant strain PHB-4/pJRDEE32d13 with a dry cell weight of 128-138 g/L and an acceptable PHA content of 71-74% (w/w). Results, ranging between 0.72 to 0.76 g-PHA per g-soybean oil utilized, have demonstrated that soybean oil can be a sustainable economical sole carbon hotspot for the production of PHA.

The study presented by Shen *et al.* (2015) researched the practicality and innovation to harvest poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) by blended culture. Chen *et al*., 2001; Qiu *et al*., 2003 previously concentrated on the fermentation production procedure of the copolymer by pure strain while this study investigated the synthesis from the activated sludge. Sodium laurate was used as the sole carbon substrate for sludge acclimation and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) production. Batch experiments were directed to research the effect of the nitrogen, carbon, phosphorus and supply on poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) production. Acclimated excess sludge 88989could deliver poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), and the extreme yield of 505.6 mg/L poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) containing 6.34 mol% hydroxyhexanoate was accomplished with states of continuous aeration, nitrogen and phosphorus constraint, and satisfactory carbon source executed by pulse feeding of 0.5 g/L sodium laurate each 4 h. Structure and composition of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from sludge were discovered identical to that from pure culture, as indicated by literature, FTIR and NMR spectra. High-throughput sequencing procedures portrayed that phylum *Chlorobi* and genus *Leadbetterella* were seen to be critical groups for poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) synthesis in the sludge community.

The study by Bhuwal *et al.* (2013) presented the use of paper, pulp, and cardboard sludge and waste water for the isolation and screening of polyhydroxyalkanoates producing bacteria and production of cost-effective poly(3- hydroxybutyrate) utilizing cardboard industry squander water. Total of 42 isolates indicated black- blue granules when stained with Sudan black B, which is the primary screening agent for lipophilic compounds while 15 isolates indicated positive outcomes with Nile blue A staining, a more explicit dye for PHA granules. The NAP11 and NAC1 isolates demonstrated a greatest PHA production of 79.27% and 77.63% with polymer concentration of 5.236 g/L and 4.042 g/L with cardboard waste water as a substrate. Both of the chosen isolates NAP11 and NAC1, were grouped up to genus level by studying their morphological and biochemical qualities and were observed to be *Enterococcus* sp., *Brevundimonas* sp. The isolates *Enterococcus* sp. NAP11 and *Brevundimonas* sp. NAC1 were considered as candidates for PHA production from cardboard industry waste water. It was reported for the first time the usage of the cardboard waste water as a cultivation medium for poly(3- hydroxybutyrate) production.

Nath *et al.* (2008) demonstrated the study on *Methylobacterium* sp. ZP24 accumulating polyhydroxybutyrate from disaccharides like sucrose and lactose. *Methylobacterium* sp. ZP24 indicated positive growth related with PHA growth resulting in the employment of intermitted feeding procedure having ammonia sulfate and lactose at different concentrations to increase the polymer yield. This intermitted feeding procedure resulted in about 1.5-fold increase in PHB production. Further growth in PHB production by 0.8 fold was accomplished by restricting the dissolved oxygen (DO) in the fermenter. Decreased DO was thought to increase flux of acetyl CO-A towards PHB production over the tricarboxylic acid cycle (TCA). Cheddar whey, a dairy waste, was utilized in this investigation for being a rich source of utilizable sugar and other supplements. Utilization in the bioreactor as a main substrate replacing lactose, prompted further increase in the PHB production by 2.5 fold. A total of 4.58-fold increase in the polyhydroxybutyrate was achieved utilizing limiting DO conditions with processed cheese whey enhanced with ammonium sulfate in fed batch culture of *Methylobacterium* sp. ZP24.

Table 2.1 is a summary of bacteria and different plant oils and waste utilized as substrates for the production of PHA

Table 2.1 Bacteria used for production of PHA from plant oils and wastes

Strains	PHA type	Substrates	PHA content (wt %)	Reference
Alcaligenes latus DSM 1124	P(3HB)	Soya waste, malt waste	33, 71	(Chee *et al.* 2010a)
Bacillus megaterium	P(3HB)	Beet molasses, date syrup	~50	(Omar *et al.* 2001)
Burkholderia sp. USM (JCM 15050)	P(3HB)	Palm oil derivatives, fatty acids, glycerol	22- 70	(Chee *et al.* 2010a)
Comamonas testosteroni	MCL-PHA	Castor oil, coconut oil, mustard oil, cottonseed oil, groundnut oil, olive oil, sesame oil	79-88	(Thakor *et al.* 2005)
Cupriavidus necator	P(3HB)	Bagasse hydrolysates	54	(Yu and Stahl 2008)
Cupriavidus necator H16	P(3HB-co-3HV)	Crude palm kernel oil, olive oil, sunflower oil, palm kernel oil, cooking oil, palm olein, crude palm oil, coconut oil + sodium propionate	65-90	(Lee *et al.* 2008)
Cupriavidus necator DSM 545	P(3HB)	Waste glycerol	50	(Cavalheiro *et al.* 2009)
Recombinant *Cupriavidus necator*	P(3HB-co-3HHx)	Palm kernel oil, palm olein, crude palm oil, palm acid oil	40-90	(Loo *et al.* 2005)
Recombinant *Escherichia coli*	P(3HB-co-3HHx-co-3HO)	Soybean oil	6	(Cavalheiro *et al.* 2009)
Pseudomonas aeruginosa IFO3924	mcl PHA	Palm oil	39	(Marsudi *et al.* 2008)
Pseudomonas aeruginosa NCIB 40045	mcl PHA	Waste frying oil	29	(Fernández *et al.* 2005)
Pseudomonas guezennei biovar. tikehau	mcl PHA	Coprah oil	63	(Simon-Colin *et al.* 2008)
Thermus thermophilus HB8	P(3HV-co-3HHp-co-3HNco-3HU)	Whey	36	(Pantazaki *et al.* 2009)

Comamonas testosteroni produced highest PHA yield of 79-88% followed by the use of crude palm kernel oil, olive oil, sunflower oil, palm kernel oil, cooking oil, palm olein, crude palm oil, coconut oil and sodium propionate substrates using *Cupriavidus necator* H16 with PHA yield of 65-90%. Soybean oil substrate using recombinant *E. coli* gave the lower 6% yield. Plant oils gave a remarkable PHA yield and due to sufficient availability and non-commercial importance these natural residues have created attention for production of cost effective bio-plastics (Rehm 2007; Jain and Tiwari 2015).

Various taxonomically-related microscopic organisms have been distinguished which accumulate poly(hydroxyalkanoate) copolymers comprising essentially 3-hydroxyvalerate (3HV) monomer units from a range of irrelevant single carbon sources Haywood *et al.* (1991) presents. One of these, *Rhodococcus* sp. NCIMB 40126, was further studied and appeared to accumulate a copolymer containing 75 mol% 3HV and 25 mol% 3-hydroxybutyrate from glucose as sole carbon source. Polyesters containing both 3HV and 3HB monomer units, together with 4-hydroxybutyrate, 3-hydroxyhexanoate or 5-hydroxyvalerate were additionally produced by this organism from accumulating substrates. A 99 mol% 3HV poly (3-hydroxyvalerate) was formed with valeric acid as substrate. ^{13}C and ^{1}H NMR analysis affirmed the composition of these polyesters. The thermal properties and atomic weight of the copolymer formed from glucose were similar to those of PHB delivered by *Alcaligenes eutrophus*.

2.3. Production of PHA using enzymes

Energy storage compound composition polyhydroxyalkanoate relies upon the PHA synthases, the source of carbon and the metabolic C (Madison and Huisman 1999; Rehm and Steinbüchel 1999). Different metabolic pathways have been depicted for the biosynthesis of PHA_{SCL} and PHA_{MCL}. They catalyze the change of 3-hydroxyacyl-CoA (3HACoA) substrates into PHA with the affiliated release of CoA (Rehm and Steinbüchel 1999). PHA synthases, which utilize CoA thioesters of (R)-3-hydroxyalkanoates as substrates and catalyze the polymerization of these monomers to PHA with corresponding release of CoA, symbolize the key enzymes of PHA biosynthesis (Qi and Rehm 2001). More than 40 PHA synthase genes have been assigned and portrayed (Rehm and Steinbüchel 1999; Rehm and Steinbüchel 2001), and their protein products can be extensively orchestrated into three distinct classes dependent on their subunit composition and substrate specificities. Class I synthases are dynamic towards short-chain-length (R)- hydroxyacyl-CoA, comprising of three to five carbon atoms, and are signified by the PHA synthase of *Ralstonia eutropha*. Class II is signified by the PHA synthase of *Pseudomonas aeruginosa* which is dynamic towards medium-chain-length (R)-3-hydroxyacyl-CoA, containing 6 to 14 carbon atoms. PHA synthases of class I and class II are made out of a single subunit, though class III PHA synthases are made out of two non-identical subunits. Class III is signified by the *Allochromatium vinosum* PHA synthase, comprising of subunits PhaC and PhaE, and displaying activity towards short-chain-length (R)-3-hydroxyacyl-CoA. Moreover, couple of bacteria, for example, *Aeromonas punctate* (Fukui, Shiomi and Doi 1998) and *Rhodococcus ruber* (Haywood *et al.* 1991) have been reported to have PHA synthases that are made out of one subunit which displays specificity for both short-and medium-chain-length (R)- 3-hydroxyacyl-CoA. Albeit various PHA synthase genes have been cloned and assigned, just a few PHA synthases have been purified and enzymatically characterized, e.g. PHA synthases from *P. aeruginosa*, *A. vinosum* and *R. eutropha* (Gerngross *et al.* 1994; Liebergesell *et al.* 1994; Qi, Steinbüchel and Rehm 2000; Rehm and Steinbüchel 2001).

Jia *et al.* (2000) revealed polyhydroxybutyrate synthases catalyze the change of β-hydroxybutyryl coenzyme A (HBCoA) to PHB. These enzymes required a functioning site cysteine nucleophile for covalent catalysis. A protein BLASTp search utilizing the Class III *Chromatium vinosum* synthase sequencing showed a high homology to prokaryotic lipases whose crystal structures were known. The homology was exceptionally persuading in the α-β-elbow (with the dynamic site nucleophile)- α-β structure and residues 131−175 of the synthase.

In the study by Müh *et al.* (1999) presents polyhydroxyalkanoate synthase from *Chromatium vinosum* catalyzes the transformation of 3-hydroxybutyryl-CoA (HB-CoA) to polyhydroxybutyrate and CoA. The synthase was made out of a 1:1 blend of two subunits, *PhaC* and *PhaE*. Size-exclusion chromatography showed that in solution *PhaC* and *PhaE* existed as large molecular weight aggregates. The holo-enzyme, *PhaEC*, had an explicit activity of 150 units/mg. Every subunit was cloned, expressed, and purified as a $(His)_6$-tagged construct. The *PhaC*-$(His)_6$ protein catalyzed polymerization with an explicit activity of 0.9 unit/mg and the *PhaE*-$(His)_6$ protein was inactive with an explicit activity of <0.001 unit/mg. The expansion of *PhaE*-$(His)_6$ to *PhaC*-$(His)_6$ expanded the activity several 100-folds. Priming step investigation of the polymerization procedure, the *PhaEC* was incubated with a trimer of HB-CoA in which the terminal hydroxyl was supplanted with tritium ([^{3}H]-sT-CoA). [^{3}H]-sT-synthase with HB-CoA incubation produced [^{3}H]-polymer. The extracted polymer was characterized with HPLC and ion trap mass spectrometry.

Qi *et al*. (1998) investigated the heterologous expression of the *phaC1* gene from *Pseudomonas aeruginosa*, which encoded one of the polyhydroxyalkanoic acid synthases, in *Escherichia coli* impaired in fatty acid β-oxidation lead to polyhydroxyalkanoic acid production when cells were cultivated on fatty acids. The utilization of the fatty acid β-oxidation inhibitor acrylic acid as an instrument to channel intermediates of β-oxidation to polyhydroxyalkanoic acid synthesis was evaluated. Different *E. coli* strains influenced in fatty acid metabolism and the wild-type strain harboring plasmid pBHR71 were investigated with respect to polyhydroxyalkanoic acid production in the presence of acrylic acid, an inhibitor of fatty acid β-oxidation. *E. coli fadR* mutant RS3097 revealed the high polyhydroxyalkanoic acid production of 45.7 mol% PHA content. The ideal inhibitory concentration of acrylic acid was seen to be 0.24 mg ml^{-1} and caused effective directing of intermediates of β-oxidation to polyhydroxyalkanoic acid

synthesis. Under these conditions decanoate *E. coli* RS3097 harboring plasmid pBHR71 showed that polyhydroxyalkanoic acid production yielded around 60% of cell dry weight.

Langenbach *et al*. (1997) studied the capability of the production of polyhydroxyalkanoates in the recombinant *E. coli*, comprising of medium-chain-length hydroxyfatty acids (C6−C14). *E. coli* mutants pretentious in fatty acid degradation and de novo fatty acid synthesis were utilized. Functional expression of the *Pseudomanas aeruginosa* PHA synthase gene *phaC1* was establish. The coding area of *phaC1* was subcloned by means of PCR into vector pBluescript SK^- and the subsequent plasmid pBHR71 permitted functional articulation of *phaC1* under *lac* promoter control and deliberated synthesis and growth of PHA to different strains of *E. coli*. Analysis of PHA synthesis was examine with respect to the carbon source in different *E. coli fad* and *fab* mutants. This investigation gave confirmation that intermediates of the fatty acid β-oxidation can be directed to PHA synthesis and that 3-hydroxydecanoyl-CoA is the fundamental substrate for PHA synthase *PhaC1* from *P. aeruginosa*. *E. coli fadB* mutant LS1298 comprising plasmid pBHR71 and cultivated in LB medium containing 0.5% (w/v) decanoates showed accumulation of PHA adding to about 21% of the cell dry weight, which was composed of 20 mol % 3-hydroxyoctanoate, 5 mol% 3-hydroxydodecanoate, 72.5 mol % hydroxydecanoates and 2.5 mol % 3-hydroxyhexanoate.

The study conducted by Brandl *et al.* (1988) studied *Pseudomonas oleovoans* developed utilizing formate to decanoate as the sole carbon sources in the homogeneous media containing n-alkanoic acid. Hexanoate and higher n-alkanoic acid showed the accumulation of intracellular poly-β-hydroxyalkanoates compare to other sources. A 30% yield of the cell dry weight was the highest isolated polymer when accumulated in either nonanoates or octanoate. The polymer had a similar chain length as the n-alkanoic acid utilized for development, yet units with two carbon atoms less or more than the acid utilized as a carbon source were produced in the formed polyester. Copolymers containing six unique types of β-hydroxyalkanoate units were formed. The average molecular weights of the poly-β-hydroxyalkanoate copolymers created by *P. oleovorans* went from 90000 to 370000. Regardless of the higher cell production obtained with nonanoate, and octanoate the utilization of heptanoate and hexanoate produced polymers of higher molecular weight.

The study by Qi *et al*. (2000) for the first time showed purification success of the type II polyhydroxyalkanoate synthases *PhaC1* and *PhaC2* from *Pseudomonas aeruginosa* by using N-terminal His_6-label fusion and metal chelate affinity chromatography. The activity of PHA synthase in *vivo* His_6-labeled was affirmed by functional articulation of the corresponding genes in *E. coli*, and the activity of PHA synthase was measured with the enzymes in *vitro*. The enzyme activity of PHA synthases *PhaC1* and *PhaC2* produced 0.039 U mg^{-1} and 0.035 U mg^{-1} protein, respectively. Kinetics studies have demonstrated a lag phase for both PHA synthases when utilizing (R, S)-3-hydroxydecanoyl-CoA as substrate. Specific enzyme was protein improved to 0.055 U mg^{-1} when the phasin GA24 from *Ralstonia eutropha* was supplemented to the assay, additionally CoA inhibited PHA synthase action and K_i of 85 μM was obtained. A two enzyme system was conventional, utilizing industrially accessible acyl-CoA synthetase and PHA synthase, which permitted the in *vitro* de novo PHA granule development and the in *vitro* synthesis of poly(3-hydroxydecanoate) showing a weight molar mass of 9.8 x 104 g mol^{-1}, and which occurred autonomously of pre-existed PHA granules.

Rehm and Steinbüchel (2001) investigated the acyl-acyl carried protein (ACP) thioesterase cDNA from the plant *Umbellularia californica*. The coexpression of the PHA synthase genes from *Pseudomonas aeruginosa* and *Ralstonia eutropha*, or just the PHA synthase gene from, respectively, demonstrated PHA_{MCL} growth when type II PHA synthase was formed. Both wild-type *E. coli* and *fad* were researched, and when the β-oxidation pathway was reduced PHA_{MCL} production from gluconate was achieved, about 60% of cell dry weight was contributed. Coexpression of type II PHA synthase gene with cDNA encoding the medium-chain acyl-ACP thioesterase from *U. californica* formed another PHA_{MCL} bio-amalgamation pathway, involving fatty acid *de novo* bio-synthesis with fatty acid β-oxidation, utilizing a non-related source of carbon.

2.4. Formation of PHAs in the cell

The study by Beeby *et al.* (2012) evaluated the bacterium *Ralstonia eutropha* forming cytoplasmic granules of polyhydroxybutyrate that are the source of biodegradable thermoplastic. Jendrossek (2005b, 2009) suggested that PHB granules are generated in the membrane, Tian *et al.* (2005) suggested that the granules are generated in the scaffolds and Gerngross *et al.* (1993) suggested that the granules are generated as the micelles in the cytoplasm. Electron cryotomography was employed to monitor granule genesis and development in 3 dimension in a near-native, frozen-hydrated state in intact *Ralstonia eutropha* cells. It was observed that neither nascent granules within the cell membrane nor scaffolds were seen, instead granules of all sizes resided towards the center of the cytoplasm along the length of the cell and exhibited a discontinuous surface layer more consistent with a partial protein coating than either a lipid mono- or bilayer. Fusing granules were also observed, this suggested that small granules were continually generated, produced and then merged. This observation supported a model of biogenesis wherein granules formed in the cytoplasm coated not by phospholipid but by protein. Thin-section electron microscopy, fluorescence microscopy and atomic force microscopy showed differences in nucleoid condensation and specimen preparation.

CHAPTER **3**

THEORETICAL FRAMEWORK

3.1 Compositional Analysis

3.1.1 Theory

Lignocellulose materials do not contain easily accessible monosaccharides and low molecular mass chemicals but rather polymers and polysaccharides, which need to be hydrolyzed to release smaller compounds. Also, lignocellulose material is composed of cellulose, hemicellulose and lignin that needs to be separated before processing of the components. Compositional analysis of lignocellulosic material is critical in providing information for energy, fuels and biochemical production using biomass. Methods established for the characterization of biomass were originally based on gravimetric and/or colorimetric analysis. Martens (2000) and Martens and Loeffelmann (2002) indicated the need for more appropriate methods to be developed for the characterization of biomass. The methods by Technical Association of Pulp and Paper Industry (TAPPI) were developed for characterizing wood for usage in the pulp and paper industry. It has been demonstrated that these techniques are not necessarily applicable for either lignocellulosic residues particularly when dealing with herbaceous and grass feed-stocks such as fodder (Mkhize 2016).

The compositional analysis of biomass feedstock enables the determination of reaction yields. The approximate carbohydrate content in a biomass is crucial as it is directly proportional to the bioethanol yield in a biochemical conversion process (Aden *et al.* 2002). Livestock, like human beings, require energy, protein, minerals and vitamins from their food. While fresh fodders as a whole provide these essential items, conserved forage on the other hand may be deficient in one or two of them, hence, the aim of the conservation process is to preserve as many of the original nutrients as possible. Preserved fodder contains a substantial amount of organic and inorganic compounds, which makes it suitable for bioconversion utilization.

The composition of different feedstocks can vary greatly because of the intricate and heterogeneous nature of biomass. The composition of corn stover, depends on the morphology and are a reflection of changing physiological roles and functional specializations of the cell

types existing in each tissue under an explicit set of agronomic conditions (Templeton *et al.* 2009). Structural carbohydrates (e.g. cellulose) are of little significance in silage fermentation, although hemicellulose may make a minor contribution to the provision of water-soluble carbohydrates. The possibility of storage carbohydrates having a role in silage fermentation is strengthened by the isolation of lactic acid bacteria with the ability to hydrolyze starch (Woolford and Pahlow 1998). There have been relatively few attempts to predict the potential of a material for silage on the basis of its chemical composition other than on the basis of the content of fermentable sugar. Biomass feedstock variability influences process cost; therefore, powerful, reliable, accurate and high-throughput methods are critical in determining the composition of different feedstocks. Tables 3.1 -3.4 lists the compositional analysis of various lignocellulosic biomass feedstock.

Table 3.1 Procedures for cellulose determination in lignocellulosic biomass

Cellulose		
Method	Reference	Description
Sulphuric acid (H_2SO_4)	Grohmann *et al.* (1984)	Based on 64% H_2SO_4 and 2 h hydrolysis at ambient temperature. A 2-step dilution and heating is utilized for complete dissolution. Measurement of released glucose is measured enzymatically (colorimetric fluorometric detection using glucose assay kit)
	Saeman *at al*. (1944)	72% H_2SO_4 hydrolysis for 3 h at ambient temperature, dilution to 4% and 1 h at 100°C. The release glucose measured by UV/Vis spectrophotometry.
Mono-ethanolamine	Nelson and Leming (1957)	Yields total cellulose by gravimetric method (hemicellulose and the lignin dissolved and cellulose is filtered and weighed)

Source: (Walford 2008)

Table 3.2 Procedures for hemicellulose determination in lignocellulosic biomass

Hemicellulose		
Method	Reference	Description
Trifluoroacetic acid (TFA)	Fengel and Wegener (1979)	Diluted TFA, otherwise similar to cellulose method (Mono-ethanolamine method)
Hydrochloric acid (HCl)	Moore and Jung (2001)	30% HCl hydrolysis followed by conversion of pentosans to furfural and quantified by UV/Vis spectrophotometry

Source: (Walford 2008)

Table 3.3 Procedure for total carbohydrate determination in lignocellulosic biomass

Total Carbohydrates		
Method	Reference	Description
Trifluoroacetic acid (TFA)	Fengel and Wegener (1979)	Undiluted TFA utilized. The reaction periods of the method for differing levels of lignin. All sugars hydrolysis and measured by HPLC.
Sulphuric acid (H_2SO_4)	Foyle *et al*. (2007)	Based on the Grohmann cellulose method (1984) with modification of hydrolysis times. Sugars measured by HPLC.

Source: (Walford 2008)

Table 3.4 Procedures for lignin determination in lignocellulosic biomass

Lignin		
Method	Reference	Description
Sulphuric acid (H_2SO_4)	Brauns (1952)	Gravimetric method in which lignin is isolated as the insoluble material from acid hydrolysis
	Saeman *et al*. (1944)	Gravimetric method based on 74% acid cellulose hydrolysis method (72% H_2SO_4 hydrolysis for 3 h at ambient temperature, dilution to 4% for 1 h at 100°C. The released glucose measured by UV/Vis spectrophotometry)
Acid detergent Neutral detergent	Van Soest (1963)	A gravimetric method developed for fibre and lignin content in forage samples. Lignin is quantified by solubilizing by H_2SO_4 after extraction with acid or neutral detergent.
Nitobenzene Acidolysis Thiocidolysis	Lapierre *et al*. (1989)	A gravimetric method in which oxidation of lignin using these chemicals gives different degradation products leading to structural analysis.
Acetyl bromide	Lu and Ralph (1997)	
Permanganate	Tasman and Berzins (1957)	Based on the oxidation of lignin by the addition of excess $KMnO_4$. Basis of the Kappa number test used in the paper industry
Thioglycolate	Hatfield and Fukushima (2005)	Relies on complete solubilisation of lignin followed by spectroscopic measurement in solution

Source: (Walford 2008)

3.1.2. Experimental methods for CS nutritional value determination

The compositional analysis of the biomass components was carried out according to the methods described by the Association of Official Analytical Chemists (AOAC) (William 1980; Chemists and Horwitz 1990).

3.1.2.1. Moisture content

Agricultural biomass contains large amounts of moisture, which can change when exposed to the air therefore the moisture and dry matter content of the test material is required for use in all calculations. Dry matter refers to the material remaining after removal of water, and the moisture content reflects the quantity of water existing in the test material. It is a significant analysis, in that the concentration of other nutrients is usually expressed on the dry matter basis. A standard convection oven (Naberthern GmbH, Germany) set at 105°C was utilized to determine the moisture content of the CS and the dry matter or oven dry weight obtained by difference. This procedure is used for determining the amount of dry matter and moisture remaining after oven drying of the biomass (known as the dry sample) using equations 3.1 and 3.2, respectively.

$$\text{Dry matter (DM)}\% = \frac{\text{Mass (dry pan + dry sample) − Mass (dry pan)}}{\text{Mass sample as received}} \text{ x } 100 \quad (3.1)$$

$$\text{Moisture } \% = 100 - (\% \text{ Dry matter}) \quad (3.2)$$

3.1.2.2. Ash content

The measure of inorganic constituents is expressed as the ash content. It is the percentage of the residue remaining after oxidation between 550 to 600°C in a muffle furnace of the dried sample. All results are reported relative to the 105°C oven dry weight (ODW) of the dry sample using equations 3.3 and 3.4.

$$\text{ODW} = \frac{\text{Weight (air dry sample) x \% Dry Matter}}{100} \quad (3.3)$$

$$\% \text{ Ash } = \frac{\text{Weight (cruicible+ash)} - \text{Weight (cruicible)}}{\text{Oven dry weight (ODW)}} \text{ x } 100 \qquad (3.4)$$

3.1.2.3. Fat content

Fats are esters made when an alcohol, glycerol condenses with carboxylic acids known as fatty acids (Figure 3.1). When the esterification involves a single fatty acid the compound produced is a simple triglyceride.

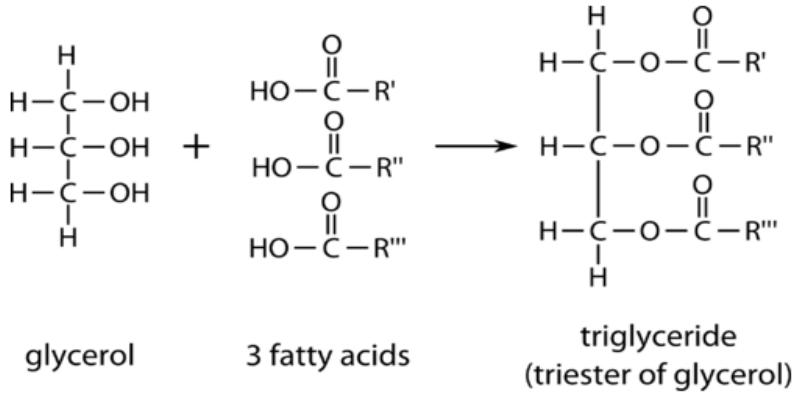


Figure 3.1 Production of triglyceride from glycerol and fatty acids (Díaz *et al.* 2014).

Fats are the group of organic substances which are insoluble in water but soluble in solvents such as ether, chloroform and benzene. They are used in metabolism by living organisms. They are quantified by dissolving a dried sample with petroleum ether (extraction flask and fat) and then the ether is evaporated in a soxhlet apparatus, with the fat remaining in the extraction flask as the fat content. The results are reported using equation 3.5.

$$\text{Fat content } (\%) \ = \frac{\text{Weight (extraction flask+fat)} - \text{Weight (extraction flask)}}{\text{Sample weight}} \text{ x } 100 \qquad (3.5)$$

3.1.2.4. Fibre

Carbohydrates are aldehyde or ketone derivatives of polyhydric alcohols. Structural carbohydrates consist largely of polysaccharides which are polymers of monosaccharides joined by glycosidic links, hemicellulose (non-cellulose matrix polysaccharides other than pectin). Examples of polysaccharides forming part of hemicellulose: arabinose, xylose, cellulose (polymer of D-glucose unit).

3.1.2.4.1. Neutral detergent fibre

A neutral detergent solution is used to remove all digestible components from agricultural residues, while the truly indigestible components are recovered in the neutral detergent fibre (NDF) residue (structural carbohydrates i.e. cellulose, hemicellulose and lignin). The sample is weighed after extraction with the neutral detergent reagent followed by treatment with α-amylase in a buffer solution to dissolve the remaining starch. The residue is referred to as the cell wall fraction and consists of cellulose, hemicellulose and lignin. The results are reported relative to the 105°C dry matter (DM) of the dry sample using equation 3.6.

$$\text{NDF content (\% DM)} = \frac{\text{Weight (crusible+dry sample after using detergent solution)}-\text{Weight (crusible)}}{\text{Weight (DM)}} \text{ x } 100 \qquad (3.6)$$

3.1.2.4.2. Acid detergent fibre

The cell wall material is treated with an acid detergent which removes hemicellulose. The acid detergent fibre (ADF) residue consists of mainly cellulose and lignin. The dried NDF sample is further extracted with an acid detergent solution. The loss in weight resulting from digestion of the dried residue corresponds to the weight of ADF. The results are reported relative at 105°C relatively to the dry matter (DM) of the dry sample using equation 3.7.

$$\text{ADF content (\%DM)} = \frac{\text{Weight (crusible+dry sample)}-\text{Weight (crusible)}}{\text{Weight (DM)}} \text{ x } 100 \qquad (3.7)$$

The amount of hemicellulose is obtained from the difference of the ADF and NDF and is calculated using equation 3.8.

$$\text{Hemicellulose} = \text{NDF} - \text{ADF} \quad (3.8)$$

3.1.2.4.3. Lignin

The procedure utilizes a two way step acid hydrolysis to fractionate the CS into simpler compounds of lignin that are identified and quantified. The lignin fractionates into acid soluble lignin (ASL) and acid insoluble lignin (AIL). The AIL may also contain protein and ash, which needs be accounted for in gravimetric analysis. The ASL is measured at maximum wavelength for lignin which is 240 nm by UV-Vis spectroscopy. The percentage of AIL; ASL and total lignin is calculated utilizing equations 3.9, 3.10 and 3.11, respectively.

$$\text{AIL content (\%DM)} = \frac{\text{Weight (crusible+dry sample)} - \text{Weight (crucible+ash)}}{\text{Weight (DM)}} \text{ x } 100 \quad (3.9)$$

$$\text{\% ASL (\%MD)} = = \frac{\text{UV Absorbance x Filtrate Volume x Dlution factor}}{\text{Absorbtivity (e) x ODW(sample) x Pathlength}} \text{ x } 100 \quad (3.10)$$

$$\text{\% Total Lignin} = (\text{\% AIL} + \text{\%ASL}) \quad (3.11)$$

3.1.2.5. Starch content

Starch is a polymer of glucose made up of α (1 – 4) linear and α (1 – 6) branching linkages, present in many plants as a reserve carbohydrate. Routine analysis method of starch samples generally involves initial washing of the sample with hot 80 % ethanol to remove soluble sugars, followed by solubilisation of starch using acetate buffer. This is followed by the breakdown of the polymer to dextrins and oligosaccharides through enzyme treatment facilitating the quantitative conversion to glucose monomer which is then quantitatively determined through calorimetry analysis using UV-Vis spectrophotometry at 570 nm which is the maximum wavelength for glucose absorption. A heat stable α-amylase and amyloglucosidase are utilized to hydrolyse the starch found in the finely grounded plant

material. The starch content is reported relative to the 105°C dry matter (DM) of the dry sample using the equation 3.10.

$$\text{Starch content (\%MD)} = \frac{0.4555 * \text{Absorbance of sample} * 0.9}{\text{sample weight} * \text{Absorbance of glucose standard}} \quad (3.12)$$

3.1.2.6. Protein

Proteins are polymers of amino acids. In common with carbohydrates and fats, they contain nitrogen, hydrogen and oxygen. They are found in all living cells, where they are intimately connected with all activity that constitutes the life of the cell. All the amino acids present in the protein structure have an L-configuration of the carbon atom. Nitrogen is determined by total combustion of the sample at 1100°C in the presence of oxygen where the nitrogen is converted to NO_x gas. The NOx gas is reduced to N_2 which is measured in a thermal conductivity cell. The percent protein is calculated by multiplying the reported nitrogen by 6.2500 (the assumption is that proteins of typical contain 16% nitrogen in average). The results are reported using equation 3.13.

$$\text{Protein (\%DM)} = \text{\% Nitrogen x Protein Factor (6.2500)} \quad (3.13)$$

3.1.2.7 Total sugars

One of the main components of food and food products are sugars and they are considered to be hydrates of carbon. They are compounds composed of the polyhydroxy ketones, aldehydes, alcohols, acids, their simplified derivatives, and their polymers having polymeric linkages of the acetal type. Mono and disaccharides available in food are referred to as simple carbohydrates. The plant material is hydrolysed with H_2SO_4, followed by mixing the aliquots of the hydrolysate with copper reagent for the determination of reducing sugars. This is followed by the addition of arsenomolybdate reagent to completely dissolve the cuprous oxide by vigorous stirring until the evolution of CO_2 ceases. The blue colour is allowed develop for

2 h and the released sugars are measured by UV/Vis spectrophotometry at 750 nm. The results are reported using equation 3.14.

$$Total\ sugars\ (\%) = \frac{Absorbance\ sample * 555.555}{Absobance\ standard} \quad (3.14)$$

3.1.2.8. UV/Vis Spectroscopy

Optical density (OD) provides the most relevant information to make the measurements in bioprocess and fermentation monitoring. This effect can be utilized to determine the concentration of biomass in turbid samples using UV-Vis spectroscopy by measuring the intensity of light passing through a sample (Figure 3.1), and compares it to the intensity of light before it passes through the sample. The light beams that passes through the sample compartment are detected in the detector and quantified. It is also used for sugar quantification. Figure 3.2 is the schematic representation of a UV-Vis spectrophotometry. It is used for lignin quantification and analyse PHA production by *T. thermophilus* HB8.

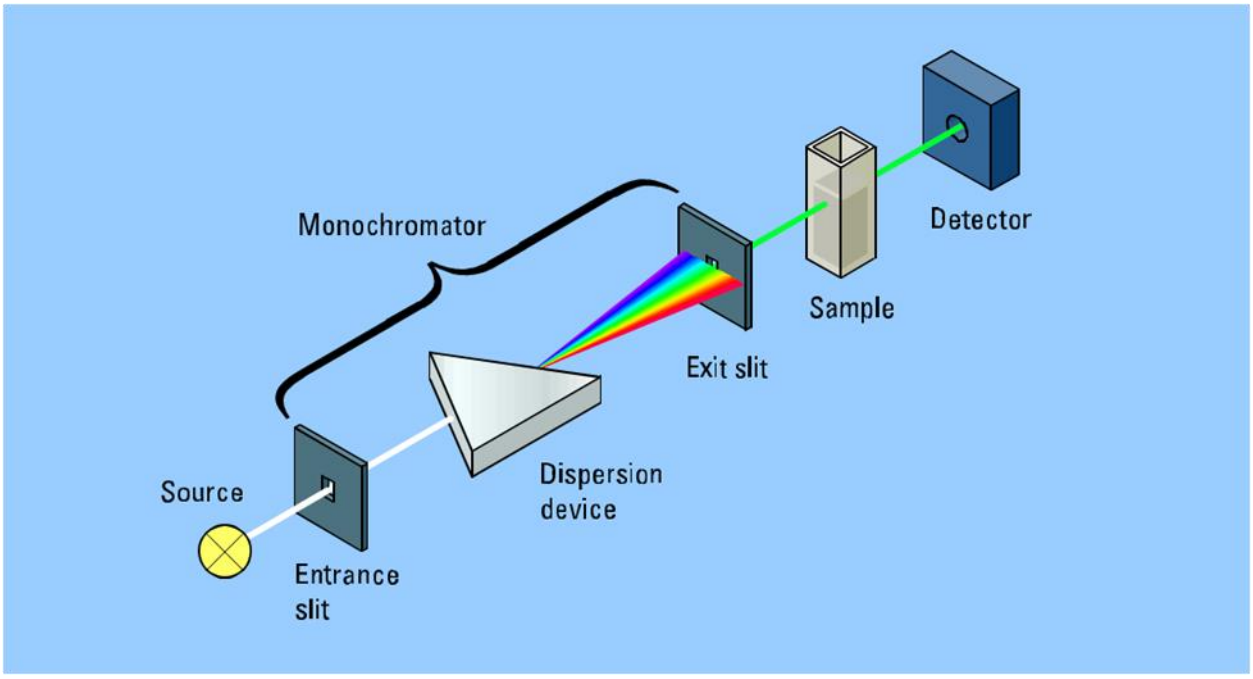


Figure 3.2 Schematic representation of a UV/Vis spectrophotometer used for the determination of optical density.

3.1.2.9. Inductive coupled plasma−Optical Emission Spectroscopy (ICP−OES)

The technique depends upon the spontaneous emission of photons from atoms and ions that have been excited in a radio frequency discharge. Liquid and gas samples may be introduced directly into the instrument, while solid samples need extraction or acid digestion so that the analyte will be present in a solution. The sample solution is transformed to an aerosol and directed into the central channel of the plasma. At its core, the inductively coupled plasma (Figure 3.3) sustains a temperature of approximately 10000 K, for the aerosol to be quickly vaporized. Analyte elements are liberated in the gaseous state as free atoms. Further collisional excitation inside the plasma conveys extra energy to the atoms, encouraging them to excited states. Sufficient energy is often obtainable to convert the atoms to ions and subsequently promote the ions to excited states. Both the atomic and ionic excited state species may then relax to the ground state via the emission of a photon. These photons contain characteristic energies that are determined by the quantized energy level structure for the ions or atoms. Thus, the wavelength of the photons can be utilized to classify the elements from which they originated. The total number of photons is directly proportional to the concentration of the originating element in the sample. Mineral elements are classified as essential because they have possible metabolic roles in mammalian tissues. These comprise of macro elements and micro elements. All the essential mineral elements, both major and traces have one or more catalytic function in the cell. Some mineral elements are firmly bound to the proteins of enzymes, while others are present in prosthetic groups in chelated form. They are quantified by ICP-OES where an ashed sample is dissolved in acid and diluted with water. Figure 3.3 is a schematic representation of a ICP-OES.

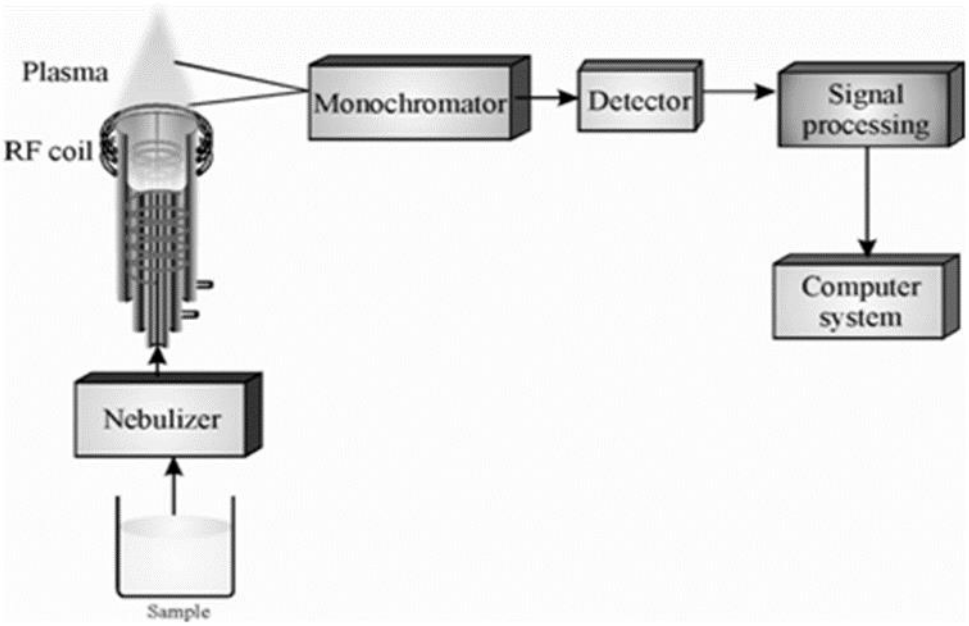


Figure 3.3 Schematic representation of an ICP−OES used for the quantification of minerals in CS.

3.1.2.10. Protein Analyzer (TruMac N-Leco)

Combustion (Duma) method for nitrogen analysis are based on sample being combusted at 1100°C in an induction furnace (Figure 3.4) in the presence of O_2 to form H_2O, SO_2, CO_2, NOx, and N_2 utilizing helium carrier gas. After the formation of the gases, removal of SO_2 and CO_2 takes place and NOx is reduced to N_2. Total N_2 of the plant material is measured by using thermal conductivity detector. Nitrogen to-protein conversion factor is utilized to calculate the total protein content of the sample. Figure 3.4 is a schematic representation of a TruMac N (Leco, Germany).

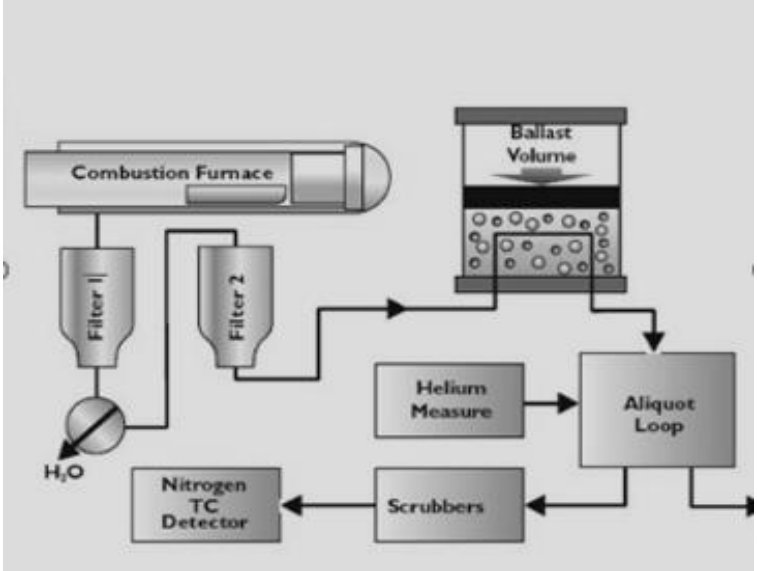


Figure 3.4 Schematic representation of Leco TruMac N combustion nitrogen analyzer for the quantification of crude protein.

3.1.2.12. Near-infrared Spectroscopy

It is based on the absorption of electromagnetic (EM) radiation at wavelengths in the range 780 to 2500 nm and provides significant complex structural information of the plant material related to the vibration conduct of blends of bonds. The light interacts with the sample and the detector measures its transmittance and absorbance. The record of NIR district of the electromagnetic spectrum includes the reaction of the molecular bonds N−H, C−O, C−H and O− H. These bonds are subject to vibrational energy changes when irradiated by NIR frequencies, and two vibration patterns exist in these bonds, including stretch vibration and bent vibration. The energy absorption of organic molecules in NIR region occurs when molecules vibrate or is translated into an absorption spectrum within the NIR spectrometer**.** NIR is utilized for the determination of structural cellulose and lignin present in the raw CS.

3.1.2.13. Fourier Transform Infra-red (FT-IR) Spectroscopy

FTIR is a fast, non-destructive, time saving method that detect a range of functional groups present in the unknown sample by placing the sample into the lens (Figure 3.5) exposing it to different wavelengths of infrared light and measuring wavelengths absorbed. It is sensitive to changes in molecular structure. It provides information on the basis of chemical composition and physical state of the sample and it is primarily used for the definitively identification of compounds such as polymers. Figure 3.5 is a schematic representation of a FT-IR. In this work it was used to identify functional peaks in the PHAs.

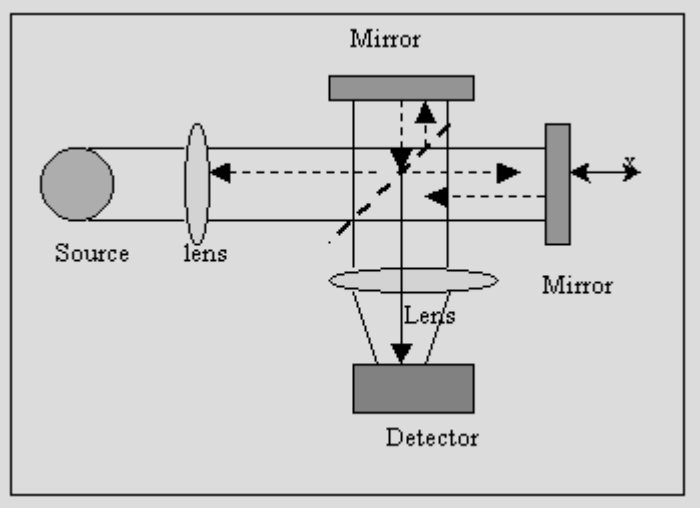


Figure 3.5 Schematic representation of FT-IR spectrometer for qualitative analysis of PHAs.

3.1.2.1.4. Gas chromatography – Flame Ionization Detector (GC-FID)

GC involve passing a mobile phase, containing the sample, through a stationary phase which slows the mobile phase to varying degrees dependent on how strongly the components interact. This causes the components to be separated in the column (Figure 3.6). The mobile phase is the sample which has been vaporised and carried in an inert gas, through a column which contains the stationary phase which can either be a solid or liquid coated on a solid support which retards the passage of the mobile phase. Samples are introduced by direct injection, after solvent extraction or from a headspace sampling system. This separating technique will be utilized to separate the different β-hydroxyalkanoatic acid methyl ester after transesterification. Figure 3.6 is the schematic representation of a GC-FID.

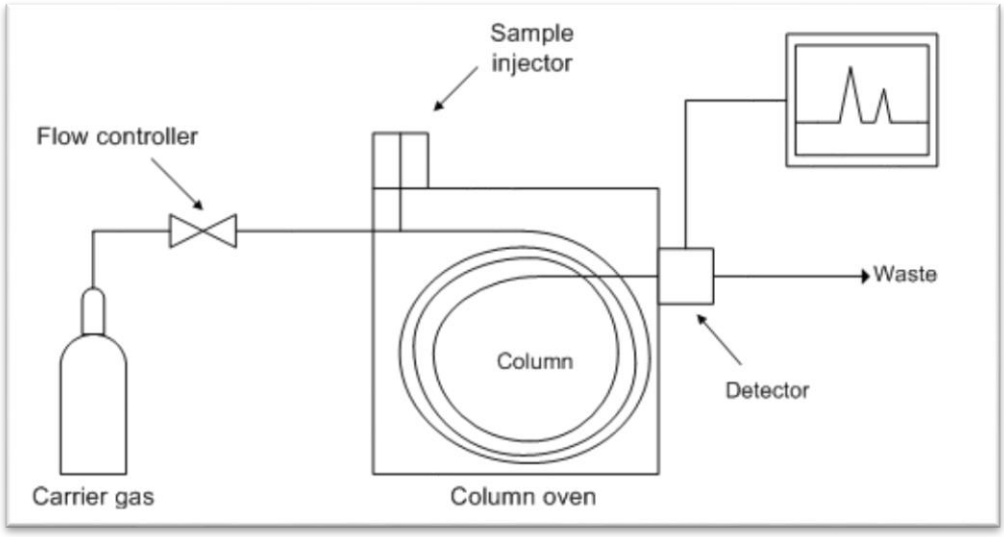


Figure 3.6 Schematic representation of GC-FID for the identification of polyhydroxyalkanoic acid methyl esters.

3.1.2.14. Gas chromatography–mass spectrometry (GC-MS)

Gas Chromatography-Mass Spectrometry separates organic mixtures in the GC component and identifies the components at a molecular level in the MS component. It is the most accurate tools for analysing unknown samples. A mixture separates into individual compounds when heated in the injector pot (Figure 3.6). The heated gases are carried through a column with an inert gas (such as helium). As the separated substances emerge from the column opening, they flow into the MS. Mass spectrometry identifies compounds by the mass of the analyte molecule. A library of known mass spectra, covering several thousand compounds, is stored on a computer. Mass spectrometry is considered the only definitive analytical detector. GC-MS will be utilized to characterize the polymer produced from CS. Figure 3.7 is the schematic representation of a GC-MS.

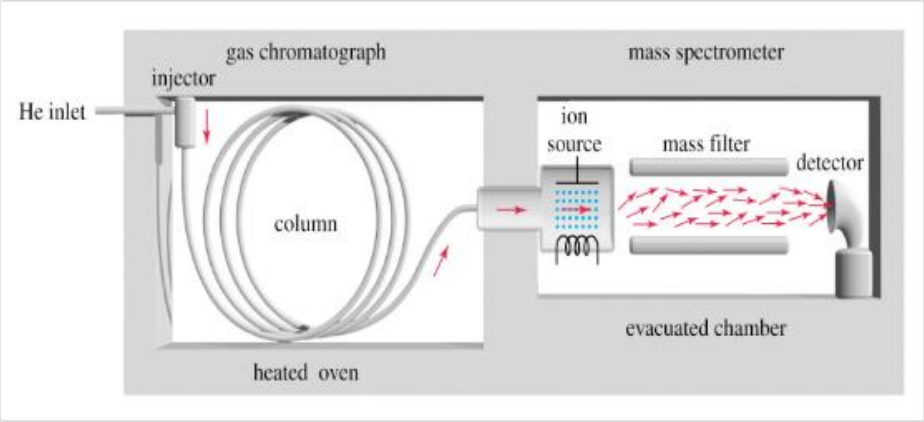


Figure 3.7 Schematic representation of GC-MS for qualitative identification of polyhydroxyalkanoic acid methyl esters.

3.2. Microorganisms

Prokaryotic microorganisms respond to sudden increases in crucial nutrients in their regularly hostile environment by storing important nutrients for survival during lengthy starvation. Such storage compounds such as PHAs produced when carbon sources are in abundance. The carbon sources are absorbed, converted into hydroxyalkanoate (HA) compounds and lastly polymerized into high molecular weight PHAs and stored as water insoluble granules in the cell cytoplasm. PHAs are exceptional storage compounds because their existence in the cytoplasm, even in large quantities does not interrupt the osmotic pressure of the cell. These insoluble granules may be enclosed by a phospholipid monolayer that contains explicit granule associated proteins such as PHA synthase, depolymerases, regulator proteins and also cytosolic proteins. These may be non-specifically attached to the granules by means of hydrophobic interactions.

These storage polymers are synthesized not only in Gram-negative bacteria but also in an extensive variety of Gram-positive bacteria, anaerobic (non-sulfur and sulfur purple bacteria) and aerobic photosynthetic bacteria (cyanobacteria), and additionally in archaebacteria (Sudesh *et al*. 2000). PHAs are a group of optically-active biological polyesters which comprise of (R)-3HA monomer units. The 3-hydroxyalkanoic acids are all in the R configuration because of the stereospecificity of the polymerizing enzyme, PHA synthase. PHAs chemical diversity is large; of which PHB is the most well-known and widely-produced form, containing repeat

units of (R)-3HB (Sudesh *et al.* 2000; Suriyamongkol *et al.* 2007). PHA production, including bacterial fermentation, isolation and physico-chemical characterization has been studied extensively for the past decades but knowledge on the regulatory mechanisms at the molecular level is relatively constrained. The primary biological attack is an enzymatically-catalyzed hydrolysis of ester, amide or urethane bonds in the polymers. This primary step of depolymerization is a surface erosion process and leads to water soluble intermediates, which are assimilated by microbial cells and thereby metabolized (Müller *et al*. 2001). Regulation of PHA metabolism can take place at different levels: I) Activation of *pha* gene expression because of specific environmental signals such as nutrient shortage; II) activation of the PHA synthetic enzymes by explicit cell components or metabolic intermediates; III) inhibition of metabolic enzymes of competing pathway and therefore enrichment of required intermediates for PHA synthesis; or IV) A combination of chose (Kessler and Witholt 2001).

3.3. PHA recovery and purification

Efficient recovery of PHA essentially influences the overall economics of PHA production. In all techniques, it is essential to concentrate cells to achieve a high yield of PHA. Centrifugation of cells in the broth, cross-stream filtration, and flocculation are the techniques of choice. Subsequently, either solvent-based extraction or water-based separation can be chosen as isolation methods. In the first technique, the cells need to be lysed and various chemical additives added to digest non-PHA material. Solvent-based methods are not appropriate for large-scale manufacturing of PHA, as the solvent is potentially hazardous to the environment and to humans (Chee *et al.* 2010b). Cell lysis by hypochlorite represents an excellent isolation method allowing optimal and cheap liberation of PHA (Zinn *et al*. 2001). PHA recovery is reported using equation 3.15.

$$\text{PHA } \% = \frac{\text{weight of PHA (g/L)}}{\text{dry cell weight (g/L)}} \text{ x } 100 \qquad (3.15)$$

CHAPTER **4**

EXPERIMENTAL

4.1. Materials

The corn silage samples used in this study were obtained from cedara in KwaZulu Natal, South Africa. The photograph of the corn silage sample is shown in Figure 4.1.

Figure 4.1 Corn silage sample milled at a particle size of 1 mm at 25°C.

Corn silage samples were dried overnight in an oven at 75°C to remove moisture. Samples were allowed to cool down then milled using a Wiley laboratory mill at a particle size of 1 mm.

The chemicals used in this study were purchased from Merck, Novozymes and Sigma Aldrich. Table 4.1 gives the list of chemicals used, the suppliers and purity. The structures of the bio-plastics polymer poly [(R)-3-hydroxyalkanoate] is shown in Figure 4.2.

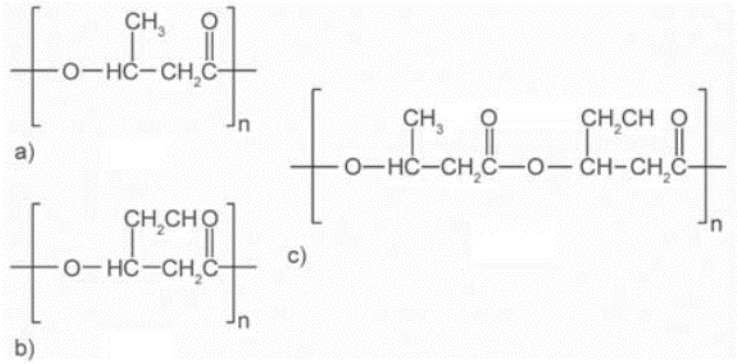


Figure 4.2 Typical structures of poly[(R)-3-hydroxyalkanoates] a) poly(3-hydroxybutyrate), b) poly(3-hydroxyvalerate), c) poly(3-hydroxybutyrate-co-3-hydroxyvalerate).

Table 4.1 Summary of chemicals used

Name	**Supplier**	**Purity**
Tryptone	Merck	-
Yeast Extract		-
NaCl		≥99%
$MgSO_4$		≥99.5%
NaClO		6% w/w
H_2SO_4		
Ethanol		99.9%
Termamyl (α amylase) enzyme	Novozymes South Africa	-
Amyl glucosidase enzyme	Novozymes South Africa	-
poly(3-hydroxybutyrate)	Sigma Aldrich	-
poly(3-hydroxybutyrate-co-3-hydroxyvalerate)	Sigma Aldrich	-
Thermus thermophiles HB8	Riken BRC, Japan	-
Neutral detergent solution	Prepared by dissolving 12.4 g ethylenediaminetetraacetate disodium salt (EDTA) and 4.35g sodium borate 10-hydrate in deionized water into 500 ml volumetric flask. Followed by add 20 g sodium lauryl sulphate and 6.7 ml ethylene glycol and 3.4g sodium hydrogen phosphate to the volumetric flask while stirring until dissolved.	-
Acidic detergent solution	Prepared by dissolving 10 g cetyl trimethylammoniumbrimide	-

	(CTAB) in deionized water into 500 ml volumetric flask. Followed by addition of 14 ml conc. sulphuric acid.	
Acetate buffer	Prepared by mixing 74 ml of 0.2 M acetic acid and 176 ml of 0.2 M sodium acetate in deionized water into 500 ml volumetric flask	-
Copper reagent	Solution A: prepared by dissolving 30g sodium carbonate (anhydrous), 20g sodium hydrogen carbonate, 15g Potassium sodium tartrate 4-hydrate and 180g sodium sulphate (anhydrous) in deionized water into 1000ml volumetric flask. Solution B: prepared by dissolving 45g sodium sulphate (anhydrous) and 5g copper (II) sulphate 5-hydratein in deionized water into 250 ml volumetric flask. Reagent was prepared by mixing mix 4 volumes of solution A with 1 volume of solution B	-
Arsernomolybdate	Prepared by dissolving 10 g ammonium molybdate 4-	-

	hydrate in deionized water. Followed by addition of 42 ml concentrated sulphuric acid and 6 g sodium arsenate7-hydrate into 1 l. The reagent was incubated at 37ºC for 48 hours.	

4.2. Compositional Analysis

Corn silage was analysed using the AOAC methods of analysis (Horwitz *et al.* 1970; Chemists and Horwitz 1990; Cheeke 1991; Jain and Tiwari 2015). Moisture content of the undried corn silage was determined following AOAC 7.003 method (Chemists and Horwitz 1990). The chemical composition of corn silage was carried out following AOAC 978.18 (Chemists and Horwitz 1990). Compositional analysis was carried out in triplicate.

4.2.1. Moisture content

1 g of the sample was weighed into a pre-dried weighing dish and placed into a convection oven at 105°C for two hours. The sample was removed from the oven and allowed to cool to room temperature in a desiccator. The dish containing the oven-dried sample was weighed to the nearest 0.1 g and the mass recorded. The above steps were repeated until a constant weight was achieved. The percentage dry matter calculated on the dry weight basis according to equations 3.1 and 3.2.

4.2.2. Ash content

0.5 g of corn silage was weighed into a pre-weighed crucible in triplicate. The crucible with the sample was placed in a Naberthern programmable muffle furnace (Naberthern GmbH, Germany) (Figure 4.4) and heated overnight at 500°C, and thereafter crucibles were placed in a desiccator for cooling. The crucible with samples were weighed and the mass recorded. The

samples were placed back into the muffle furnace and ashed to constant weight which reflected by a less than ± 0.3 mg change in the weight upon one hour of re-heating the crucible. The percentage ash was calculated according to equations 3.3 and 3.4.

4.2.3. Neutral detergent fibre

0.5 g corn silage sample was weighed into a pre-weighed sintered glass crucible (34 x 2.8 mm, porosity 2). A 50 ml volume of neutral-detergent fibre solvent was added into the pyrex crucible holders containing glass beads. The crucible holders with the NDF solution was placed in the reflexing block until foam ceased to form. The crucible was placed into the crucible holder in the refluxing block, a 1 ml of Termamyl (α amylase) was added into the crucible and refluxed for 70 min at 110°C (Figure 4.5). The crucible holders were then removed from the block and the crucibles with sample were removed from the holders for washing. The indigestible sample was washed five times with boiling water and then washed with acetone. The crucible sample was dried overnight at 100°C, removed from the oven and allowed to cool to room temperature in a desiccator. The crucible with samples were weighed and the mass recorded. The percent of the cell wall residue (NDF) was calculated according to equation 3.6.

4.2.4. Acidic detergent fibre

The crucible with NDF (a new indigestible NDF sample) was placed into the crucible holder in the refluxing block containing acidic detergent solution and glass beads. The sample was refluxed for 70 min at 110°C. The crucible holders were then removed from the block and the crucibles with sample were removed from the holders for washing. The new indigestible sample was washed five times with boiling water under vacuum and then washed with acetone. The crucible with sample was dried overnight at 100°C, removed from the oven and allowed to cool to room temperature in a desiccator (Figure 4.3). The crucible with samples were weighed and the mass recorded. The percent of the cell wall residue (ADF) was calculated according to equation 3.7 and percent hemicellulose was calculated according to equation 3.8.

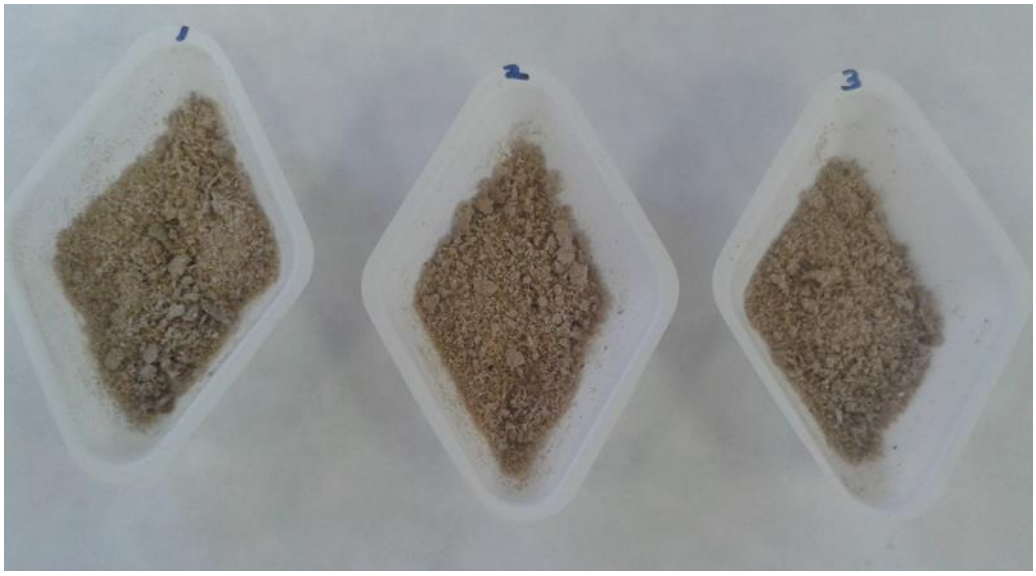


Figure 4.3 ADF samples after detergent pre-treatment at 110°C for 70 min.

4.2.5. Lignin

AIL was quantified by solubilizing the ADF sample with 72% H_2SO_4 after the extraction with neutral and acid detergent solvents. Crucible containing ADF samples were half filled with 72% H_2SO_4 and stirred with a glass rod (Figure 4.7). The crucibles were filled with acid as acid drains away and stirred at hourly intervals for three hours. The ASL content of the sample was quantified by measuring the UV-Vis absorption at 240 nm wavelength of the acid hydrolysis supernatant. The remaining sample in the crucible after a two stage hydrolysis was washed with boiling deionized water until free from acid while testing with blue litmus paper (Figure 4.7). The crucibles with the samples were dried overnight at 100°C, removed from the oven and weighed after cooling to room temperature in a desiccator. The crucibles with samples were placed in a furnace at 500°C for two hours, placed in a desiccator to cool and the crucible was reweighed (AIL). The total lignin was quantified as the sum of ASL and AIL. The percent of total lignin content was calculated using equations 3.9, 3.10 and 3.11.

Figure 4.4 ADL and AIL pretreatment with 72% H_2SO_4 after NDF and ADF extraction.

4.2.6. Fats

1 g of sample was placed on a S&S Rund 595, 90 mm filter paper. The filter paper with the sample was placed in watmman cellulose extraction thimbles (single thickness) (Merck). Petroleum ether was added to the pre-weighed extraction flask until ¾ full. The cellulose thimbles containing the sample was placed in the soxhlet extractor. The sample was extracted on the heating mantle at 100°C for 3 h. The thimbles were removed, allowing ether to clear by continuing to reflux for 10 min. The extraction flask was placed overnight in an oven at 105°C, the extraction flask was placed in a desiccator to cool and weighed. The percent of fat content was calculated using equation 3.5.

4.2.7. Starch

A 1 g of the sample was weighed into a test tube and 5 ml volume of 80% ethanol was added. The mixture was vortexed then incubated at 80°C for 30 min. Ethanol was aspirated out of the mixture leaving the sample in the test tube. The washing step was repeated twice for complete removal of sugars. Test tube with the sample was placed back in the oven at 80°C for until all the ethanol completely evaporated. A 10 ml of acetate buffer was added into the test tube followed by 200 µl of Termamyl α amylase enzyme. The mixture was vortexed and incubated for 30 min at 90°C, and allowed to cool. After cooling, 200 µl of amyl glucosidase was added to the mixture in the test tube and gently shaked. The test tube with the mixture was incubated at 60°C for 8 h. The sample was diluted in a 200 ml volumetric flask using deionized water and filtered through watmman filter paper no 541. A 5 ml volume of copper reagent was added into 3 ml of the filtrate in the test tube. A 5 ml volume of the arsernomolybdate reagent was added into the test tube, shaked and allowed to stand for 90 min. The starch content of the sample was determined by measuring the UV absorption at 750 nm wavelength (Rasmussen *et al*. 1990). The amount of starch was calculated using equation 3.12.

4.2.8. Protein

0.2 g of the sample was weighed in a ceramic boat, then deposited into the ceramic horizontal furnace at 1100°C (Figure 3.4). Conversion factor of nitrogen-to-protein was used to calculate the total (crude) protein content of the sample (Moore *et al.* 2010). The amount of the protein was calculated using equation 3.13.

4.2.9. Total reducing sugars

A sugar standard was prepared by weighing out 0.6667 g glucose and dissolving in deionized water diluting to 200 ml. Then 1 ml of the sugar standard was mixed with 5 ml of 0.05 N H_2SO_4. 1 ml of the sample was measured and transferred into a test tube. 5 ml of 0.05 N H_2SO_4 was added into the sample and mixed using a vortex mixer. The standard, blank and sample mixtures were boiled at 100°C for 30 min. After boiling, the mixtures were allowed to cool to room temperature. The mixtures were transferred into 250 ml volumetric flask with deionized water. The mixtures were filtered using whatman no1 filter papers. 3 ml sample of each filtrate

was transferred into clean test tubes and mixed with 3 ml copper reagent. The mixtures were incubated into a water bath at 100°C for 20 min. The mixture was allowed to cool to room temperature and 3 ml of arsenomolybdate reagent was added into the mixture and mixed until no bubbles were formed. The mixture was allowed to stand for 90 min until the colour was fully developed. The solution was transferred into 200 ml volumetric flasks using deionized water. The samples were transferred into cuvettes and absorbance was read at 750 nm using UV-Vis spectroscopy (Marais *et al*. 1966). The percent of the total sugars after hydrolysis was calculated using equation 3.14.

4.3. Macro and micro minerals

0.5 g mass of sample was weighed in a crucible and ashed overnight at 500°C. 2 ml volume of 32% HCl was added into the crucible after ashing and heated on the steam bath for 1 hour until all solid material dissolved. After heating, 25 ml of 10% HCl was added into the crucible. The mixture was stirred using a glass rod. The mixture was then filtered using whatman no1 filter papers. A 5 ml volume of the filtrate was transferred into 25 ml volumetric flask using deionized water. The sample was analysed using ICP for macro and micro minerals.

4.4. Near-infrared spectroscopy

The ADF was used to characterize cellulose and lignin by NIR System 6500 (Berthold). Spectroscopic measurements of treated CS samples were carried out in a static ring cup sample cell and spectra were recorded on NIR System 6500 with an integrating sphere for sampling in diffuse reflectance mode in the 1100-2500 nm region. All the spectral data were collected with a 8 nm spectral resolution, and 32 scans were collected and averaged into a single average spectrum to ensure an adequate signal-to-noise ratio (Li *et al.* 2015).

4.5. Bacterial strain and growth

4.5.1 Cultivation of *Thermus thermophilus* HB8

T. thermophilus HB8, was obtained from Rike BioResource Center, Japan. It was grown at 70°C in DSMZ-74 medium containing 8 g/l tryptone, 4 g/l yeast extract and 2 g/l NaCl. For PHAs production, nitrogen-limited cultivation was carried in a 2 L Erlenmeyer flask containing 8 g/l tryptone, 4 g/l yeast extract and 2 g/l NaCl and 1% (w/v) glucose the carbon source. Figure 4.5 is a photograph of *T. thermophiles* HB8 growing in glucose under different pH conditions.

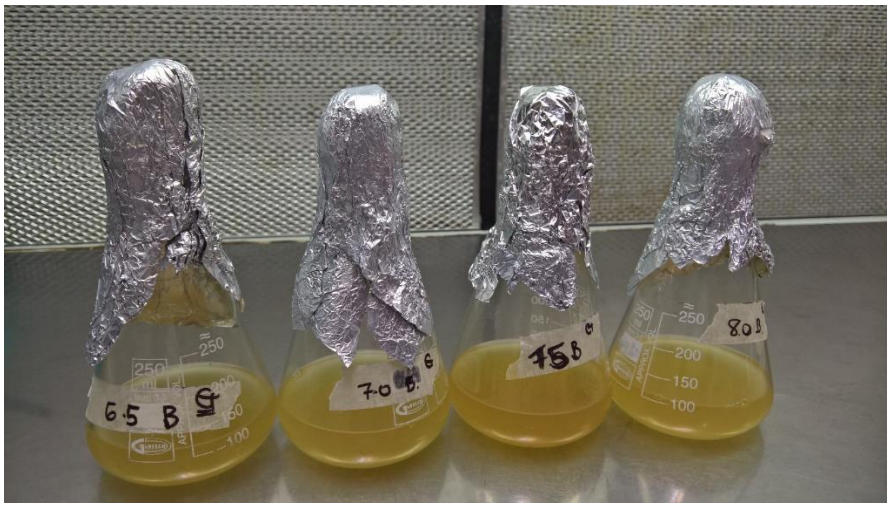


Figure 4.5 *T. thermophilus* HB8 growing in glucose at different pH conditions at 70°C.

The medium contained 6, 12, 24 and 48% (v/v) CS supernatant as a cheap carbon source. A 1% (v/v) inoculum was added to 700 ml of production medium and incubated at 70°C for 50 h on a shaker at 200 rpm. Media with different initial pH (6.5, 7.0, 7.5 and 8.0) were prepared for optimization purposes. The pH of the medium was adjusted using 1N HCl and 1N NaOH.

4.5.2. PHA production

As part of initial preliminary identification of PHA in the cells, gram staining was done. The cells were tested for PHAs production by using the lipophilic stain Sudan black B. A loopful of culture was mixed with 20% brine and smeared on a clean glass slide. The smear was air dried and fixed by immersing in 2% acetic acid for 5 min. The slide was then removed, dried, covered with Sudan black B solution (0.3 g of Sudan black in 100 ml of ethanol) and kept at room temperature for 15 min. The excess stain was drained off. The slide was dried by blotting thoroughly and then cleared by adding xylene. The slide was blotted and 0.5% aqueous safranin was used as a counter stain for 10 s. The slide was then washed with deionized water, blotted, dried and examined under a light microscope (Selvakumar *et al.* 2011). Stained samples were observed under oil immersion at 1000 × magnification with direct bright-field illumination using an Olympus CX31 microscope (Scientific Laboratory Supplies, New York) with a Lumenera infinity 2 CCD camera. Cells were also stained with Nile blue A, 1% aqueous solution of Nile blue A (Sigma), was heated in a 100°C using a water bath, and the solution was filtered before use. Smears of cells were heatfixed and stained with the Nile blue A solution at 55°C in a water bath for 10 min. After staining, slides were washed twice with tap water followed by an 8% acetic acid solution for 1 min, and finally the stained smear was blotted dry with bibulous paper and covered with a glass cover slip. The samples were examined with a Leica DM5000B fluorescence microscope and observed with blue excitation wavelengths (filter BP 450-490) (Meyer Instruments, Texas) (Legat *et al.* 2010). Figure 4.11 is a photograph of Olympus CX31 microscope with a Lumenera infinity 2 CCD camera.

4.5.3 Cell growth

Cell growth was monitored by removing 1 ml aliquots of the culture at 6 h intervals and measuring the absorbance at 600 nm for 50 h using a Perkin-Elmer spectrophotometer (Varian, England). The bacteria were harvested at the end of the logarithmic phase by centrifugation at 6000 x g and cells were washed twice with phosphate buffered saline solution. All experiments and measurements were performed in triplicate. Figure 4.6 shows a photograph of *T. thermophiles* HB8 growing at different concentrations of CS.

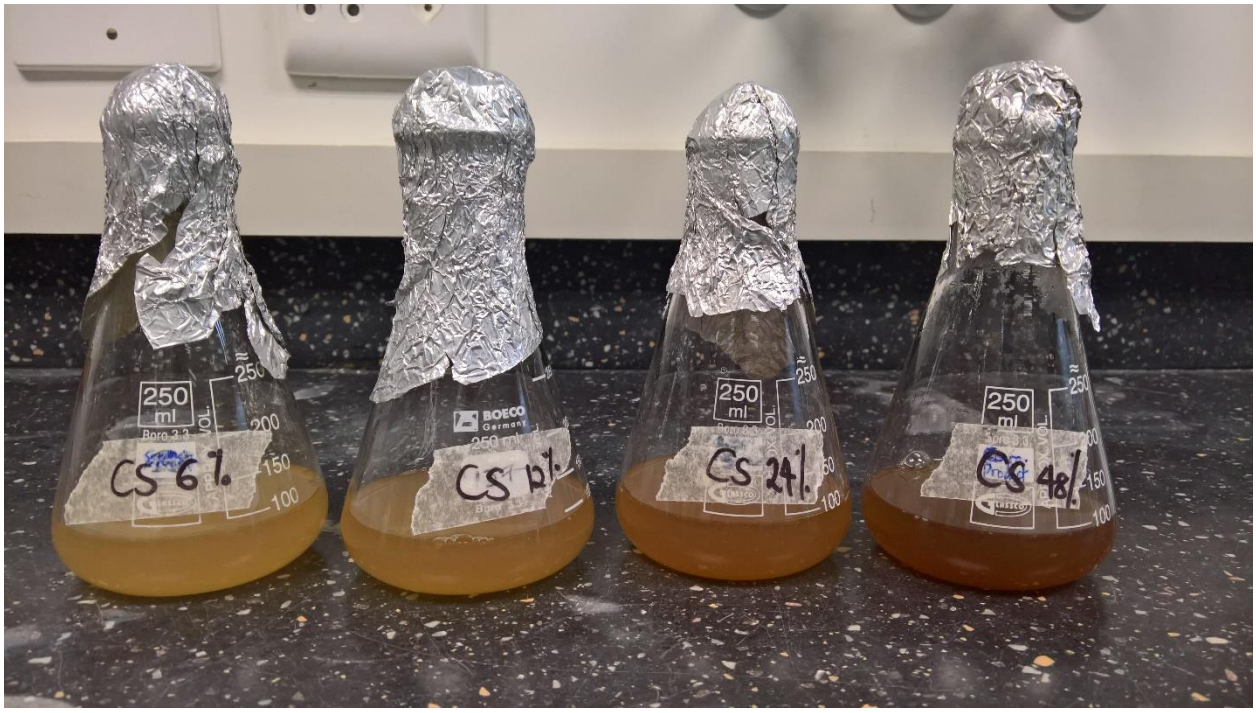


Figure 4.6 Growth of *T. thermophilus* HB8 at different concentrations of CS supernatants at 70°C.

4.5.4 Cell concentration determination

Cell concentration defined as dry weight of cells per litre of culture broth, was determined using 50 ml sterilized falcon tubes. 45 ml volume of culture broth was transferred into the falcon tubes and centrifuged at 8000 x g for 15 min. The supernatant was discarded and the pellet was collected and washed using 10 mM $MgSO_4$ and then washed with deionized water. The pellet was dried using a freeze dryer with the ice condenser set at -80°C and vacuum set at 1.0 mbar. The dried cells were weighed. The pellet samples were stored in the bio-freezer until PHA extraction was carried out (Rohini *et al*. 2006; Pantazaki *et al.* 2009).

4.5.5 PHA recovery and estimation

The PHA content defined as the ratio of PHA concentration to cell concentration was calculated as a percentage. The PHA produced by *T. thermophilus* cells was recovered by using a solution of sodium hypochlorite and chloroform digestion method after 50 h of fermentation. Biomass (0.5 g) was treated with a solution of 20 ml of 6% sodium hypochlorite and 20 ml of chloroform. The mixture was incubated for 1 h at 37°C, then the dispersion was centrifuged at 4000 rpm for 10 min. Three separate phases were obtained, the upper phase was hypochlorite solution, the middle phase contained some undisrupted cells among others, and the bottom phase was the chloroform phase containing PHAs (Rohini *et al*. 2006; Pantazaki *et al.* 2009). Figure 4.7 is showing cell lysis using hypochlorite and recovery of polymer using chloroform.

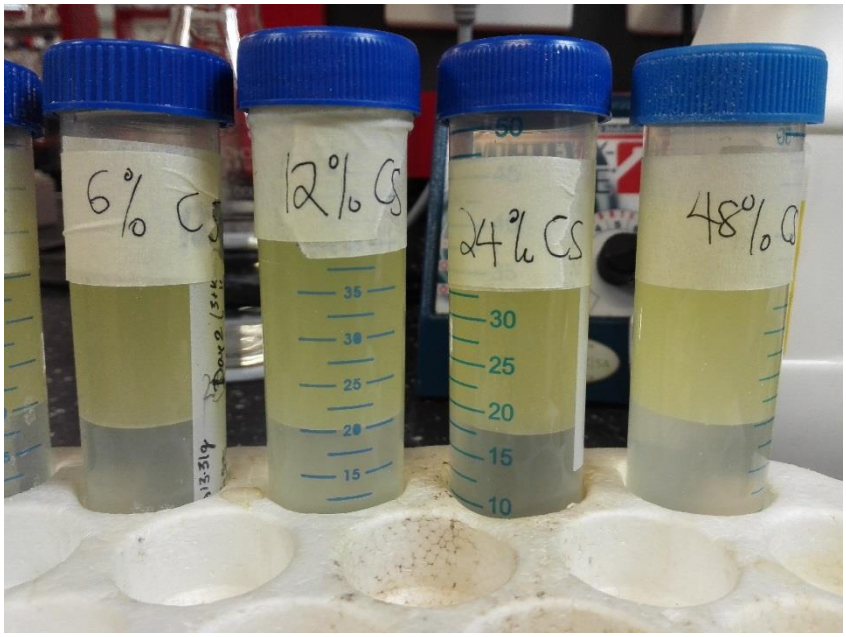


Figure 4.7 Cell lysis using hypochlorite and recovery of extracted polymer using chloroform at 37°C.

The two upper phases were removed using plastic pipettes, then the lower chloroform phase that contained the solubilised polymer was recovered. The PHA polymer was precipitated by addition of 4x volumes of cold methanol (Rohini *et al*. 2006; Pantazaki *et al.* 2009). The precipitated polymer was washed with deionised water and air dried in the desiccator and used

for physical characterization. PHA yield was calculated using equation 3.15. Figures 4.8 and 4.9 show the photographs of the extracted PHA.

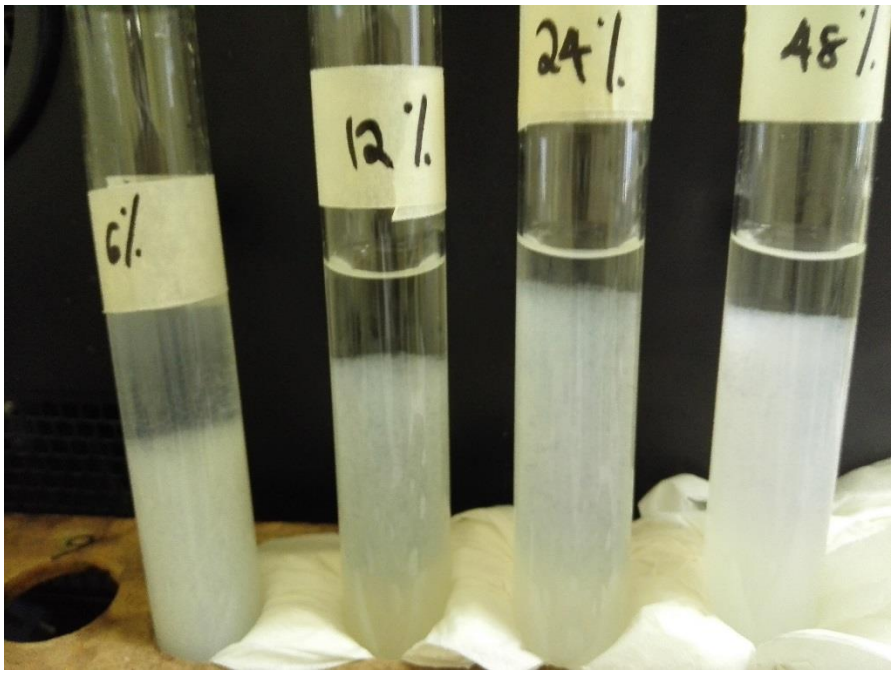


Figure 4.8 Precipitation of the extracted polymer using methanol at 25°C.

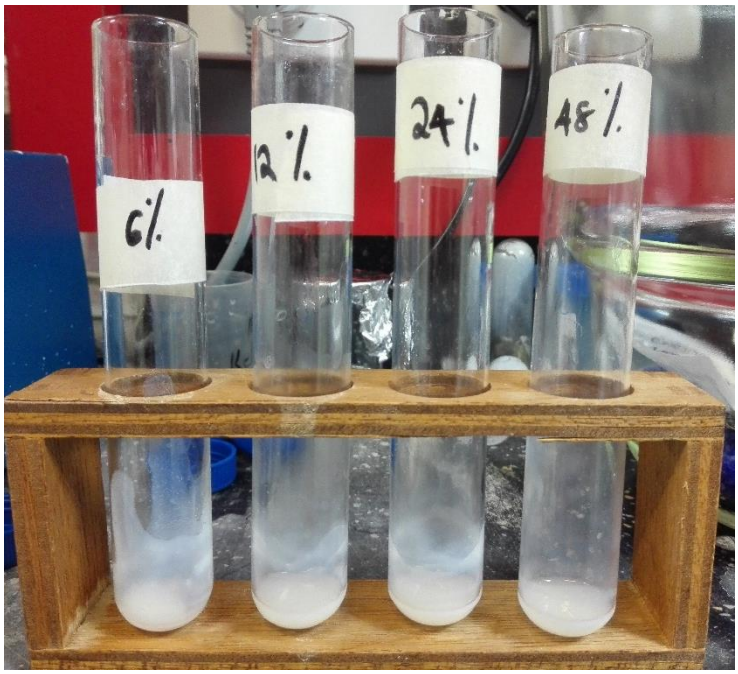


Figure 4.9 Drying of the extracted polymer at room temperature.

4.6. Corn silage medium

Pre-treatment of CS was performed in two ways i.e. water extraction and acid treatment.

4.6.1 Unhydrolysed CS

CS was collected from Cedara Collage of Agriculture (Department of Agriculture and Rural Development, KZN). Silage solution was prepared by suspending 400 g CS in 1 L of deionized water. The mixture was stirred for 24 h at room temperature for complete extraction. Whole silage solution was acidified with 5 N HCl solution until a pH 4.5 was obtained to remove excess protein present in the solution. The solution was autoclaved at 121°C for 15 min and then cooled to room temperature. The mixture was then centrifuged at 11000 x g in sterilized tubes for 15 min to remove aggregates. The supernatant (silage supernatant) was passed through whatman No. 3 filters and was utilized as the main ingredient and substrate at concentrations of 6%, 12%, 24% and 48% (v/v) (Pantazaki *et al.* 2009).

4.6.2 Hydrolysed CS

CS substrate was hydrolysed with 2.5% v/v sulphuric acid (solid: liquid, 1:10). The mixture was autoclaved for 30 min at 121°C. The hydrolysed mixture was cooled to room temperature and filtered through whatman No. 1 filter paper. The supernatants were neutralized using 6 N NaOH. Reducing sugars content was quantified by photometric method (Marais *et al*. 1966). The growth media was prepared utilizing these filtrates at concentrations of 6%, 12%, 24% and 48% (v/v).

4.7 UV-VIS characterization

10 mg of the extracted PHA was dissolved in 4 ml of chloroform. The mixture was mixed using the vortex for 1 min and allowed to stand for 30 min at room temperature for complete solubilisation. A sample was transferred into a glass cuvette and the absorbance from 800 nm to 200 nm was measured. Chloroform was used as the blank (Selvakumar *et al.* 2011; Alarfaj *et al.* 2015).

4.8 FT-IR characterization

The FT-IR spectrum of the polymer extracted from *T. thermophilus* HB8 was compared with the pure poly(3HB-co-3HV) obtained from Sigma-Aldrich. The structural analysis was performed using a FTIR spectrophotometer Perkin Elmer 537 spectrophotometer. The extracted polymer was placed on the sample holder and the spectra were recorded using attenuated total reflectance (ATR) disc. Samples were scanned from 4000-400 cm^{-1} and the absorption frequencies were expressed as vmax cm^{-1} (Singh *et al.* 2013).

4.9 GC-FID characterization

The monomer composition of the polymer was determined by gas chromatography (GC) Varian 2100T GC–FID (Thermo). The Varian 2100T gas chromatograph was equipped with a ZB-5MS (Phenomenex) column (30 m-length, 0.25 mm-internal diameter, and 0.25 μm -film thickness) using a flame ionization detector. 5 mg of extracted polymer was subjected to methanolysis in small screw-cap test tubes with a solution containing 1 ml of chloroform, 0.85 ml of methanol and 0.15 ml of sulphuric acid. The mixture was incubated in the water bath at 100°C for 1 h and cooled down to room temperature. After the reaction, 0.5 ml of deionized water was added and the tubes were vortexed for 1 min. After the phase separation, the heavier phase containing chloroform solution with methyl ester of β-hydroxyl acid from PHA hydrolysis was removed and transferred to a small screw cap vial. Analysis was performed by injecting 2 μl of sample into the injector pot. The carrier gas (nitrogen) inlet pressure was 109.2 kPa. The temperature program was used for efficient separation of the esters [60°C for 1 min, temperature increase 25°C /min to 230°C (230°C for 1 min)]; Flow controller: split, total flow 301.3 ml/min, column flow 1.48 ml/min; the temperature of the injector and detector were 200 and 230°C, respectively (Pantazaki *et al.* 2009). Different β-hydroxyalkanoatic acid methyl ester standards were used as references.

4.10 GC-MS characterization

GC-MS analysis was performed using a Varian 2100T GC–MS (Thermo). The Varian 2100T gas chromatograph was equipped with a ZB-5MS (Phenomenex) column (30 m-length, 0.25 mm-internal diameter, and 0.25 µm -film thickness). 5 mg of extracted polymer was subjected to methanolysis in a small screw-cap test tubes with a solution containing 1 ml of chloroform, 0.85 ml of methanol and 0.15 ml of sulphuric acid. The mixture was incubated in the water bath at 100°C for 1 h and cooled down to room temperature. After the reaction, 0.5 ml of distilled water was added and the tubes was vortexed for 1 min. After the phase separation, the heavier phase containing chloroform solution with methyl ester of β-hydroxyl acid from PHA hydrolysis was removed and transferred to a small screw cap vial. Analysis was performed by injecting 2 µl of sample into the injector pot. The carrier gas (nitrogen) inlet pressure was 109.2 kPa. The temperature program was used for efficient separation of the esters [60°C for 1 min, temperature increase 25°C/min to 230°C (230°C for 1 min)]; flow controller: split, total flow 301.3 ml/min, column flow 1.48 ml/min; the temperature of the injector and detector were 200 and 230°C, respectively (Pantazaki *et al.* 2009). Different β-hydroxyalkanoatic acid methyl ester standards were used as references.

4.11 Blending of polymer and film casting

Conventional solvent cast technique was used for preparation of polymer blend sheets. The polymer powder extracted from *T. thermophilus* strain HB8 was mixed with soluble starch in a ratio of 4:1 (w/w) and then dissolved in 20 ml of chloroform, respectively. The mixture was placed on the hot plate with continuous stirring for 15 min. The solution was poured into an open flat glass petri plate and allowed to evaporate slowly at room temperature. The polymer blend sheet was then used for tensile testing and cut into small pieces used for degradation studies (Singh *et al.* 2013). Figure 4.10 is a photograph of blended bioplastics.

Figure 4.10 Blended bioplastics using the extracted polymer powder and starch in chloroform.

4.12. Tensile testing

The tensile properties of CS films were determined according to ASTM D638 using the Instron 3369 tensile testing instrument. To observe the effect of starch on the tensile strength and strain of the plastic, the poly(3HB-co-3HV) film was prepared in three different ratios (4:1; 4:2 and 4:3). The films were cut into 6 x 1.5 cm rectangular strips. Stress-strain (engineering) analysis was performed at a crosshead speed of 100 mm/min at room temperature. The initial distance of separation and velocity were adjusted to 40 mm and 1 mm/s, respectively. Tensile stress at break, and elongation at break of each sample was recorded.

4.13 Scanning electron microscope (SEM)

A small piece of the sample (approx. 0.5 x 0.5 cm) was cut and mounted to an SEM aluminium stub with double sided carbon tape. The samples were then gold sputter coated with a Quorum Q150R ES Sputter coater. Samples were imaged on the Zeiss Ultra Plus FEG SEM. The EDX analysis was performed with an Oxford X-Max 80mm Silicon Drift Detector.

4.14 Determination of biodegradability

Biodegradability testing of polymer sheets was performed by the soil burial method. Pre-weighed polymer sheets were buried in 200 g of freshly-collected soil, compost and industrial sludge samples in wide mouth glass beakers. The beakers were filled with 200 g of soil, compost and sludge. The mouths of the beakers were kept open and incubated at room temperature up to 60 days. Moisture content of 30% in the samples was maintained by adding sterile water to study the degradation of poly(3HB-co-3HV) films. Degradation of poly(3HB-co-3HV) was recorded as the loss of the weight after definite period of incubation (Singh *et al.* 2013).

CHAPTER **5**

RESULTS

5.1. Compositional analysis

5.1.1. Moisture content of whole CS

The moisture content of whole CS was determined according to the AOAC, (1990) procedure. All experiments we repeated three times. The average moisture content was found to be 2.92% relative to the oven dried weight. Tables 5.1 describes the moisture content of CS samples.

Table 5.1 Moisture content of CS samples

Sample No	Mass of crucible (g)	Mass before sample drying (g)	Mass after drying (g)	Mass of air dried sample (g)	Water content (wt. %)
1	30.77	31.79	31.77	1.03	2.92
2	30.78	31.80	31.77	1.02	2.94
3	30.76	31.81	31.78	1.05	2.85
Average	30.77 ± 0.01	31.79 ± 0.02	31.76 ± 0.02	1.03 ± 0.02	2.92 ± 0.06

5.1.2. Total reducing sugars of hydrolysed CS juice

A calorimetric method was used to analyse reducing sugars after CS hydrolysis by the controlled acid hydrolysis procedure. The sugar content values are shown in Table 5.2. The average sugar content was found to be 14.26 % after hydrolysis.

Table 5.2 Total reducing sugars after CS hydrolysis

Sample Number	Total sugar (%)
1	12.79
2	14.56
3	15.42
Average	14.26 ± 1.34

5.1.3. Lignin content of whole CS

Sulphuric acid was utilized for CS hydrolysis to determine the insoluble and soluble lignin content. The reaction was conducted in a water bath at 30℃ for 1 h in 4 replicates. Table 5.3 shows the lignin values. The AIL was found to be 4.11%, ASL was found to be 2.43% and the total lignin 6.54%.

$$\% \text{ ASL} = \frac{\text{UV Absorbance x Filtrate Volume x Dilution factor}}{\text{Absorbtivity (e) x ODW(sample) x Pathlength}} \text{ x } 100$$

$$= \frac{0.082 \text{ x } 87.0 \text{ x } 10}{30 \text{ x } 97.08 \text{ x } 1} \text{ x } 100$$

$$= 2.45\%$$

$$\% \text{ AIL} = \frac{\text{Weight (crusible+dry sample)} - \text{Weight (crusible+ash)}}{\text{Weight (sample)}} \text{ x } 100$$

$$= \frac{47.8110 - 47.7912}{0.5006} \text{x } 100$$

$$= 4.12\ \%$$

$$\% \text{ Total lignin} = \% \text{ AIL} + \%\text{ASL}$$

$$= 2.45 + 4.12$$

$$= 6.57\%$$

Table 5.3 Amounts of ASL, AIL and total lignin in CS

Sample Number	Absorbance nm	Acid Soluble Lignin (ASL) %	Acid Insoluble Lignin (AIL) %	Total Lignin %
1	0.082	2.45	4.12	6.57
2	0.080	2.41	4.10	6.51
3	0.081	2.44	4.11	6.55
4	0.082	2.41	4.12	6.53
Average	0.082 ± 0.001	2.43 ± 0.021	4.11 ± 0.010	6.54 ± 0.026

5.1.4. Cellulose, hemicellulose, lignin, ash, fat, protein, starch and minerals in whole CS

The compositional analysis of the main components (cellulose, hemicellulose, lignin, ash, protein, fat, starch and minerals) that make up the raw whole CS were analysed and the values are shown in Figure 5.1. The hemicellulose was found to be 20.70%, cellulose 27.0%, lignin 6.54%, ash 3.7%, fat 1%, protein 9.28%, starch 26.89% and minerals 4.01%. Tables 5.4-5.9 shows the compositional analysis data of the CS samples.

Table 5.4 Starch content of the CS samples

Sample Number	Sample weight g	Sample Absorbance nm	Standard Absorbance nm	Starch %
1	1.0005	10.1	0.304	27.2
2	1.0002	9.98	0.301	26.9
3	1.0008	10.0	0.306	26.9
Average	1.0005 ± 0.01	10.03 ± 0.06	0.304 ± 0.01	27.0 ± 0.17

Table 5.5 Protein content of the CS samples

Sample Number	Sample Weight g	Nitrogen %	Protein factor	Protein %
1	0.2005	1.44	6.25	9.0
2	0.2008	1.46	6.25	9.1
3	0.2001	1.46	6.25	9.1
Average	0.2002 ± 0.01	1.45 ± 0.01		9.1± 0.06

Table 5.6 Ash content of the CS samples

Sample Number	Sample weight g	Crucible + Dry sample g	Crucible g	Ash %
1	0.5005	30.8012	30.7652	3.7
2	0.5002	30.8018	30.7665	3.6
3	0.5008	30.8016	30.7649	3.7
Average	0.5005 ± 0.02	30.8015 ±0.04	30.7655 ± 0.02	3.7 ± 0.06

Table 5.7 Fat content of the CS samples

Sample Number	Sample weight g	Flask + Dry sample g	Flask g	Fat %
1	1.0003	72.9301	72.9186	1.1
2	1.0007	72.9339	72.9201	1.6
3	1.0009	72.9304	72.9190	1.3
Average	1.0006 ± 0.02	72.9315 ±0.05	72.9192 ± 0.01	1.3 ± 0.17

Table 5.8 Cellulose content of the CS samples

Sample Number	Sample weight g	Crucible + Dry sample g	Crucible g	Cellulose %
1	0.5002	48.4242	48.2904	26.7
2	0.5009	48.4248	48.2908	26.8
3	0.5006	48.4245	48.2906	27.1
Average	0.5006 ± 0.02	48.4245 ± 0.06	48.2948 ± 0.02	26.9 ± 0.17

Table 5.9 Macro and micro minerals of the CS samples

Sample Number	Sample weight g	Total macro minerals %	Total micro minerals %	Total minerals %
1	0.5002	2.33	1.68	4.01
2	0.5004	2.35	1.66	4.00
3	0.5006	2.32	1.69	4.01
Average	0.5004 ± 0.01	2.33 ± 0.06	1.68 ± 0.03	4.01± 0.02

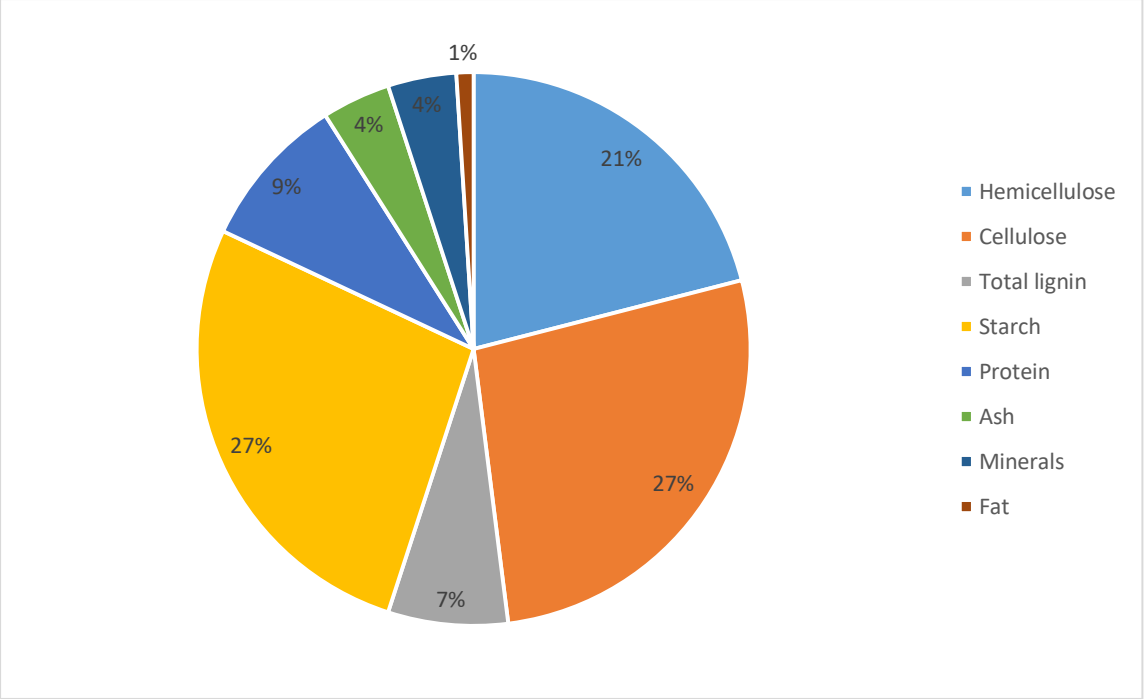


Figure 5.1 Cellulose, hemicellulose, lignin, ash, fat, protein, starch and minerals content of whole CS.

5.1.5. NIR scan for cellulose and lignin components in CS

The scans below (Figure 5.2) shows the NIR profile of the CS. The profiles were recorded at wavelength range of 1100 – 2500 nm region at 25°C. A broad and weak peak around 1200 nm reflects C-H stretching (2nd overtone). Absorption signals around 1400-1600 nm region are assigned to O-H stretching (1st overtone). Absorption bands in 1620-1780 region are associated with C-H group (1st overtone). The band at around 1920 nm is assigned to –C=O and –OH groups. Signal peaks around 2100-2150 nm are attributed to the combination of C-H and O-H stretching vibrations. Signal peaks at around 2300-2350 nm are assigned to the C-H stretching and deformation group frequencies of polysaccharides. Table 5.9 shows the absorption bands and functional groups of cellulose and lignin.

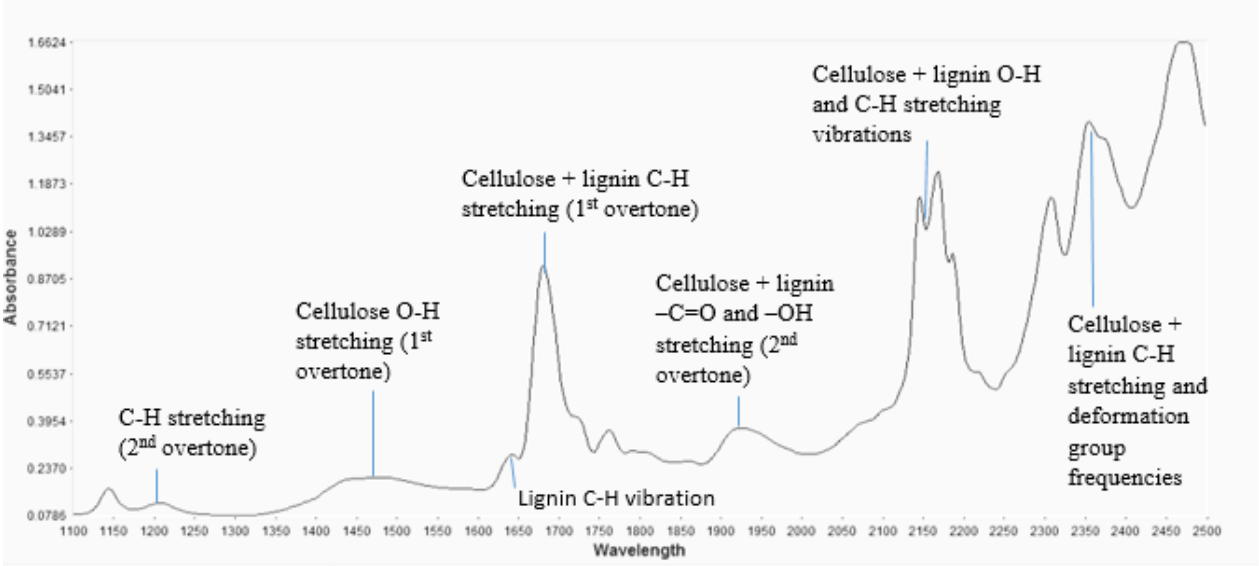


Figure 5.2 Cellulose and lignin structural information after ADF treatment using NIR.

Table 5.10 Relative intensity bands in the NIR spectra of cellulose and lignin in CS

NIR absorption band	**Cellulose**	**Lignin**
C-H stretching and deformation of $–CH_3$	1380	1380
O-H stretching (1^{st} overtone)	1400-1660	-
O-H stretching (1^{st} overtone)	-	1404
C-H vibration	-	1646; 1672; 1702
C-H stretching (1^{st} overtone) of $–CH_2$	1725	1725
C=O stretching (2^{nd} overtone) of $–CO_2H$ and the combination of O-H stretching and deformation vibrations, respectively	1898; 1927	1898; 1927
Combination of O-H stretching and C=O stretching (2^{nd} overtone)	-	1996
Combination of O-H and C-H and C-H stretching vibrations	2100	2100
C-H stretching and deformation group frequencies	2280; 2322	2280; 2322

5.2. Optimization of *T. thermophilus* HB8 growing conditions

T. thermophilus HB8 was grown at different pH conditions at 70°C to achieve the maximum growing environment of the bacteria. From Figure 5.4 it can be seen that alkaline medium at pH 7.5 showed that the maximum growth of the bacteria was obtained with maximum OD of 2.44 nm. Figure 5.3 shows the growth of bacteria at 600 nm at different pH mediums using glucose as a carbon source.

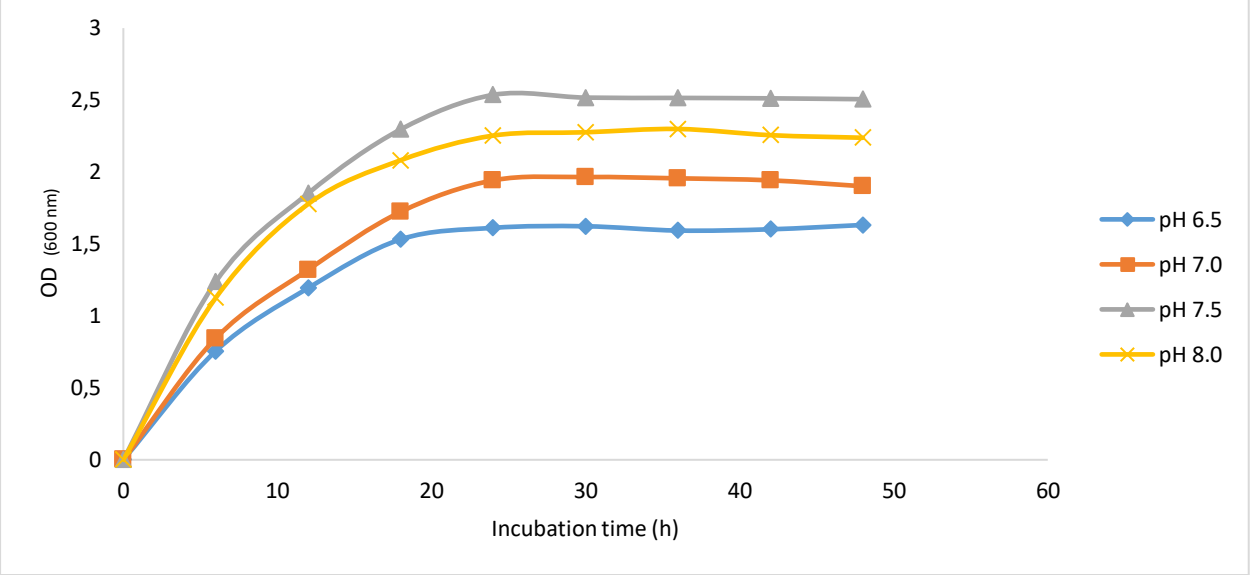


Figure 5.3 Growth profile of *T. thermophilus* at 600 nm in media containing glucose as a carbon source, at different pH values.

5.3 Utilization of CS for PHA production by *T. thermophilus* under nitrogen limitation

The microorganism was subsequently grown in cultures containing the agriculture by-product corn silage substrate, a more complex carbohydrate-containing substrate for PHA production under nitrogen-limited conditions. To observe the effect on bacterial growth and polymer production, unhydrolysed and hydrolysed CS supernatant was utilized at varying concentrations, 6, 12, 24 and 48% (w/v). 24% of unhydrolysed CS supernatant concentration showed high OD of 2.435 nm, followed by 48% (2.197 nm), 12% (1.995 nm), and lastly 6% (1.827 nm). 48% of hydrolysed CS supernatant concentration showed high OD of 2.925 nm, followed by 24% (2.695 nm), 12% (2.285 nm), and lastly 6% (1.952 nm). Figure 5.4 shows

the representation of *T. thermophilus* at 600 nm containing different strengths of unhydrolysed CS. Figure 5.5 shows the growth time profile of *T. thermophilus* at 600 nm containing different strengths of acid hydrolysed CS.

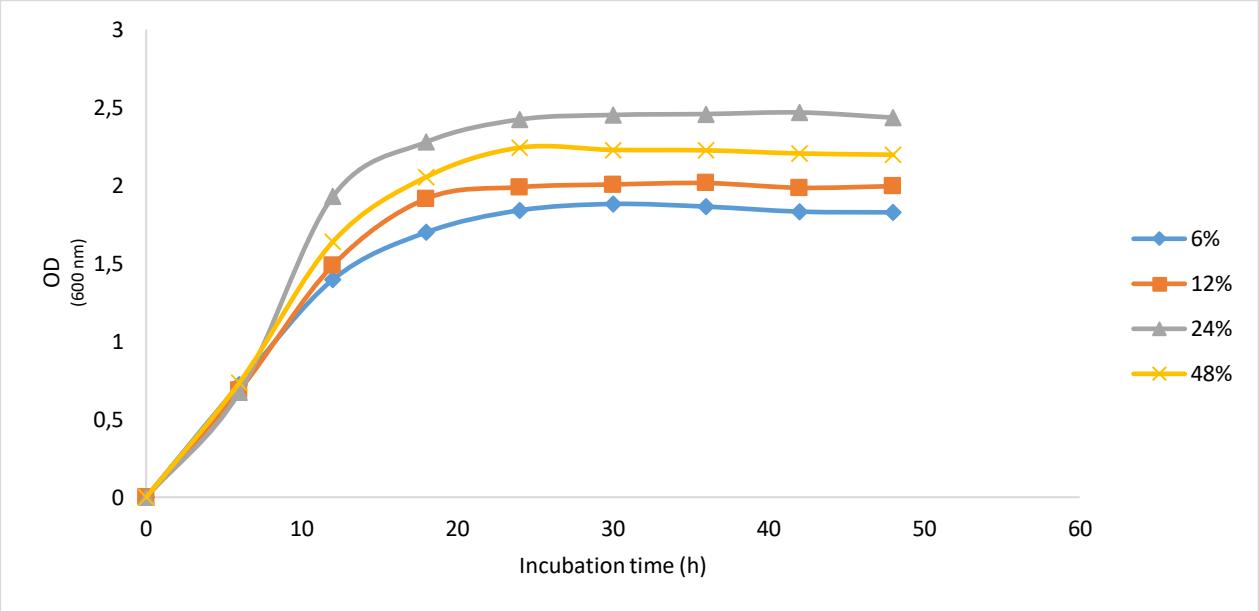


Figure 5.4 Growth profile of *T. thermophilus* at 600 nm in unhydrolysed CS as a carbon source at different strengths.

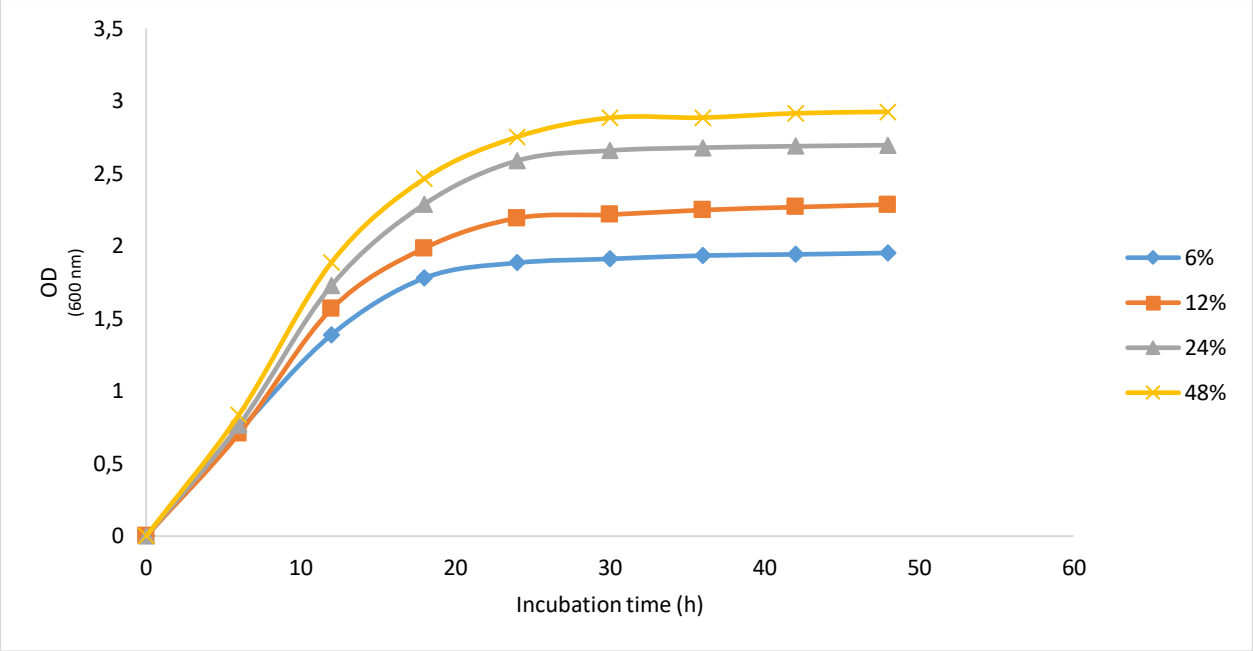


Figure 5.5 Growth profile of *T. thermophilus* at 600 nm in hydrolysed CS as a carbon source at different strengths.

5.4 PHA production screening

PHA granules in the cells were stained with Sudan Black B revealing PHA granules as blue–black droplets and a pink cytoplasm and the more precisely Nile Blue A showing golden yellow fluorescence due to the presence of PHA granules in the cytoplasm. The lipophilic staining with Sudan Black B and Nile Blue A showed a high sensitivity in PHA screening. Figure 5.6 (A) showed light micrograph of strain *T. thermophilus* HB8 stained with Sudan Black B; magnification 1000×, (B) fluorescent micrograph of strain *T. thermophilus* HB8 stained with Nile Blue A at wavelength 460 nm.

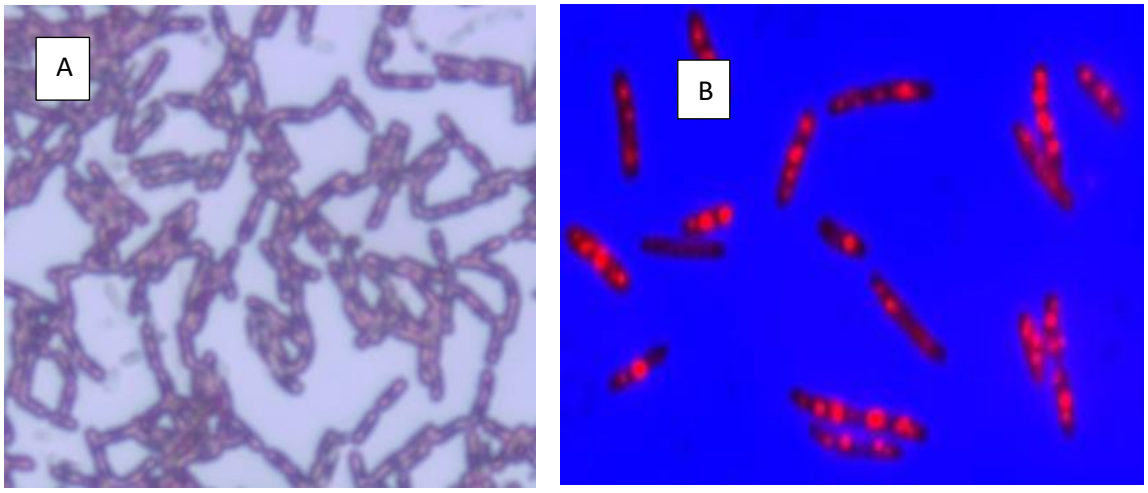


Figure 5.6 (A) Light micrograph of strain *T. thermophilus* HB8 stained with Sudan Black B; magnification 1000×. **(B)** Fluorescent micrograph (wavelength 460 nm) of strain *T. thermophilus* HB8 stained with Nile Blue A.

5.5 Extraction and estimation of PHAs

The PHAs produced by *T. thermophilus* cells was extracted by dispersions of chloroform and sodium hypochlorite solution for recovery of microbial PHA after a 50 h fermentation. Unhydrolysed CS yielded the maximum of 28.48% and hydrolysed CS yielded 61.32%. Table 5.10 shows quantities of PHA produced using unhydrolysed CS at different concentrations and Table 5.11 shows the quantities of PHA produced using unhydrolysed CS at different concentrations.

Table 5.11 Effect of unhydrolysed CS supernatant concentration on biomass and polymer production after 48 hrs of cultivation

% CS in media	Biomass (g/L)	Polymer (g/L)	% PHA content (w/w)
6	2.8983 ± 0.106	0.3725 ± 0.121	12.85 ± 1.65
12	3.6364 ± 0.202	0.7432 ± 0.263	20.44 ± 2.26
24	4.2261 ± 0.172	1.2014 ± 0.325	28.42 ± 2.65
48	4.8216 ± 0.220	0.8992 ± 0.221	18.65 ± 1.51

Table 5.12 Effect of hydrolysed CS supernatant concentration on biomass and polymer production after 48 hrs of cultivation

% CS in media	Biomass (g/L)	Polymer (g/L)	% PHA content (w/w)
6	4.1461 ± 0.235	1.7413 ± 0.242	42.23 ± 2.25
12	4.2614 ± 0.182	2.0888 ± 0.222	49.53 ± 1.20
24	5.4673 ± 0.161	3.0617 ± 0.176	56.41 ± 3.21
48	5.6890 ± 0.211	3.4702 ± 0.192	61.32 ± 2.01

5.6 Characterization of PHAs

5.6.1. FT-IR spectroscopy

To confirm the presence of PHA, powdered PHA samples were analysed by FTIR to conclude if the characteristic functional groups of PHA exist in the extract. The peaks showed existence of ester group (1720 cm^{-1}), methylene group (2934 cm^{-1}) and terminal hydroxyl group (3405 cm^{-1}) which are present in the polymeric structure of PHAs. The FTIR analysis confirmed the presence of PHA in the extract by showing the presence of characteristic functional groups of PHAs. Figure 5.7 shows the FT-IR absorption spectra of the poly(3HB-co-3HV) obtained from Sigma-Aldrich (A) and the poly(3HB-co-3HV) polymer obtained from *T. thermophilus* BH8 (B).

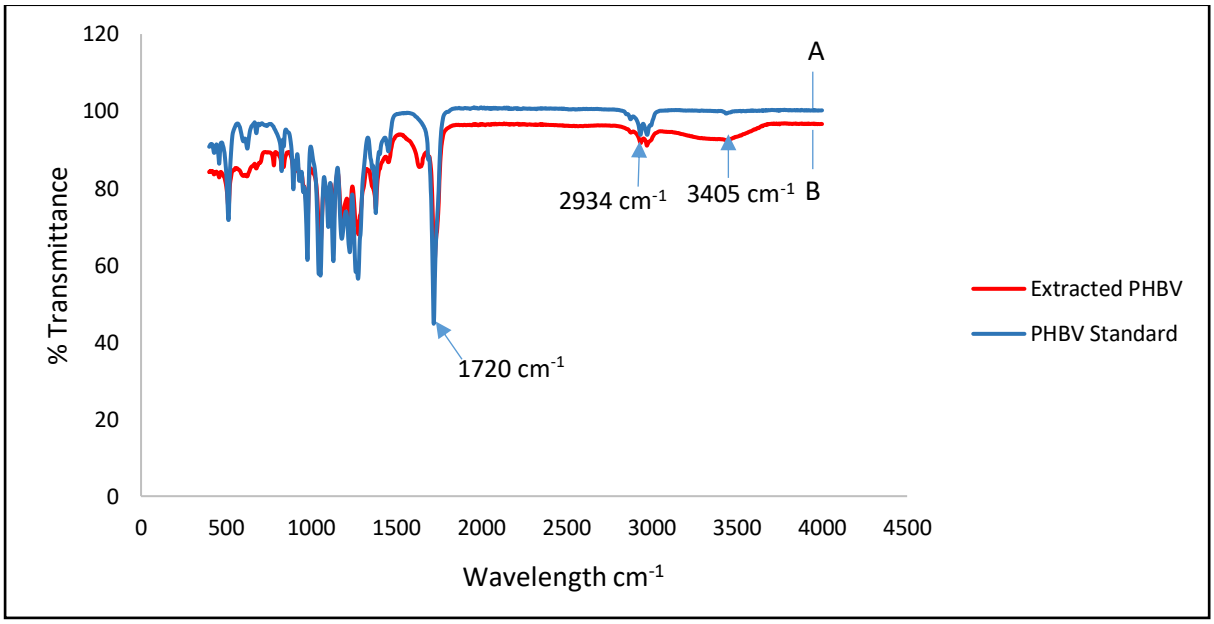


Figure 5.7 FT-IR absorption spectra of (**A)** poly(3HB-co-3HV) Sigma-Aldrich (standard) and (**B)** poly(3HB-co-3HV) polymer obtained from *T. thermophilus* BH8.

5.6.2 UV-Vis spectroscopy

UV-Vis spectrophotometry was utilized to analyse the PHA produced by *T. thermophilus* and the poly(3HB-co-3HV) standard obtained from Sigma-Aldrich using chloroform as the blank. The reference standard in this analysis also showed identical absorption at around the same wavelength and the extracted polymer. Figure 5.8 showed UV-Vis absorption spectra of A. poly(3HB-co-3HV) obtained from Sigma-Aldrich and B. poly(3HB-co-3HV) polymer obtained from *T. thermophilus* HB8.

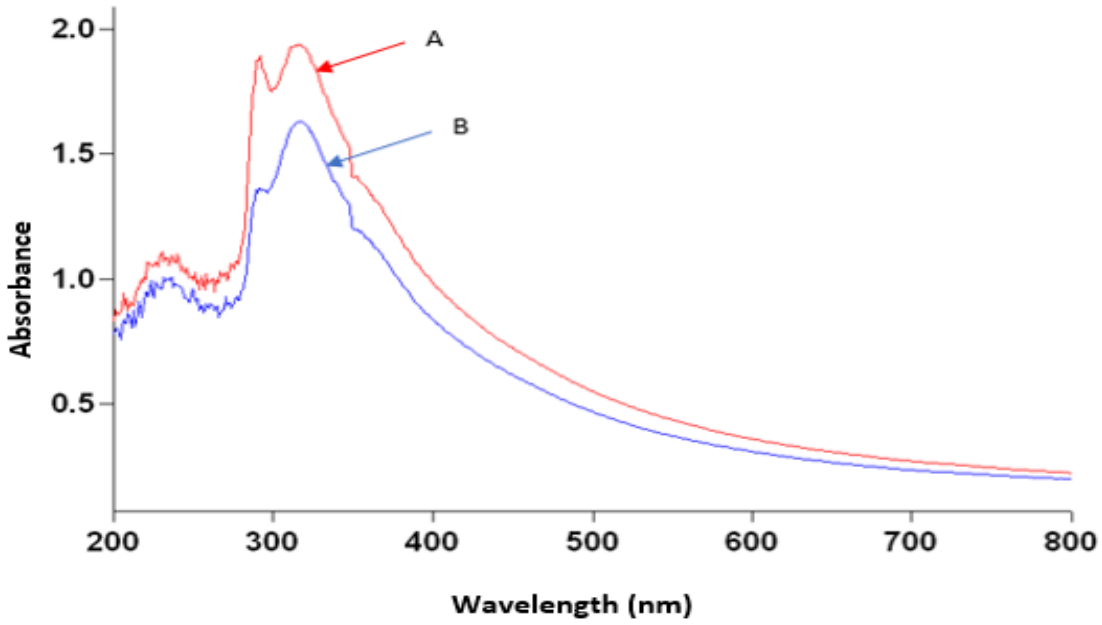


Figure 5.8 UV-Vis absorption spectra of (**A**) poly(3HB-co-3HV) Sigma-Aldrich (Standard) and (**B**) poly(3HB-co-3HV) polymer obtained from *T. thermophilus* HB8.

5.6.3 GC-FID

PHA methyl esters monomer composition was determined by GC-FID. The extracted methyl esters were analysed to characterize and confirm the PHA monomers synthesised by *T. thermophilus*. The peak profiles for 3HB and 3HV were detected at 5.77 min and 6.81 min [Figure 5.9 (a, b)], respectively based on the flame ionization analysis.

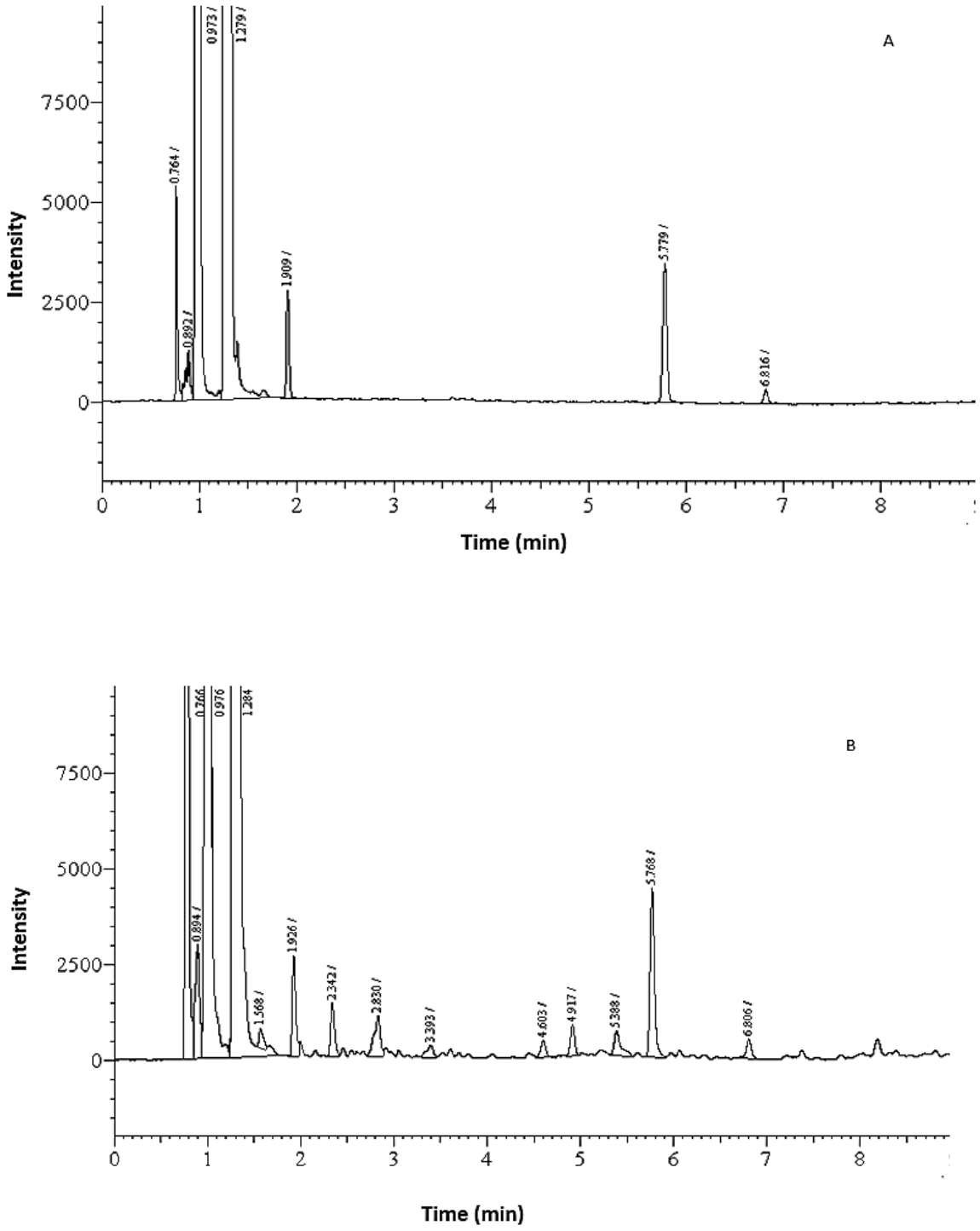
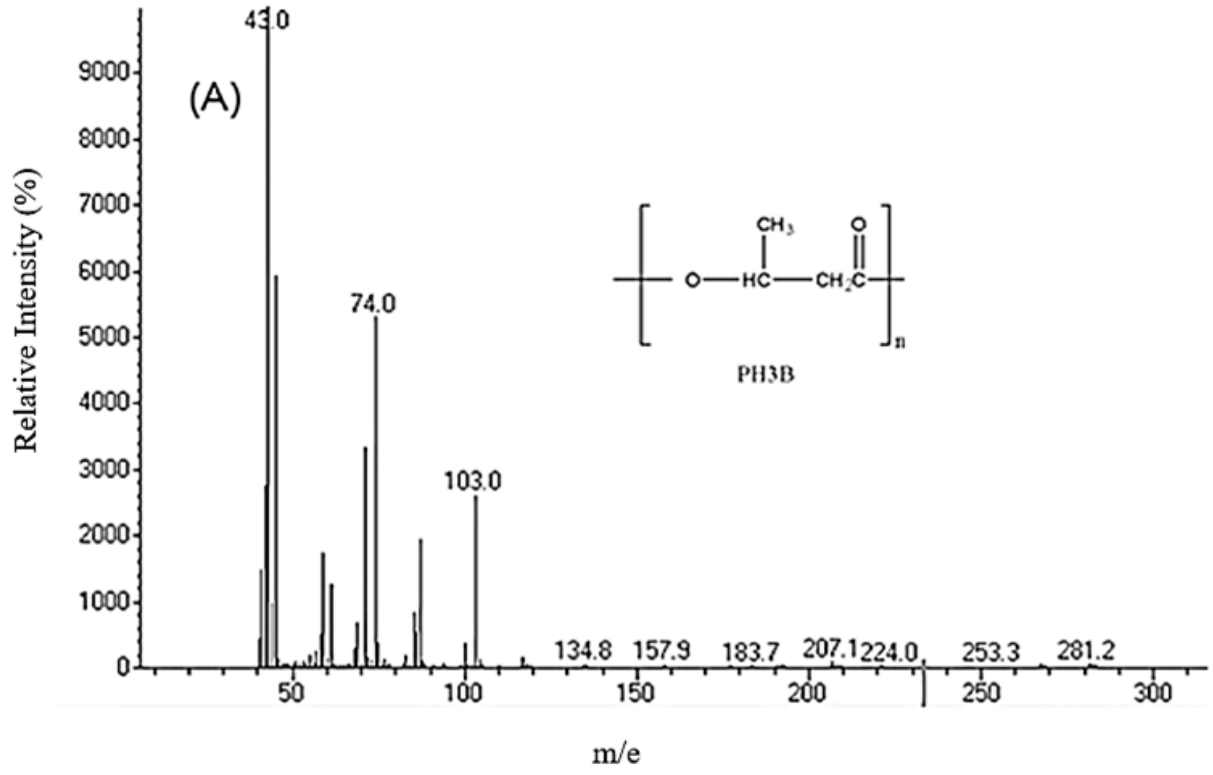


Figure 5.9 GC-FID chromatogram of the **(A)** poly(3HB-co-3HV) standard (Sigma-Aldrich), **(B)** and the poly(3HB-co-3HV) polymer obtained from *T. thermophilus* HB8.

5.6.4 GC-MS

Mass spectra analysis for methyl esters further confirm the presence of 3HB and 3HV methyl esters by showing the fragment m/e 131 formed by α-cleavage of the hydroxyl function group. Figure. 5.10 shows mass spectra of electron ionized methyl-esters of 3-hydroxyalkanoates: (A) 3HB and (B) 3HV obtained from *T. thermophilus* HB8.



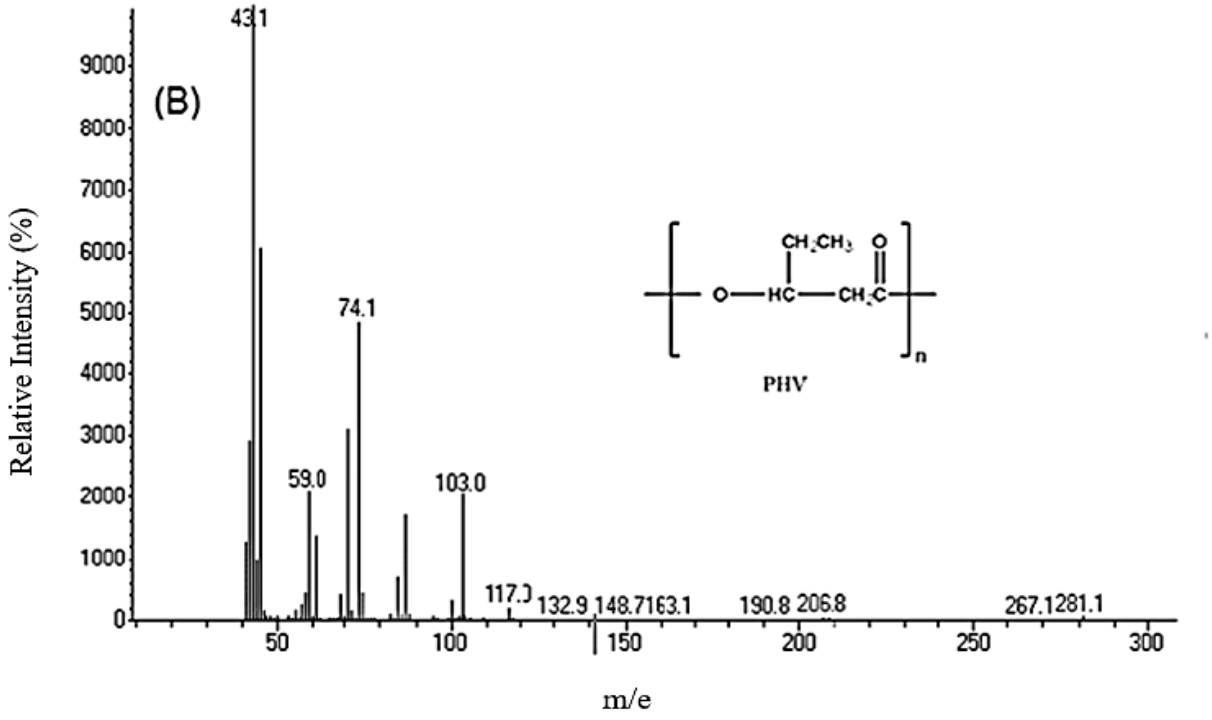


Figure. 5.10 Mass spectra of electron ionized methyl-esters of 3-hydroxyalkanoates: (**A**) 3HB and (**B**) 3HV obtained from *T. thermophilus* HB8.

5.7. Tensile Testing

Different polymer: starch (4.1, 4.2 and 4.3) bioplastics were tested for tensile strengths and elongation at break to determine their breaking points. 4.1 ratio showed high tensile strength of 11.9 MPa, followed by 4:2 ratio (11.5 MPa) and lastly 4:3 ratio (10.9 MPa). Table 5.12 shows the tensile strengths and elongation at break for different bioplastics.

Table 5.13 Tensile properties of biodegradable film

Plastic ratio Polymer : starch	**Tensile Strength at break (MPa)**	**Elongation at break (%)**
4:1	11.9 ± 0.7	24
4:2	11.5 ± 0.6	23
4:3	10.9 ± 0.9	20

*Error is reported as standard deviation of triplicate samples.

5.8 Scanning Electron Microscope (SEM)

To monitor the deviations in appearance of samples produced at different polymer: starch ratios, the surface area on top of the polymer films were observed by SEM. The polymer blends showed structural and morphological changes. 4:1 ratio showed a smooth intact surface, 4.2 ratio showed rough flaky network surface with holes, and 4:3 ratio showed rougher flaky network surface with more visible holes. Figure 5.11 shows SEM images of blended films at different polymer: starch ratios (a) 4:1, (b) 4:2, (c) 4:3.

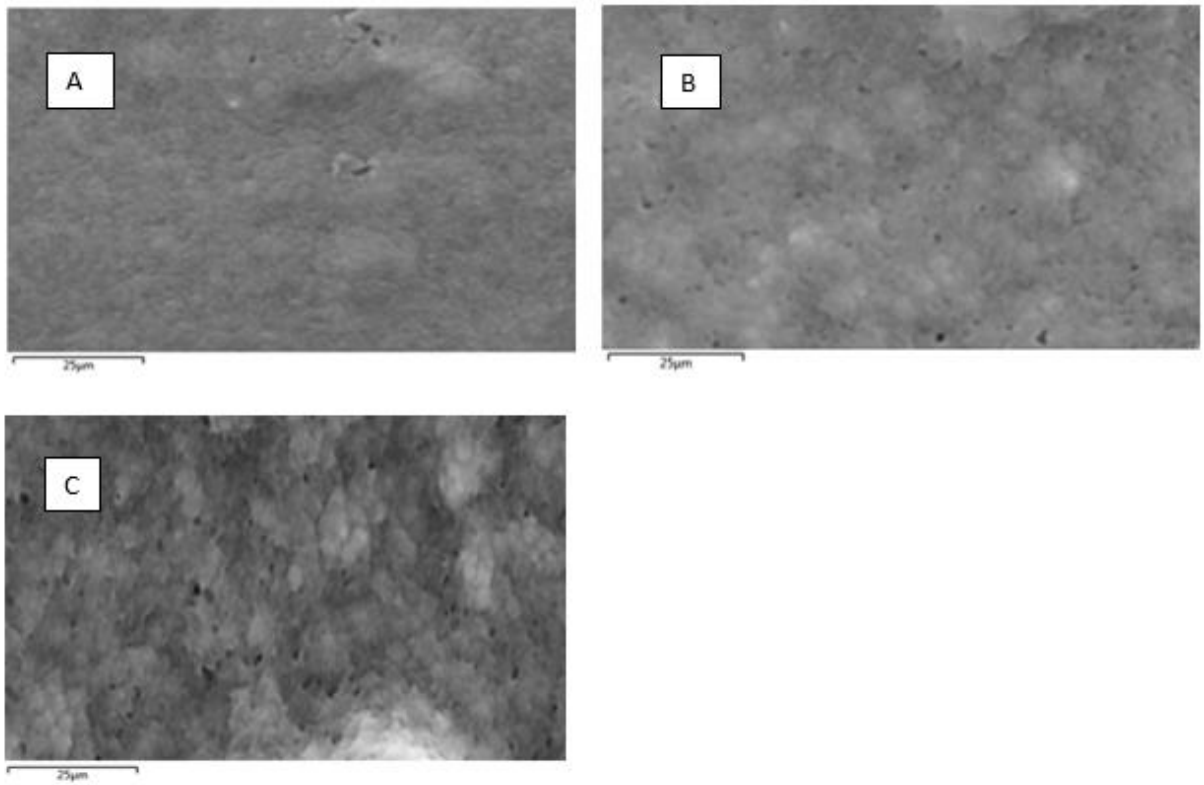


Figure 5.11 SEM images of blended films at different starch ratios (a) 4:1, (b) 4:2, (c) 4:3. As the starch: polymer ratio was increased, the pore size of the blended polymers became larger.

5.9 Biodegradability of the polymer sheet

Determination of the film samples biodegradation was measured by weight loss of the films after buried the polymer in natural environmental samples. There was a marked difference in the degradation profile for the three different environmental samples used. The soil sample showed maximum degradation of 47% after 60 days, followed by the compost (30%) and lastly the industrial sludge (10%). Figure 5.12 shows degradation percentages of poly (3HB-co-3HV) films up to 60 days.

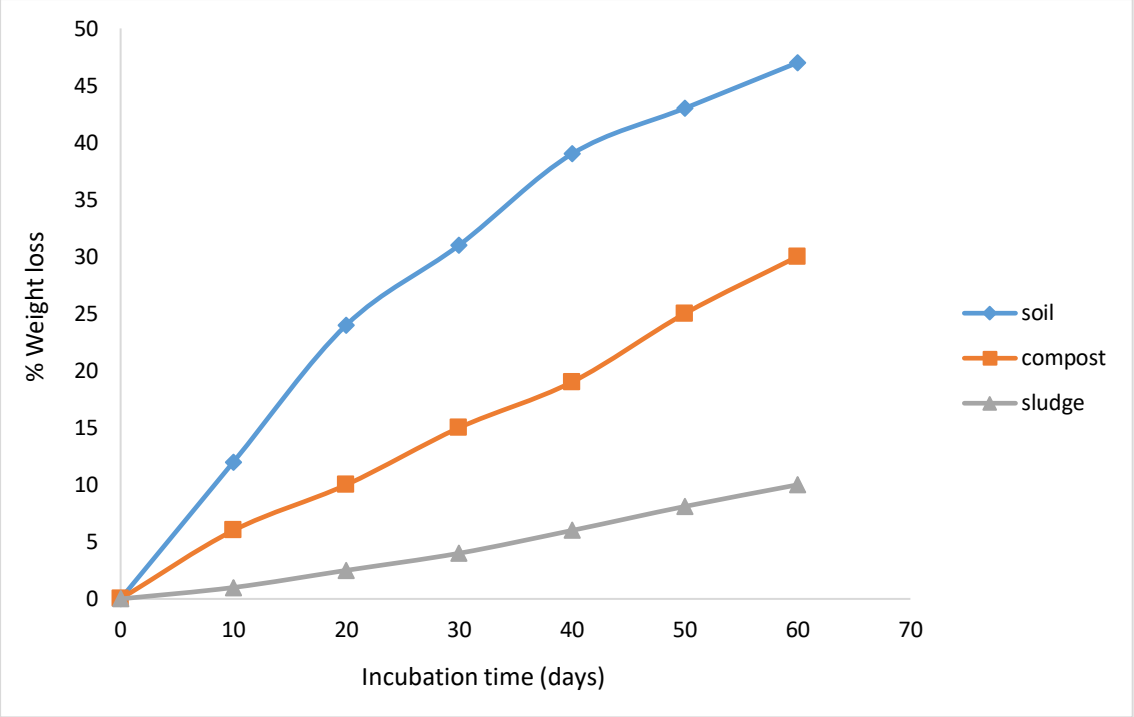


Figure 5.12 Degradation percentage of poly (3HB-co-3HV) films by the three environmental samples up to 60 days.

CHAPTER **6**

DISCUSSION

Bacterial strains that have the ability to utilize low-cost carbon feedstock to produce large quantities of PHAs are required for economic production of PHA. The agricultural sector generates low-cost biomass waste which are a rich source of carbohydrates and with abundant availability. These residues have little economic value hence they are used as animal feed. In this work, the agricultural by-product corn silage has been valorized in a novel way for the microbial production of biodegradable polymers (PHAs). The use of microbial strains for the production and accumulation of these novel polyesters requires a suitable carbon source. Also, identification of cheap substrates is of importance for the replacement of costly chemicals currently used for the production PHAs. This study demonstrates the successful use of corn silage using *T. thermophilus* HB8 for the production of significant levels of PHA under nitrogen-limiting conditions. *T. thermophilus* HB8 has the capacity to use such diverse and cheap residues as it possesses hydrolytic enzymes capable of metabolizing complex residues (Gowda and Shivakumar 2014). Numerous studies have indicated the usage of various carbon sources by different bacterial strains for the production of PHAs. To confirm the ability of utilizing agricultural residues to replace glucose in PHA production by *T. thermophilus* HB8, dry cell weight and PHA accumulation was determine on CS substrate (6%, 12%, 24% and 48% w/v). The *T. thermophilus* HB8 strain showed poly(3HB-co-3HV) production from CS, which indicated its nutritional versatility and amylolytic potential.

6.1 Compositional analysis

Agricultural starchy food wastes and by-products such as CS and whey (Pantazaki *et al.* 2009) are of interest in this century in the production of biodegradable bioplastics. These aliphatic biopolymers have ability to be to be produced through fermentation from natural resources like starch, fatty acids and sugars (Reddy *et al*. 2003). Figure 5.1 represent nutrient analysis of CS, which yielded greater amounts of carbohydrates as compared to other nutrients with cellulose (27.24%), starch (27.0%), hemicellulose (20.70%), lignin (6.54%), protein (9.1%), ash (3.7%),

fat (1.3%) and minerals (4%) (Tables 5.3-5.9). Compositional analysis yielded 100% because lignocellulosic biomass accounts for 100% of the original material (Sluiter *et al.* 2010). Literature indicated that CS contains average amounts of cellulose (28.4%), starch (21.78%), hemicellulose (20.9%), lignin (9.6%), protein (8.6%), ash (3%), fat (3.7%) and minerals (5.5%) (Htet *et al.* 2016; Euken 2018). Nutrients obtained in this study were comparable to literature, which indicated similar behavior of the plant from growth to harvesting for CS. The absorption of minerals by the plant was slightly lower compared to that reported in literature due to soil tillage during plant season. Soil tillage affect soil profile resulting in the poor uptake of minerals by the plant (Gaj *et al*. 2015). Also, CS produced from agricultural corn and corn stover as a by-product has great amounts of sugars present, which makes it a suitable substrate for PHAs production (Mishra and Rai 2006). Sugar rich waste or by-products from raw materials are readily available for bioconversion into high value products. Thus, CS can be an alternative substrate for industrial scale up due to its abundance and its carbohydrates availability. It is estimated that more than 1.9 million tons of CS are produced annually in South Africa for animal feed (Wilkinson and Toivonen 2003) and are readily available for further conversion.

6.2 NIR spectra analysis

NIR characterization of CS was performed to qualitatively analyse the presence of carbohydrates in CS. A significant relationship between cellulose and lignin functional groups was achieved (Figure 5.3), which signifies the relationship between structural carbohydrates present in CS. The absorbance bands around 1380 nm, shared by cellulose and lignin, correspond to C–H stretching and deformation of $–CH_3$ (Sun *et al.* 2011; Li *et al.* 2015). Absorption bands in the region of 1400 – 1660 nm correspond with O–H stretching (1^{st} overtone) for cellulose (Li *et al.* 2015). As for lignin, the absorbance characteristic of 1404 nm is associated with O–H stretching (1^{st} overtone) and the absorbance at 1646 nm, 1672 nm and 1702 nm are connected with C–H vibration (Lande *et al.* 2010; Sun *et al.* 2011). Lignin aromatic functionalities were identified, characteristic wavelength of 1143 nm is associated with C–H stretching (2^{nd} overtone), 1445 nm and 1678 nm are connected to C-H stretching (1^{st} overtone) and 2136 nm is connected to C=C, C–H combinations (Osborne and Fearn 2000). The absorbance signal approximately 1725 nm, which appears in the shared characteristic wavelengths for cellulose and lignin, may relate to the C–H stretching (1^{st} overtone) of $–CH_2$.

The common characteristic wavelengths of 1898 nm and approximately 1927 nm for cellulose and lignin are ascribed to C=O stretching (2^{nd} overtone) of $–CO_2H$ and the combination of O–H stretching and deformation vibrations, respectively. The shared wavelength around 1996 nm for cellulose and lignin correspond to the combination of O–H stretching and C=O stretching (2^{nd} overtone) (He and Hu 2013a). The signal around 2100 nm is connected with the combination of O–H and C–H stretching vibrations for cellulose and lignin (Wójciak *et al.* 2014). Wavelengths approximately 2280 nm and 2322 nm of cellulose and lignin belong to C–H stretching and deformation group frequencies (He and Hu 2013b). Availability of carbohydrates in CS was further analysed by NIR and these findings makes CS a useful renewable carbon source.

6.3 PHA production screening

Depending on the microbial strain and growing conditions, PHAs are polymerized into high molecular weight polymers in the range of 200000 to 3000000 Da (Sudesh *et al*. 2000). They occur as discrete inclusions or intercellular granules that are 0.2 ± 0.5 mm in diameter contained in the cytoplasm and were visualized with a microscope because of their high refractivity (Dawes and Senior 1973). Sudan Black B and Nile Blue A have proved to be selective dyes and were used for inclusive PHA staining (Mesquita *et al.* 2015**).** After staining with Sudan Black B, cells of *T. thermophilus* showed prominent blue-black granules inside the cytoplasm of the cell (Figure. 5.6a) (Williamson and Wilkinson 1958; Kallio and Harrington 1960). Following Nile Blue A staining, fluorescence microscopy at excitation wavelength of 460 nm PHAs exhibited orange fluorescing granules (Figure. 5.6b). The obtained results identified *T. thermophilus* as the PHA producer. According to literature, Legat *et al.* (2010) reported similar findings in the identification of PHA in *Halococcus*. Also, study by Mayeli *et al*. (2015) reported similar results after staining *Bacillus axaraqnsis* BIPC01 cells for the PHAs production using Sudan Black B and Nile Blue A.

6.4 Optimization of *T. thermophilus* HB8 growth conditions

In this study, *T. thermophilus* HB8 was selected for PHA production based on several factors, including the microorganism's capability to use inexpensive carbon sources, its growth rate, its polymer synthesis rate, and the extent of polymer accumulation (Pantazaki *et al.* 2009). For optimum growth, *T. thermophilus* cells were grown at different pH values at 70°C to improve cell density during one-step cultivation process using glucose as a carbon source. pH 7.5 showed to be the best growing environment with the maximum of optical density (OD) of 2.506 nm (Figure 5.3) after 48 h for this culture and. This showed that *T. thermophilus* grows adequately in weak alkaline medium hence it was used in all fermentation processes. As a cheap carbon source, substituting glucose CS was utilized in two different forms: unhydrolysed (Figure 5.4) and acidic hydrolysed (Figure 5.5) supernatants to achieve substantial PHA yields. Both CS supernatants supported PHA production, where 24% unhydrolysed CS concentration yielded maximum optical density of 2.435 nm, followed by 48% (2.197 nm), 12% (1.995 nm) and lastly 6% (1.827 nm). 24% unhydrolysed CS concentration produced higher cell biomass compared to other concentrations because it contained balance amount of carbon that allowed effective mitosis, whereas 48% unhydrolysed CS concentration produces lesser cell biomass. This indicated that as the concentration of the substrate increases, the availability of salts increase hence it reduced the cell production. On the other hand, 48% hydrolysed CS concentration yielded maximum optical density of 2.925 nm, followed by 24% (2.695 nm), 12% (2.285nm) and lastly 6% (1.953 nm). 48% hydrolysed CS concentration produced higher biomass compare to other concentrations because of higher amounts of carbon allowing significant cell mitosis. According to literature, CS has been used mainly for animal feed but not for the production of PHAs. It is for the first time in this study that CS is used for this purpose hence the cell growth using this substrate was significant and accepted.

Some agro-industrial residues like babassu, soy cake, potato starch (Oliveira *et al*. 2004) whey (Pantazaki *et al.* 2009), cane molasses (Ahn *et al.* 2001) have been studied for PHA production. Plant oils such as corn oil, olive oil and palm were found to be profitable substrates for *R. eutropha* for PHB production (Fukui *et al.* 1998). On the other hand, Thakor *et al*. (2005) studied the coconut oil as carbon source for *Comamonas testosterone.* Rusendi and Sheppard (1995) reported a 77% of biomass dry weight using potato starch hydrolysate for PHB production. Similar levels of PHB was achieved by utilizing jackfruit powder and potato starch,

the study showed the maximum production of 0.69 g/L using hydrolyzate prepared from 2% jackfruit seed powder. However, previous studies has stated that bacteria such as *A. latus* and a mutant *Azotobacter vinelandii* (Ojumu *et al*. 2004) and *A. vinelandii* UWD (Chou *et al.* 1997) have an ability to synthesis PHAs under non-limiting conditions.

6.5 PHA extraction and estimation

This extraction protocol was used based on PHAs ability to be soluble in chloroform and insoluble in methanol (Kessler *et al.* 2001). Hypochlorite was used for cell lysis following cell growth. This was followed by the isolation of extracted PHA using chloroform, which was followed by methanol precipitation. Other lipophilic components and lipids in the bacteria were removed during PHA isolation in chloroform. Although this method yields high amounts of PHA, but a great amount of hazardous solvent is required for mass production to repeat the same process thus this makes this protocol environmentally unfriendly and not suitable for mass production. Solvent extraction method has proved to be demanding because of solvent resistance to evaporation during drying stage causing the extracted polymer to be viscous before completely drying. After 48 h of fermentation, the 24% CS supernatant yielded the optimum PHA concentration of 1.2 g/L and accumulated PHA up to 28% (Table 5.11), followed but 12% unhydrolysed CS substrate (0.7 g/L; 20.44%), 48 % unhydrolysed CS substrate (0.9 g/L; 18.65%) lastly 6% unhydrolysed CS substrate (0.34 g/L; 12.8%). 48% unhydrolysed CS substrate show a highest biomass compared to other concentrations but the polymer concentration and PHA yield was less. This means cells were able to grow with less PHA accumulation ability the cell were not exposed to stress as compared to 24% and 12% (Aslan *et al.* 2016). The maximum PHA yield of 28.4% was significant based on the fact that agricultural raw materials require hydrolysis to realise crude sugars. On the other hand, 48% hydrolysed CS substrate yielded the optimum PHA concentration of 3.4 g/L and accumulated PHA up to 61.3% (Table 5.12), followed but 24% hydrolysed CS substrate (3.1 g/L; 56.4%), 12% hydrolysed CS substrate (2.1 g/L; 49.5%) lastly 6% unhydrolysed CS substrate (1.7 g/L; 42.2%). Substrate pre-treatment promoted enhancement of high amount of carbon while reducing nitrogen amounts hence significant amounts of PHAs was obtained. Substrate pre-treatment sustained significant cell growth, PHA concentration and PHA yield. Koller *et al.* (2005) obtained 5.5 g/L of poly[3(hydroxybutyrate-co-hydroxyvalerate)] from hydrolysed whey agricultural waste. Sawant *et al*. (2015a) obtained up to 72.5% yield and production

yielded 9.71 g/L from corn stover hydrolysate. Literature shows that agricultural waste or by-products produces prominent amounts of polymers particularly corn stover. Acceptable amount of polymer was obtained in this study although it was less compared to previous studies. Previous studies have shown that the most PHAs produced are poly(3HB-co-3HV) and poly(3HB).

Scl-PHA homopolymers (C_3–C_5), such as PHB, form stiff crystalline materials, which are brittle and breaks when extended. A wide range of application of scl-PHA homopolymers is limited by the lack of flexibility. Processing of PHB homopolymer consist solely of C_4 monomer is challenging, due to the fact that degradation takes place at temperature slightly above its melting point (Koning 1995). Other type of polymers composed of mcl-PHA (C_6–C_{14}) which are semi-crystalline thermoplastic elastomers and reinforcement is needed to enhance the mechanical properties of these types of polymers. But polymers consist of either scl- or mcl monomer units, have a wider range physical properties, depending on the mol% composition of the different monomers in the copolymer (Matsusaki *et al*. 2000). Also, scl-mcl-copolymers composed of mostly C_4 monomers, with a small quantity of C6 monomer, have properties similar to polypropylene (Abe and Doi 2002). The copolymer of HB and HV poly (3HB-co-3HV) has reduced crystallinity and melting point, leading to improved flexibility, strength and easier processing (Suriyamongkol *et al.* 2007). On the other hand, poly (3HB-co-3HV) shows better properties, which includes reduced brittleness. Vieira *et al.* (2011) indicated that poly (3HB-co-3HV) has proven to be a copolymer compound that is more useful commercially because its melting point can be lowered, and its mechanical properties and thermoplastic characteristics can be greatly improved by increasing the ratio of 3-hydroxyvalerate to 3-hydroxybutyrate repeating units. *T. thermophilus* organism in the presence of CS carbon source produced a copolymer poly (3HB-co-3HV) without addition of precursor.

6.6 UV-Vis spectroscopy

UV-Vis scanning indicated similarities between the spectrum of the reference standard and the extracted polymer and similarities between the spectrums indicated the occurrence of the PHAs (Figure 5.8). Chloroform was utilized as the polymer solubilizing solvent and hence it was used a blank (Selvakumar *et al.* 2011; Alarfaj *et al.* 2015). Both spectrums of both standard and extracted polymer indicated a peak around 240 nm and one at 340 nm indicating the present of poly(3HB-co-3HV) (Shah 2014). In this study UV-VIS was used as preliminary identification of the extracted polymer. Similarities between both spectrums indicated the presence of poly (3HB-co-3HV) compound in the isolated polymer. GC was used as a further confirmation of the extracted polymer.

6.7 FT-IR spectroscopy

Polymer extracted from *T. thermophilus* was subjected to FT-IR analysis (Figure 5.7). From the spectrum obtained it was inferred that the band at 3375 cm^{-1} indicated a strong O-H group found corn cobs presence of in CS. Similar results have been reported literature (Gumel *et al.* 2012; Getachew and Woldesenbet 2016). Comparable absorption bands attained from bacterial poly(3HB-co-3HV) and the commercial poly(3HB-co-3HV) standard were obtained. The absorption bands of the standard showed high transmittance compared to the extracted polymer particularly the ester group. This indicated that the extracted polymer lack purity due to traces of interferences emanated from the extraction process. Absorption bands at 1719.6 and 1275.1 cm^{-1} are typical of poly(3HB-co-3HV) and correspond to C=O and C–O stretching, respectively (Misra *et al.* 2000). Absorption at 1719.6 cm^{-1} band represents the ester carbonyl group C=O stretch in PHA located in the chain of exceedingly ordered crystalline structures (Valappil *et al.* 2007). The absorption bands around 2934, 2974.9 cm^{-1} represent the symmetric and asymmetric CH_3 stretching, $–CH_2$, and –CH groups and these bands associated to the monomeric units in the lateral chain (Masood *et al.* 2012; Montanheiro *et al.* 2016; Flores-Sánchez *et al.* 2017). Absorption band at 1450.6 cm^{-1} represent asymmetrical C-H bending vibration in CH_3 group while 1378.6 cm^{-1} represent C-O-H (Flores-Sánchez *et al.* 2017). In one study by Masood *et al.* (2012), characterization of poly(3HB-co-3HV) from *Bacillus cereus* FA11 obtained from trinitrotoluene-contaminated soil was conducted and similar results

were reported. In another study, Mumtaz *et al.* (2010) reported similar results of poly(3HB-co-3HV) produced from *Comamonas* sp. EB.

6.8 GC-FID

Extracted polymer was further characterized by GC after the depolymerization and derivatization to methyl ester, through methanolysis. GC was used to determine the monomeric compositions of the extracted polymer. The retention time of the reference standard were comparable to retention time of the extracted polymer (Figure 5.9a, b). The peaks at 5.779 min presents PHB and 6.816 min presents PHV, respectively. This confirmed that the extracted polymer is poly(3HB-co-3HV). *T. thermophilus* was able to produce poly(3HB-co-3HV) and other types of PHAs from 2.3 min to 5.5 min (Figure 5.9b). *T. thermophilus* has been reported to produce copolymer from food waste (Pantazaki *et al.* 2009; Nielsen *et al.* 2017). This indicates that growth of *T. thermophilus* in CS substrate produced copolymer. Peaks at 1.568 min and 1.926 min are by-products formed during transesterification reaction using sulphuric acid and methanol (Godbole 2016). Riis and Mai (1988) suggested derivatization method of 3-hydroxyakanoic acid with HCl and propanol to a propyl ester as it is more lipophilic when compared to methyl ester hence partitioning coefficient improves. This theory was confirmed by Jan *et al.* (1995) and Hahn *et al.* (1999). Similar results were obtained by Flores-Sánchez *et al.* (2017), where poly(3HB-co-3HV) was produced from Mexican avocado by *Cupriavidus necator* ATCC 17699. PHB eluted at 5.516 min and PHV eluted at 7.150 min. Results obtained corroborate with literature.

6.9 GC-MS

Monomer composition of the extracted polymer was determined by GC-MS after methanolysis. 3HB methyl ester spectrum (Figure 5.10a), the dominating peak at m/e 43 caused by a α cleavage of bonds between C_3 and C_4 of the saturated 3-hydroxy alkanoic acids. Additionally, the secondary m/e 74 ions designated the carbonyl end of the molecule instigated from McLafferty arrangement after bond cleavage between C_3 and C_4, both of 3HB and 3HV methyl esters (McLafferty, Tureček and Turecek 1993), and the exclusion of methanol from m/e 103 might have produced the ion with m/e 71.0 (Wallen and Rohwedder 1974; Huijberts *et al.*

1994; He and Hu 2013b). The m/e 59 peak indicated the hydroxyl end of the PHA molecule, presenting C3 and C4 bond cleavage in the mass spectrum of 3HV methyl ester (Figure 5.10b). Low signal intensities of some ion are due to the incapability of 3-hydroxyalkanoic acids methyl esters to absorb the large amount of energy (70 eV) used in electron impact (EI)-MS. To achieve higher signal intensity de Rijk *et al.* (2005) advised on the use of chemical ionization (CI)-MS of the available methyl esters. de Paula *et al.* (2017) concludes that fragment m/e 87 is associated with 3HB and fragment at m/e 101is related to 3HB. Results obtained in this study showed a shift in electron ionization compared to the study conducted by de Paula *et al.* (2017), because propyl-esters of poly(3HB-co-3HV) were produced through propanol and HCl transesterification resulting in more carbon groups formed and in this study methyl-esters of poly(3HB-co-3HV) were produced through methanol and H_2SO_4 and lesser carbon groups of the final compound. NMR can be further used for the exact position and cis/trans configuration of the double bonds since MS cannot determine such information (McLafferty *et al*. 1993).

6.10 Tensile Testing

PHAs in previous studied have proven to have identical mechanical properties to conventional plastics like polypropylene or polyethylene. The selection of media was not only vital for supplying optimal conditions for PHA production but also to achieve higher volumetric productivity to provide a final product that is economically competitive with the traditional plastics. Tensile strength (TS) of plastic material is a vital parameter because these PHA bioplastics are utilized for packing films, cosmetic products, disposable items (utensils, hygiene products), in agricultural sector they are used for seed encapsulation and crop protection (Saxena and Tiwari 2011). Lately, these bioplastics are utilized as mobile phone castings and for E-devices (Varsha and Savitha 2011). Degree of TS allows this material to resist the typical stress that packaging materials encounter during food handling and transportation (Sadegh-Hassani and Nafchi 2014). Previous tensile studies had to compromise on the sample dimensions (i.e. width 4 mm, thickness 0.1 mm, probably due to a lack of polymer availability (Ouyang *et al.* 2007). Using ASTM D638 standard methods, 4:1 (11.9 MPa) ratio showed high tensile strength as compared to 4:2 (11.5 MPa) and 4:3 (10.9 MPa) (Table 5.13). Also, 4:1 ratio showed a better elongation at break of 24% compared to 4:2 (23%) and 4:3 (22%). As the starch ratio increased, the resistance to stress and elongation decreased.

Starch did not prove to be an effective toughening agent in this study mainly because of its powder-like structural characteristics. The polymer content of 4 g was selected based on literature (Singh *et al.* 2013) but the increase in polymer could increase the tensile strength and elongation of the blends. In the study by Zhao *et al*. (2019), natural rubber was utilized as plasticizer of the poly(3HB-co-3HV) polymer. The addition of the rubber increased the flexibility and elongation by 40-100 %. Study conducted by Mumtaz *et al.* (2010), solvent-casting technique using chloroform as the plasticizer was employed. Tensile strength of 29.51 MPa and 8.63 % elongation. Results obtained in this study were lower than that reported in previous studies. This is due to powder plasticizer that increases the biofilm thickness while liquid-like plasticized decreases the secant module (Zhao *et al*. 2019).

6.11 Scanning Electron Microscope (SEM)

Mechanical behaviour of the bioplastics and the SEM technique was used to study the morphology at different polymer: starch ratios (4:1; 4:2; 4:3) (Figure 5.11). The poly(3HB-co-3HV) films experienced structural and morphological changes with the increase in starch content present in the blend. The smooth intact surface was observed in 4:1 ratio due to the dominating polymer ratio in the bioplastic and little starch ratio present. Poly(3HB-co-3HV) typically produces smooth surface film (Zhao *et al*. 2019). Rougher flaky network surfaces with holes were observed in 4:2 ratio but more in 4:3 ratios (Figure 5.11b, c) which indicated the improved ductility and difference in fracturing mechanisms of the two polymers (Zhao *et al*. 2019). This indicate that high plasticizer content changes the homogeneity of the film surface. Results are in agreement with Zhao *et al.* (2019) that as the rubber ratio increases in the poly(3HB-co-3HV): rubber blends the surface becomes rougher due to their improved ductility. Mumtaz *et al.* (2010) observed flaky network structure on Poly(3HB-co-3HV) biofilm due to the polymer extraction protocol.

6.12 Biodegradation of plastics

In natural environments, polymer degradation takes place through thermal, mechanical, hydrolysis, and photochemical destructions and also biodegradation. As PHAs contains vital properties of being biodegradable the biodegradability test was conducted in natural environmental samples i.e. soil, compost and sludge. Weight loss of the poly(3HB-co-3HV) starch blend sheets took place during incubation, also the amount of degradation was greatly influenced by the environment conditions like moisture level, temperature, microbial population in a given environment, pH and inorganic composition of nutrients (Jendrossek 2005a; Khanna and Srivastava 2005). A better degradation environment for poly(3HB-co-3HV) bioplastics was found to be soil when compared to industrial sludge and compost (Figure 5.12). This finding was influenced by the fact that soil contains about 10^5-10^6 fungal colony-forming units per gram, 10^5-10^8 per gram bacteria and 10^6-10^7 per gram actinomycetes (Lawlor *et al.* 2000). Compost showed less degradability compared to soil because it is a manufactured natural environment through degradation of organic materials by activities of succeeding groups of microbes (Danon *et al.* 2008). Industrial sludge showed the least degradation because it is the natural environment resulting from wastewater treatment under aerobic conditions and significant amounts of organic contamination (Kallistova *et al.* 2014). Percentage degradation of poly(3HB-co-3HV) film after 60 days burial in soil, compost and industrial sludge was 47%, 30% and 10%, respectively. Biodegradability was greatly influenced by the activities of living microorganisms in the natural environment samples. Hence, poly(3HB-co-3HV) bioplastics have the ability to degrade into products that can be metabolized by living microbes (Rivera-Briso and Serrano-Aroca 2018). Subsequently CS as an agricultural byproduct derived from CO_2 and water and converted microbially into biodegradable PHA, its breakdown products in the environment were again CO_2 and water (Madison and Huisman 1999). Altaee *et al.* (2016), demonstrated that the degradation of polyhydroxyalkanoates under anaerobic conditions decomposes to CO_2, H_2O, and methane, whereas under aerobic they decompose to CO_2 and H_2O. A study by Singh *et al.* (2013) showed that compost yielded significant degradation rate compared to soil and industrial sludge because of high microorganism load (5.0×106 cfu/mL^{-1} to more than 3.0×109 cfu mL^{-1}/g) found in compost. However, this was not the case in this work.

CHAPTER **7**

CONCLUSION AND RECOMMENDATIONS

The biotechnological utilization of agricultural by-product corn silage as cheap substrate for the production of PHAs was achieved. Unhydrolysed sugar-based CS as a carbon source produced up to 28% (Table 5.10) PHA and hydrolyzed CS yielded 61% PHA (Table 5.11). Both CS pretreatments yielded significant amounts of polymer but for future studies alkaline and enzymatic hydrolysis will studied. PHAs production process in this study has shown to be environmentally friendly due to the utilization of agricultural feed stock and by-products. In this study, *T thermophilus* has produced a copolymer of poly(3HB-co-3HV) and other unknown PHA's, which will be identified and characterized for future studies. H_2SO_4-methanol transesterification also resulted in the production of unknown side products which reduced the amounts of the polymer hence other transesterification methods should be employed to improve the production of the polymer. Utilization of HCl-propanol or Lewis- acid boron trifloride (BF_3) transesterification methods will be employed in future studies. Starch did not show to be an effective plasticizer; this can be improved by using liquid or liquid-like structure plasticizer. Due to the comparatively high production cost, the current development of PHA applications undoubtedly aims at high value products medical and tissue engineering applications. Therefore, analytical methods like NMR should be employed for the identification of novel PHA synthesised. Discovery of novel PHAs will be vital in plastic industry due to their versatility and novel features. More studies on alternative sugar resources are needed to investigate this notion. Agricultural by-product CS showed ability of exploiting the innate enzymatic ability of *T. thermophilus* and its nutritional versatility, which makes it a promising source for producing PHA at industrial scale.

REFERENCES

Abe, H. and Doi, Y. 2002. Side-chain effect of second monomer units on crystalline morphology, thermal properties, and enzymatic degradability for random copolyesters of (R)-3-hydroxybutyric acid with (R)-3-hydroxyalkanoic acids. *Biomacromolecules*, 3 (1): 133-138.

Aden, A., Ruth, M., Ibsen, K., Jechura, J., Neeves, K., Sheehan, J., Wallace, B., Montague, L., Slayton, A. and Lukas, J. 2002. Lignocellulosic biomass to ethanol process design and economics utilizing co-current dilute acid prehydrolysis and enzymatic hydrolysis for corn stover. National Renewable Energy Laboratory, NREL/TP-510-32438.

Ahn, W. S., Park, S. J. and Lee, S. Y. 2001. Production of poly (3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant *Escherichia coli*. *Biotechnology Letters*, 23 (3): 235-240.

Alarfaj, A. A., Arshad, M., Sholkamy, E. N. and Munusamy, M. A. 2015. Extraction and Characterization of Polyhydroxybutyrates (PHB) from *Bacillus thuringiensis* KSADL127 Isolated from Mangrove Environments of Saudi Arabia. *Brazilian Archives of Biology and Technology*, 58 (5): 781-788.

Albuquerque, M., Eiroa, M., Torres, C., Nunes, B. and Reis, M. 2007. Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. *Journal of Biotechnology*, 130 (4): 411-421.

Altaee, N., El-Hiti, G. A., Fahdil, A., Sudesh, K. and Yousif, E. 2016. Biodegradation of different formulations of polyhydroxybutyrate films in soil. *SpringerPlus*, 5 (1): 762.

Amass, W., Amass, A. and Tighe, B. 1998. A review of biodegradable polymers: uses, current developments in the synthesis and characterization of biodegradable polyesters, blends of biodegradable polymers and recent advances in biodegradation studies. *Polymer international*, 47 (2): 89-144.

Amulya, K., Reddy, M. V. and Mohan, S. V. 2014. Acidogenic spent wash valorization through polyhydroxyalkanoate (PHA) synthesis coupled with fermentative biohydrogen production. *Bioresource Technology*, 158: 336-342.

Anwar, Z., Gulfraz, M. and Irshad, M. 2014. Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review. *Journal of Radiation Research and Applied Sciences*, 7 (2): 163-173.

Ashby, R. D., Solaiman, D. K. and Strahan, G. D. 2011. Efficient utilization of crude glycerol as fermentation substrate in the synthesis of poly (3-hydroxybutyrate) biopolymers. *Journal of the American Oil Chemists' Society*, 88 (7): 949-959.

Aslan, A. N., Ali, M. M., Morad, N. and Tamunaidu, P. 2016. Polyhydroxyalkanoates production from waste biomass. In: Proceedings of *IOP Conference Series: Earth and Environmental Science*. IOP Publishing, 012040.

Babel, W., Riis, V. and Hainich, E. J. P. U. K. 1990. Mikrobielle Thermpolaste: Biosyntheses, Eigenschaften und Anwendung. 37 (4): 109-115.

Beeby, M., Cho, M., Stubbe, J. and Jensen, G. J. 2012. Growth and localization of polyhydroxybutyrate granules in *Ralstonia eutropha*. *Journal of Bacteriology*, 194 (5): 1092-1099.

Bengtsson, S., Werker, A., Christensson, M. and Welander, T. 2008. Production of polyhydroxyalkanoates by activated sludge treating a paper mill wastewater. *Bioresource Technology*, 99 (3): 509-516.

Bhuwal, A. K., Singh, G., Aggarwal, N. K., Goyal, V. and Yadav, A. 2013. Isolation and screening of polyhydroxyalkanoates producing bacteria from pulp, paper, and cardboard industry wastes. *International Journal of Biomaterials*, 2013, Article ID 752821, 10. doi.org/10.1155/2013/752821.

Brandl, H., Gross, R. A., Lenz, R. W. and Fuller, R. C. 1988. *Pseudomonas oleovorans* as a source of poly (β-hydroxyalkanoates) for potential applications as biodegradable polyesters. *Applied and Environmental Microbiology*, 54 (8): 1977-1982.

Braunegg, G., Lefebvre, G. and Genser, K. F. 1998. Polyhydroxyalkanoates, biopolyesters from renewable resources: physiological and engineering aspects. *Journal of Biotechnology*, 65 (2): 127-161.

Brauns, F. E. 1952. The chemistry of lignin. Academic Press, New York: 151–177.

Bugnicourt, E., Cinelli, P., Lazzeri, A. and Alvarez, V. A. 2014. Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging. *eXPRESS Polymer Letters,* 8 (11): 791–808.

Cavalheiro, J. M., de Almeida, M. C. M., Grandfils, C. and Da Fonseca, M. 2009. Poly (3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. *Process Biochemistry*, 44 (5): 509-515.

Chanprateep, S. 2010. Current trends in biodegradable polyhydroxyalkanoates. *Journal of Bioscience and Bioengineering*, 110 (6): 621-632.

Charen, T., Vaishali, P., Kaushalya, M., Amutha, K., Ponnusami, V. and Gowdhaman, D. 2014. Isolation and identification of Polyhydroxybutyrate producing bacterial strain (*Bacillus thuringiensis* GVP) from chlorine contaminated soil. *International Journal of ChemTech Research*, 5: 3197-3202.

Chee, J.-Y., Tan, Y., Samian, M.-R. and Sudesh, K. 2010a. Isolation and characterization of a *Burkholderia* sp. USM (JCM15050) capable of producing polyhydroxyalkanoate (PHA) from triglycerides, fatty acids and glycerols. *Journal of Polymers and the Environment*, 18 (4): 584-592.

Chee, J.-Y., Yoga, S.-S., Lau, N.-S., Ling, S.-C., Abed, R. M. and Sudesh, K. 2010b. Bacterially produced polyhydroxyalkanoate (PHA): converting renewable resources into bioplastics. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, 2: 1395-1404.

Cheeke, P. R. 1991. *Applied animal nutrition feeds and feeding. Department of Animal Science Oregon State University*: Prentice Hall. Englewood Cliffs, NJ.

Chemists, A. A. and Horwitz, W. 1990. Official methods of analysis. *Vol. I. 15th ed. AOAC, Arlington,VA*.Available: https://law.resource.org/pub/us/cfr/ibr/002/aoac.methods.1.1990.pdf.

Chen, G.-Q. 2010. Plastics completely synthesized by bacteria: polyhydroxyalkanoates. In: *Plastics from Bacteria*. Springer, 17-37.

Cherubini, F. J. E. c. and management. 2010. The biorefinery concept: using biomass instead of oil for producing energy and chemicals. *Energy Conversion and Management,* 51 (7): 1412-1421.

Chou, K.-S., Chang-Ho, H. W. R. and Goodrich, P. R. 1997. Poly (hydroxybutyrate-co-hydroxy-valerate) from swine waste liquor by *Azotobacter vinelandii* UWD. *Biotechnology Letters*, 19 (1): 7-10.

Chua, A. S., Takabatake, H., Satoh, H. and Mino, T. 2003. Production of polyhydroxyalkanoates (PHA) by activated sludge treating municipal wastewater: effect of pH, sludge retention time (SRT), and acetate concentration in influent. *Water Research*, 37 (15): 3602-3611.

DAFF. 2008. Maize. Available: https://www.google.co.za/search?q=.+Livestock+uses+both+white+and+yellow+maize+to+fed+livestock+as+hay+or+corn+silage+while+yellow+maize+contributes+significantly+to+the+production+of+white+and+red+meats+and+dairy+products&rlz=1C1CHFX_enZA823ZA823&oq=.+Livestock+uses+both+white+and+yellow+maize+to+fed+livestock+as+hay+or+corn+silage+while+yellow+maize+contributes+significantly+to+the+production+of+white+and+red+meats+and+dairy+products&aqs=chrome..69i57.2869j0j8&sourceid=chrome&ie=UTF-8 (Accessed 15/10/2017).

DAFF. 2015/2016. A Profile of South African Maize Market Value Chain. Available: https://www.nda.agric.za/doaDev/sideMenu/Marketing/Annual%20Publications/Commodity%20Profiles/field%20crops/Maize%20Market%20value%20Chain%20Profile%202016.pdf (Accessed 06/01/2019).

DAFF. 2016. Trends in the Agricultural Sector. Available: https://www.google.co.za/search?rlz=1C1CHFX_enZA823ZA823&ei=miX5W6ikPI6flwTuyozQDw&q=Trends+in+the+Agricultural+Sector+2016&oq=Trends+in+the+Agricultural+Sector+2016&gs_l=psy-ab.12..0i71l8.554766.554766..557911...0.0..0.0.0.....0....1j2..gws-wiz.k6LkAOaF3Y0 (Accessed 06/11/2017).

DAFF. 2017. Trens in Agricultural Sector. Available: https://www.google.co.za/search?q=Trens+in+Agricultural+Sector+2017&rlz=1C1CHFX_enZA823ZA823&oq=Trens+in+Agricultural+Sector+2017&aqs=chrome..69i57.5566j0j8&sourceid=chrome&ie=UTF-8 (Accessed 05/10/2017).

Danon, M., Franke-Whittle, I. H., Insam, H., Chen, Y. and Hadar, Y. 2008. Molecular analysis of bacterial community succession during prolonged compost curing. *FEMS Microbiology Ecology,* 65 (1): 133-144.

Dawes, E. and Senior, P. 1973. The role and regulation of energy reserve polymers in microorganisms polyhydroxybutyrate. *Advances in Microbial Physiology*. 10:135-266.

de Rijk, T. C., van de Meer, P., Eggink, G. and Weusthuis, R. A. 2005. Methods for Analysis of Poly (3-hydroxyalkanoate)(PHA) Composition. *Biopolymers Online: Biology Chemistry Biotechnology Applications*, 3: doi.org/10.1002/3527600035.bpol3b01.

Demirbas, A. 2008. Importance of biomass energy sources for Turkey. *Energy Policy*, 36 (2): 834-842.

Díaz, G. C., Tapanes, N. d. l. C. O., Câmara, L. D. T. and Aranda, D. A. J. R. E. 2014. Glycerol conversion in the experimental study of catalytic hydrolysis of triglycerides for fatty acids production using Ni or Pd on Al2O3 or SiO2. Renewable Energy, 64: 113-122.

Doi, Y. 1990. Microbial Polyesters, VCH. *New York*: 156

Elain, A., Le Grand, A., Corre, Y.-M., Le Fellic, M., Hachet, N., Le Tilly, V., Loulergue, P., Audic, J.-L. and Bruzaud, S. 2016. Valorisation of local agro-industrial processing waters as growth media for polyhydroxyalkanoates (PHA) production. *Industrial Crops and Products*, 80: 1-5.

Euken, R. 2018. Corn Silage and Earlage Nutrient Analysis. Animal Industry Report, 664 (1): 16.

Fernández, D., Rodríguez, E., Bassas, M., Viñas, M., Solanas, A., Llorens, J., Marqués, A. and Manresa, A. 2005. Agro-industrial oily wastes as substrates for PHA production by the new strain *Pseudomonas aeruginosa* NCIB 40045: Effect of culture conditions. *Biochemical Engineering Journal*, 26 (2): 159-167.

Findlay, R. H. and White, D. C. 1983. Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*. *Applied and Environmental Microbiology*, 45 (1): 71-78.

Flores-Sánchez, A., López-Cuellar, M., Pérez-Guevara, F., Figueroa López, U., Martín-Bufájer, J. M. and Vergara-Porras, B. 2017. Synthesis of Poly-(R-hydroxyalkanoates) by *Cupriavidus necator* ATCC 17699 Using Mexican Avocado (Persea americana) Oil as a Carbon Source. *International Journal of Polymer Science*: 10. Doi.org/10.1155/2017/6942950.

Forsyth, W., Hayward, A. and Roberts, J. 1958. Occurrence of poly-β-hydroxybutyric acid in aerobic gram-negative bacteria. *Nature*, 182 (4638): 800-801.

Fukui, T., Shiomi, N. and Doi, Y. 1998. Expression and characterization of (R)-specific enoyl coenzyme A hydratase involved in polyhydroxyalkanoate biosynthesis by *Aeromonas caviae*. *Journal of Bacteriology*, 180 (3): 667-673.

Gaj, R., Budka, A. and Przybyl, J. 2015. Effect of different tillage systems on the macronutrient content and uptake in sugar beets. *Journal of Elementology*, 20 (4): 839-853.

Gerngross, T., Reilly, P., Stubbe, J., Sinskey, A. and Peoples, O. 1993. Immunocytochemical analysis of poly-beta-hydroxybutyrate (PHB) synthase in *Alcaligenes eutrophus* H16: localization of the synthase enzyme at the surface of PHB granules. *Journal of Bacteriology*, 175 (16): 5289-5293.

Gerngross, T., Snell, K., Peoples, O., Sinskey, A., CSuhai, E., Masamune, S. and Stubbe, J. 1994. Overexpression and purification of the soluble polyhydroxyalkanoate synthase from *Alcaligenes eutrophus*: evidence for a required posttranslational modification for catalytic activity. *Biochemistry*, 33 (31): 9311-9320.

Getachew, A. and Woldesenbet, F. 2016. Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low cost agricultural waste material. *BMC Research Notes*, 9 (1): 509.

Gobi, K. and Vadivelu, V. 2014. Aerobic dynamic feeding as a strategy for in situ accumulation of polyhydroxyalkanoate in aerobic granules. *Bioresource Technology*, 161: 441-445.

Godbole, S. 2016. Methods for identification, quantification and characterization of polyhydroxyalkanoates. *International Journal of Bioassays*, 5 (4): 4977-4983.

Gowda, V. and Shivakumar, S. 2014. Agrowaste-based Polyhydroxyalkanoate (PHA) production using hydrolytic potential of *Bacillus thuringiensis* IAM 12077. *Brazilian Archives of Biology and Technology*, 57 (1): 55-61.

Grohmann, K., Himmel, M., Rivard, C., Tucker, M., Baker, J., Torget, R. and Graboski, M. 1984. Chemical-mechanical methods for the enhanced utilization of straw. In: Proceedings of *Proceedings, Sixth Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, Tennessee, USA*, May 15-18, 1984. John Wiley, 137-157.

Gumel, A. M., Annuar, M. S. M. and Heidelberg, T. 2012. Biosynthesis and characterization of polyhydroxyalkanoates copolymers produced by *Pseudomonas putida* Bet001 isolated from palm oil mill effluent. *PLoS One*, 7 (9): e45214.

Haas, R., Jin, B. and Zepf, F. T. 2008. Production of poly (3-hydroxybutyrate) from waste potato starch. *Bioscience, Biotechnology, and Biochemistry*, 72 (1): 253-256.

Hadar, Y. 2013. Sources for lignocellulosic raw materials for the production of ethanol. In: *Lignocellulose conversion*. Springer, 21-38.

Hahn, J., Eschenlauer, A. C., Sleytr, U. B., Somers, D. A. and Srienc, F. 1999. Peroxisomes as sites for synthesis of polyhydroxyalkanoates in transgenic plants. *Biotechnology Progress*, 15 (6): 1053-1057.

Halami, P. M. 2008. Production of polyhydroxyalkanoate from starch by the native isolate *Bacillus cereus* CFR06. *World Journal of Microbiology and Biotechnology*, 24 (6): 805-812.

Hall, D. and Scrase, J. 1998. Will biomass be the environmentally friendly fuel of the future. *Biomass and Bioenergy*, 15 (4): 357-367.

Hall, D. O., Rosillo-Calle, F., Williams, R. H. and Woods, J. 1993. Biomass for energy: supply prospects. King's College, Strand, London WC2R 2LS, UK: 593-651

Harmsen, P., Huijgen, W., Bermudez, L. and Bakker, R. 2010. Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. Wageningen UR Food & Biobased Research.

Hashimoto, K., Tsuboi, H., Iwasaki, S. and Shirai, Y. 1993. Effect of pH on the Production of Poly-β-Hydroxybutylate by Photosynthetic Bacteria, *Rhodospirillum rubrum*. *Journal of Chemical Engineering of Japan*, 26 (1): 56-58.

Hassan, M. A., Shirai, Y., Kubota, A., Karim, M. I. A., Nakanishi, K. and Hashimoto, K. 1998. Effect of oligosaccharides on glucose consumption by *Rhodobacter sphaeroides* in polyhydroxyalkanoate production from enzymatically treated crude sago starch. *Journal of Fermentation and Bioengineering*, 86 (1): 57-61.

Hassan, M. A., Shirai, Y., Kusubayashi, N., Karim, M. I. A., Nakanishi, K. and Hashimoto, K. 1996. Effect of organic acid profiles during anaerobic treatment of palm oil mill effluent on the production of polyhydroxyalkanoates by *Rhodobacter sphaeroides*. *Journal of Fermentation and Bioengineering*, 82 (2): 151-156.

Hassan, M. A., Shirai, Y., Kusubayashi, N., Karim, M. I. A., Nakanishi, K. and Hasimoto, K. 1997. The production of polyhydroxyalkanoate from anaerobically treated palm oil mill effluent by *Rhodobacter sphaeroides*. *Journal of Fermentation and Bioengineering*, 83 (5): 485-488.

Hatfield, R. and Fukushima, R. S. 2005. Can lignin be accurately measured? *Crop Science*, 45 (3): 832-839.

Haywood, G. W., Anderson, A. J., Williams, D. R., Dawes, E. A. and Ewing, D. F. 1991. Accumulation of a poly (hydroxyalkanoate) copolymer containing primarily 3-hydroxyvalerate from simple carbohydrate substrates by *Rhodococcus* sp. NCIMB 40126. *International Journal of Biological Macromolecules*, 13 (2): 83-88.

He, W. and Hu, H. 2013a. Prediction of hot-water-soluble extractive, pentosan and cellulose content of various wood species using FT-NIR spectroscopy. *Bioresource Technology*, 140: 299-305.

He, W. and Hu, H. 2013b. Rapid prediction of different wood species extractives and lignin content using near infrared spectroscopy. *Journal of Wood Chemistry and Technology*, 33 (1): 52-64.

He, W., Zhang, Z., Hu, P. and Chen, C. 1999. Microbial synthesis and characterization of polyhydroxyalkanoates by DG17 from glucose. *Acta Polymerica Sinica*, 6: 010.

Horwitz, W., Chichilo, P. and Reynolds, H. 1970. Official methods of analysis of the Association of Official Analytical Chemists. 11th edition. Association of Official Analytical Chemists, USA.

Htet, M. N. S., Than, N. N., Soomro, R. N., Ya-Dong, X. and Jiang-Bo, H. 2016. Comparison of Nutrients Composition, Forage and Silage Yields of Maize (Zea mays L). *Scholars Journal of Agriculture and Veterinary Sciences*, 3: 474-479.

Huijberts, G. N., van der Wal, H., Wilkinson, C. and Eggink, G. 1994. Gas-chromatographic analysis of poly (3-hydroxyalkanoates) in bacteria. *Biotechnology Techniques*, 8 (3): 187-192.

Jain, R. and Tiwari, A. 2015. Biosynthesis of planet friendly bioplastics using renewable carbon source. *Journal of Environmental Health Science and Engineering*, 13 (1): 11.

James, B. W., Mauchline, W. S., Dennis, P. J., Keevil, C. W. and Wait, R. 1999. Poly-3-hydroxybutyrate in *Legionella pneumophila*, an energy source for survival in low-nutrient environments. *Applied and Environmental Microbiology*, 65 (2): 822-827.

Jan, S., Roblot, C., Goethals, G., Courtois, J., Courtois, B., Saucedo, J. N., Seguin, J.-P. and Barbotin, J.-N. 1995. Study of parameters affecting poly (3-hydroxybutyrate) quantification by gas chromatography. *Analytical Biochemistry*, 225 (2): 258-263.

Jau, M.-H., Yew, S.-P., Toh, P. S., Chong, A. S., Chu, W.-L., Phang, S.-M., Najimudin, N. and Sudesh, K. 2005. Biosynthesis and mobilization of poly (3-hydroxybutyrate)[P (3HB)] by *Spirulina platensis*. *International Journal of Biological Macromolecules*, 36 (3): 144-151.

Jendrossek, D. 2005a. Extracellular polyhydroxyalkanoate (PHA) depolymerases: the key enzymes of PHA degradation. *Biopolymers Online: Biology• Chemistry• Biotechnology• Applications*, 3. Doi.org/10.1002/3527600035.bpol3a06.

Jendrossek, D. 2005b. Fluorescence microscopical investigation of poly (3-hydroxybutyrate) granule formation in bacteria. *Biomacromolecules*, 6 (2): 598-603.

Jendrossek, D. 2009. Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes). *Journal of Bacteriology*, 191 (10): 3195-3202.

Jendrossek, D. and Handrick, R. 2002. Microbial degradation of polyhydroxyalkanoates. *Annual Review of Microbiology*, 56 (1): 403-432.

Jensen, T. E. and Sicko, L. M. 1971. Fine structure of poly-β-hydroxybutyric acid granules in a blue-green alga, *Chlorogloea fritschii*. *Journal of Bacteriology*, 106 (2): 683-686.

Jia, Q., Xiong, H., Wang, H., Shi, H., Sheng, X., Sun, R. and Chen, G. 2014. Production of polyhydroxyalkanoates (PHA) by bacterial consortium from excess sludge fermentation liquid at laboratory and pilot scales. *Bioresource Technology*, 171: 159-167.

Jia, Y., Kappock, T. J., Frick, T., Sinskey, A. J. and Stubbe, J. 2000. Lipases provide a new mechanistic model for polyhydroxybutyrate (PHB) synthases: characterization of the functional residues in *Chromatium vinosum* PHB synthase. *Biochemistry*, 39 (14): 3927-3936.

Jiang, X. 2010. Process development for the production and separation of medium-chain-length poly (3-hydroxyalkanoates) by *Pseudomonas putida* KT2440. Queen's University (Canada).

Jung, H. J. G. 2012. Forage digestibility: the intersection of cell wall lignification and plant tissue anatomy. In: Proceedings of the 23rd annual Florida ruminant nutrition symposium: 162-174.

Kahar, P., Tsuge, T., Taguchi, K. and Doi, Y. 2004. High yield production of polyhydroxyalkanoates from soybean oil by *Ralstonia eutropha* and its recombinant strain. *Polymer Degradation and Stability*, 83 (1): 79-86.

Kai, Z., Ying, D. and Guo-Qiang, C. 2003. Effects of surface morphology on the biocompatibility of polyhydroxyalkanoates. *Biochemical Engineering Journal*, 16 (2): 115-123.

Kallio, R. and Harrington, A. A. 1960. Sudanophilic granules and lipid of *Pseudomonas methanica*. *Journal of Bacteriology*, 80 (3): 321.

Kallistova, A. Y., Pimenov, N., Kozlov, M., Nikolaev, Y. A., Dorofeev, A., Aseeva, V., Grachev, V., Men'ko, E., Berestovskaya, Y. Y. and Nozhevnikova, A. 2014. Microbial composition of the activated sludge of Moscow wastewater treatment plants. *Microbiology*, 83 (5): 699-708.

Kawas, J., Jorgensen, N. and Danelon, J. 1991. Fiber requirements of dairy cows: optimum fiber level in lucerne-based diets for high producing cows. *Livestock Production Science*, 28 (2): 107-119.

Kemnitzer, J. E., McCarthy, S. P. and Gross, R. A. 1993. Preparation of predominantly syndiotactic poly (.beta.-hydroxybutyrate) by the tributyltin methoxide catalyzed ring-opening polymerization of racemic. beta.-butyrolactone. *Macromolecules*, 26 (6): 1221-1229.

Keshavarz, T. and Roy, I. 2010. Polyhydroxyalkanoates: bioplastics with a green agenda. *Current Opinion in Microbiology*, 13 (3): 321-326.

Kessler, B., Weusthuis, R., Witholt, B. and Eggink, G. 2001. Production of microbial polyesters: fermentation and downstream processes. In: *Biopolyesters*. Springer, 159-182.

Kessler, B. and Witholt, B. 2001. Factors involved in the regulatory network of polyhydroxyalkanoate metabolism. *Journal of Biotechnology*, 86 (2): 97-104.

Kezar, W. W. 2001. Successful use of high quality corn silage for dairies in the western United States. In: Proceedings of *Proceedings, 31st California Alfalfa and forage Symposium*: 12-13.

Khanna, S. and Srivastava, A. K. 2005. Recent advances in microbial polyhydroxyalkanoates. *Process Biochemistry*, 40 (2): 607-619.

Koller, M., Bona, R., Braunegg, G., Hermann, C., Horvat, P., Kroutil, M., Martinz, J., Neto, J., Pereira, L. and Varila, P. 2005. Production of polyhydroxyalkanoates from agricultural waste and surplus materials. *Biomacromolecules*, 6 (2): 561-565.

Koning, G. D. 1995. Physical properties of bacterial poly ((R)-3-hydroxyalkanoates). *Canadian Journal of Microbiology*, 41 (13): 303-309.

Kourmentza, C., Ntaikou, I., Lyberatos, G. and Kornaros, M. 2015. Polyhydroxyalkanoates from *Pseudomonas* sp. using synthetic and olive mill wastewater under limiting conditions. *International Journal of Biological Macromolecules*, 74: 202-210.

Koutinas, A. A., Xu, Y., Wang, R. and Webb, C. 2007. Polyhydroxybutyrate production from a novel feedstock derived from a wheat-based biorefinery. *Enzyme and Microbial Technology*, 40 (5): 1035-1044.

Kuntanoo, K., Promkotra, S. and Kaewkannetra, P. 2013. Biodegradation of polyhydroxybutyrate-co-hydroxyvalerate (PHBV) blended with natural rubber in soil environment. *International Science Index*, 7: 12.

Lande, S., van Riel, S., Høibø, O. A. and Schneider, M. H. 2010. Development of chemometric models based on near infrared spectroscopy and thermogravimetric analysis for predicting the treatment level of furfurylated Scots pine. *Wood Science and Technology*, 44 (2): 189-203.

Langenbach, S., Rehm, B. H. and Steinbüchel, A. 1997. Functional expression of the PHA synthase gene *phaC1* from *Pseudomonas aeruginosa* in *Escherichia coli* results in poly (3-hydroxyalkanoate) synthesis. *FEMS Microbiology Letters*, 150 (2): 303-309.

Lapierre, C., Jouin, D. and Monties, B. 1989. Lignin characterization of wheat straw samples as determined by chemical degradation procedures. In: *Physico-Chemical Characterisation of Plant Residues for Industrial and Feed Use*. Springer: 118-130.

Lauzier, C. A., Monasterios, C. J., Saracovan, I., Marchessault, R. and Ramsay, B. A. J. T. J. 1993. Film formation and paper coating with poly ([beta]-hydroxyalkanoate), a biodegradable latex. *Tappi Journal*, 76 (5)

Lee, S. Y. 1996. Bacterial polyhydroxyalkanoates. *Biotechnology and Bioengineering*, 49 (1): 1-14.

Lee, W.-H., Loo, C.-Y., Nomura, C. T. and Sudesh, K. 2008. Biosynthesis of polyhydroxyalkanoate copolymers from mixtures of plant oils and 3-hydroxyvalerate precursors. *Bioresource Technology*, 99 (15): 6844-6851.

Legat, A., Gruber, C., Zangger, K., Wanner, G. and Stan-Lotter, H. 2010. Identification of polyhydroxyalkanoates in *Halococcus* and other haloarchaeal species. *Applied Microbiology and Biotechnology*, 87 (3): 1119-1127.

Lemoigne, M. 1926. Products of dehydration and of polymerization of β-hydroxybutyric acid. *Bulletin of the Society for Biological Chemistry*, 8: 770-782.

Li, X., Sun, C., Zhou, B. and He, Y. 2015. Determination of hemicellulose, cellulose and lignin in moso bamboo by near infrared spectroscopy. *Scientific Reports*, 5: 17210.

Liebergesell, M., Sonomoto, K., Madkour, M., Mayer, F. and Steinbüchel, A. 1994. Purification and characterization of the poly (hydroxyalkanoic acid) synthase from *Chromatium vinosum* and localization of the enzyme at the surface of poly (hydroxyalkanoic acid) granules. *The FEBS Journal*, 226 (1): 71-80.

Lim, S.-P., Gan, S.-N. and Tan, I. K. 2005. Degradation of medium-chain-length polyhydroxyalkanoates in tropical forest and mangrove soils. *Applied Biochemistry and Biotechnology*, 126 (1): 23-33.

Loo, C.-Y., Lee, W.-H., Tsuge, T., Doi, Y. and Sudesh, K. 2005. Biosynthesis and characterization of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) from palm oil products in a *Wautersia eutropha* mutant. *Biotechnology letters*, 27 (18): 1405-1410.

Lu, F. and Ralph, J. 1997. Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: protocol for analysis of DFRC monomers. *Journal of Agricultural and Food Chemistry*, 45 (7): 2590-2592.

Macrae, R. and Wilkinson, J. 1958. Poly-β-hyroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. *Microbiology*, 19 (1): 210-222.

Madison, L. L. and Huisman, G. W. 1999. Metabolic engineering of poly (3-hydroxyalkanoates): from DNA to plastic. *Microbiology and Molecular Biology Reviews*, 63 (1): 21-53.

Marais, J., De Wit, J. and Quicke, G. J. A. B. 1966. A critical examination of the Nelson-Somogyi method for the determination of reducing sugars. *Analytical Biochemistry*, 15 (3): 373-381.

Marsudi, S., Unno, H. and Hori, K. 2008. Palm oil utilization for the simultaneous production of polyhydroxyalkanoates and rhamnolipids by *Pseudomonas aeruginosa*. *Applied Microbiology and Biotechnology*, 78 (6): 955-961.

Martens, D. A. 2000. Plant residue biochemistry regulates soil carbon cycling and carbon sequestration. *Soil Biology and Biochemistry*, 32 (3): 361-369.

Martens, D. A. and Loeffelmann, K. L. 2002. Improved accounting of carbohydrate carbon from plants and soils. *Soil Biology and Biochemistry*, 34 (10): 1393-1399.

Martin, N. P., Mertens, D. R., Hall, M. B. and Lauer, J. G. 2008. Fiber digestibility and starch content of corn silage. In: Proceedings of *Idaho Alfalfa and Forage Conference. Idaho*: 26-27. Available: https://pdfs.semanticscholar.org/c7ed/b8e9ec8a01b25813fdb94bc9bb9848537851.pdf.

Martinez, G. A., Bertin, L., Scoma, A., Rebecchi, S., Braunegg, G. and Fava, F. 2015. Production of polyhydroxyalkanoates from dephenolised and fermented olive mill wastewaters by employing a pure culture of *Cupriavidus necator*. *Biochemical Engineering Journal*, 97: 92-100.

Masood, F., Hasan, F., Ahmed, S. and Hameed, A. 2012. Biosynthesis and characterization of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) from *Bacillus cereus* FA11 isolated from TNT-contaminated soil. *Annals of Microbiology*, 62 (4): 1377-1384.

Matsusaki, H., Abe, H. and Doi, Y. 2000. Biosynthesis and properties of poly (3-hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant strains of *Pseudomonas* sp. 61-3. *Biomacromolecules*, 1 (1): 17-22.

Mayeli, N., Motamedi, H. and Heidarizadeh, F. 2015. Production of polyhydroxybutyrate by *Bacillus axaraqunsis* BIPC01 using petrochemical wastewater as carbon source. *Brazilian Archives of Biology and Technology*, 58 (4): 643-650.

McLafferty, F. W., Tureček, F. and Turecek, F. 1993. *Interpretation of mass spectra*. University science books.

Menon, V. and Rao, M. 2012. Trends in bioconversion of lignocellulose: biofuels, platform chemicals & biorefinery concept. *Progress in Energy and Combustion Science*, 38 (4): 522-550.

Mesquita, D. P., Amaral, A. L., Leal, C., Oehmen, A., Reis, M. A. and Ferreira, E. C. 2015. Polyhydroxyalkanoate granules quantification in mixed microbial cultures using image analysis: Sudan Black B versus Nile Blue A staining. *Analytica Chimica Acta*, 865: 8-15.

Mishra, S. and Rai, T. 2006. Morphology and functional properties of corn, potato and tapioca starches. *Food Hydrocolloids*, 20 (5): 557-566.

Misra, A., Thakur, M., Srinivas, P. and Karanth, N. 2000. Screening of poly-β-hydroxybutyrate-producing microorganisms using Fourier transform infrared spectroscopy. *Biotechnology Letters*, 22 (15): 1217-1219.

Mkhize, T. 2016. Selective extraction of lignin from lignocellulosic biomas using ionic liquids. Available: http://hdl.handle.net/10321/1756.

Mogala, M. 2012. *Maize Market Value Chain Profile*. Available:https://www.google.co.za/search?q=Maize+Market+Value+Chain+Profiel&rlz=1C1CHFX_enZA823ZA823&oq=Maize+Market+Value+Chain+Profiel&aqs=chrome..69i57.4666j0j8&sourceid=chrome&ie=UTF-8 (Accessed 21/10/2017).

Montanheiro, T. L. d. A., Passador, F. R., Oliveira, M. P. d., Durán, N. and Lemes, A. P. 2016. Preparation and characterization of maleic anhydride grafted poly(Hydroxybutirate-co-Hydroxyvalerate)–PHBV-g-MA. *Materials Research*, 19 (1): 229-235.

Moore, J. C., DeVries, J. W., Lipp, M., Griffiths, J. C., Abernethy, D. R. and Safety, F. 2010. Total protein methods and their potential utility to reduce the risk of food protein adulteration. *Comprehensive Reviews in Food Science and Food Safety*, 9 (4): 330-357.

Moore, K. J. and Jung, H.-J. G. 2001. Lignin and fiber digestion. *Journal of Range Management*: 420-430.

Morgan-Sagastume, F., Hjort, M., Cirne, D., Gérardin, F., Lacroix, S., Gaval, G., Karabegovic, L., Alexandersson, T., Johansson, P. and Karlsson, A. 2015. Integrated production of polyhydroxyalkanoates (PHAs) with municipal wastewater and sludge treatment at pilot scale. *Bioresource Technology*, 181: 78-89.

Müh, U., Sinskey, A. J., Kirby, D. P., Lane, W. S. and Stubbe, J. 1999. PHA synthase from Chromatium vinosum: cysteine 149 is involved in covalent catalysis*. Biochemistry, 38 (2): 826-837.*

Müller, R.-J., Kleeberg, I. and Deckwer, W.-D. 2001. Biodegradation of polyesters containing aromatic constituents. *Journal of Biotechnology*, 86 (2): 87-95.

Mumme, J., Eckervogt, L., Pielert, J., Diakité, M., Rupp, F. and Kern, J. J. 2011. Hydrothermal carbonization of anaerobically digested maize silage. *Bioresource Technology*, 102 (19): 9255-9260.

Mumtaz, T., Abd-Aziz, S., Yee, P. L., Yunus, W. M. Z. W., Shirai, Y. and Hassan, M. A. 2010. Synthesis, characterization, and structural properties of intracellular copolyester poly (3-hydroxybutyrate-co-3-hydroxyvalerate) produced by *Comamonas* sp. EB 172 from renewable resource. *International Journal of Polymer Analysis and Characterization*, 15 (6): 329-340.

Munir, S. and Jamil, N. 2015. Characterization of polyhydroxyalkanoates produced by contaminated soil bacteria using wastewater and glucose as carbon sources. *Tropical Journal of Pharmaceutical Research*, 14 (9): 1605-1611.

Mussatto, S. I. and Teixeira, J. 2010. Lignocellulose as raw material in fermentation processes. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology (Méndez-Vilas, A., Ed.)*, 2: 897-907.

Nath, A., Dixit, M., Bandiya, A., Chavda, S. and Desai, A. 2008. Enhanced PHB production and scale up studies using cheese whey in fed batch culture of *Methylobacterium* sp. ZP24. *Bioresource Technology*, 99 (13): 5749-5755.

Nawrath, C., Poirier, Y. and Somerville, C. 1994. Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. *Proceedings of the National Academy of Sciences*, 91 (26): 12760-12764.

Nelson, L. and Leming, J. 1957. Evaluation of monoethanolamine method of cellulose determination for agricultural residues. *Tappi*, 40 (10): 846-850.

Nielsen, C., Rahman, A., Rehman, A. U., Walsh, M. K. and Miller, C. D. 2017. Food waste conversion to microbial polyhydroxyalkanoates. *Microbial Biotechnology*, 10 (6): 1338-1352.

Ojumu, T., Yu, J. and Solomon, B. 2004. Production of polyhydroxyalkanoates, a bacterial biodegradable polymers. *African Journal of Biotechnology*, 3 (1): 18-24.

Oliveira, F., Freire, D. and Castilho, L. 2004. Production of poly (3-hydroxybutyrate) by solid-state fermentation with *Ralstonia eutropha*. *Biotechnology Letters*, 26 (24): 1851-1855.

Omar, S., Rayes, A., Eqaab, A., Voß, I. and Steinbüchel, A. 2001. Optimization of cell growth and poly (3-hydroxybutyrate) accumulation on date syrup by a *Bacillus megaterium* strain. *Biotechnology Letters*, 23 (14): 1119-1123.

Osborne, B. G. and Fearn, T. 2000. Near-infrared spectroscopy in food analysis. *Encyclopedia of Analytical Chemistry*, 5: 4069-4082.

Ouyang, S.-P., Luo, R. C., Chen, S.-S., Liu, Q., Chung, A., Wu, Q. and Chen, G.-Q. 2007. Production of polyhydroxyalkanoates with high 3-hydroxydodecanoate monomer content by *fadB* and *fadA* knockout mutant of *Pseudomonas putida* KT2442. *Biomacromolecules*, 8 (8): 2504-2511.

Page, W. J. 1992. Production of polyhydroxyalkanoates by *Azotobacter vinelandii* UWD in beet molasses culture. *FEMS Microbiology Letters*, 103 (2-4): 149-157.

Palmeri, R., Pappalardo, F., Fragalà, M., Tomasello, M., Damigella, A. and Catara, A. F. 2012. Polyhydroxyalkanoates (PHAs) production through conversion of glycerol by selected strains of *Pseudomonas mediterranea* and *Pseudomonas corrugata*. *Chemical Engineering*, 27: 121-126.

Pantazaki, A. A., Papaneophytou, C. P., Pritsa, A. G., Liakopoulou-Kyriakides, M. and Kyriakidis, D. A. 2009. Production of polyhydroxyalkanoates from whey by *Thermus thermophilus* HB8. *Process Biochemistry*, 44 (8): 847-853.

Philip, S., Keshavarz, T., Roy, I. 2007. Polyhydroxyalkanoates: biodegradable polymers with a range of applications. *Journal of Chemical Technology and Biotechnology,* 82 (3): 233-247.

Poirier, Y., Erard, N. and Petétot, J. M.-C. 2001. Synthesis of polyhydroxyalkanoate in the peroxisome of *Saccharomyces cerevisiae* by using intermediates of fatty acid β-oxidation. *Applied and Environmental Microbiology*, 67 (11): 5254-5260.

Qi, Q. and Rehm, B. H. 2001. Polyhydroxybutyrate biosynthesis in *Caulobacter crescentus*: molecular characterization of the polyhydroxybutyrate synthase. *Microbiology*, 147 (12): 3353-3358.

Qi, Q., Steinbüchel, A. and Rehm, B. 2000. In *vitro* synthesis of poly (3-hydroxydecanoate): purification and enzymatic characterization of type II polyhydroxyalkanoate synthases *PhaC1* and *PhaC2* from *Pseudomonas aeruginosa*. *Applied Microbiology and Biotechnology*, 54 (1): 37-43.

Qi, Q., Steinbüchel, A. and Rehm, B. H. 1998. Metabolic routing towards polyhydroxyalkanoic acid synthesis in recombinant *Escherichia coli* (*fadR*): inhibition of fatty acid β-oxidation by acrylic acid. *FEMS Microbiology Letters*, 167 (1): 89-94.

Rasmussen, T. S., Henry, R. J. 1990. Starch determination in horticultural plant material by an enzymic-colorimetric procedure. *Journal of the Science of Food and Agriculture*, 52 (2): 159-170.

Reddy, C., Ghai, R. and Kalia, V. C. 2003. Polyhydroxyalkanoates: an overview. *Bioresource Technology*, 87 (2): 137-146.

Rehm, B. H. 2007. Biogenesis of microbial polyhydroxyalkanoate granules: a platform technology for the production of tailor-made bioparticles. *Current Issues in Molecular Biology*, 9 (1): 41.

Rehm, B. H. and Steinbüchel, A. 1999. Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. *International Journal of Biological Macromolecules*, 25 (1): 3-19.

Rehm, B. H. and Steinbüchel, A. 2001. Heterologous expression of the acyl–acyl carrier protein thioesterase gene from the plant *Umbellularia californica* mediates polyhydroxyalkanoate biosynthesis in recombinant *Escherichia coli*. *Applied Microbiology and Biotechnology*, 55 (2): 205-209.

Ribera, R. G., Monteoliva-Sanchez, M. and Ramos-Cormenzana, A. 2001. Production of polyhidroxyalkanoates by *Pseudomonas putida* KT2442 harboring pSK2665 in wastewater from olive oil mills (alpechin). *Electronic Journal of Biotechnology*, 4 (2): 11-12.

Riis, V. and Mai, W. 1988. Gas chromatographic determination of poly-β-hydroxybutyric acid in microbial biomass after hydrochloric acid propanolysis. *Journal of Chromatography A*, 445: 285-289.

Rivera-Briso, A. and Serrano-Aroca, Á. 2018. Poly (3-Hydroxybutyrate-co-3-Hydroxyvalerate): Enhancement strategies for advanced applications. *Polymers*, 10 (7): 732.

Robledo, A., Aguilar, C. N., Belmares-Cerda, R. E., Flores-Gallegos, A. C., Contreras-Esquivel, J. C., Montañez, J. C. and Mussatto, S. I. 2016. Production of thermostable xylanase by thermophilic fungal strains isolated from maize silage. *CyTA-Journal of Food*, 14 (2): 302-308.

Rohini, D., Phadnis, S. and Rawal, S. 2006. Synthesis and characterization of poly-β-hydroxybutyrate from *Bacillus thuringiensis* R1. *Indian Journal of Biotechnology*. 5: 276-283.

Rusendi, D. and Sheppard, J. D. 1995. Hydrolysis of potato processing waste for the production of poly-β-hydroxybutyrate. *Bioresource Technology*, 54 (2): 191-196.

Sadegh-Hassani, F. and Nafchi, A. M. 2014. Preparation and characterization of bionanocomposite films based on potato starch/halloysite nanoclay. *International Journal of Biological Macromolecules*, 67: 458-462.

Santimano, M. C. 2015. Bacterial synthesis of polyhydroxyalkanoates using renewable resources. In: *Bioprospects of Coastal Eubacteria*. Springer: 163-177.

Sawant, S. S., Salunke, B. K. and Kim, B. S. 2014. A laboratory case study of efficient polyhydoxyalkonates production by *Bacillus cereus*, a contaminant in *Saccharophagus degradans* ATCC 43961 in minimal sea salt media. *Current Microbiology*, 69 (6): 832-838.

Sawant, S. S., Salunke, B. K. and Kim, B. S. 2015a. Degradation of corn stover by fungal cellulase cocktail for production of polyhydroxyalkanoates by moderate halophile *Paracoccus* sp. LL1. *Bioresource Technology*. 194: 247-255.

Sawant, S. S., Salunke, B. K. and Kim, B. S. 2015b. Degradation of corn stover by fungal cellulase cocktail for production of polyhydroxyalkanoates by moderate halophile *Paracoccus* sp. LL1. *Bioresource Technology*. 194: 247-255.

Saxena, A. and Tiwari, A. 2011. Polyhydroxyalkonates: green plastics of the future. *International Journal of Biomedical and Advance Research*, 2: 356-367.

Schubert, P., Krüger, N. and Steinbüchel, A. 1991. Molecular analysis of the *Alcaligenes eutrophus* poly (3-hydroxybutyrate) biosynthetic operon: identification of the N terminus of poly (3-hydroxybutyrate) synthase and identification of the promoter. *Journal of Bacteriology*, 173 (1): 168-175.

Scott, G. 2000. 'Green'polymers. *Polymer Degradation and Stability*, 68 (1): 1-7.

Selvakumar, K., Srinivasan, G., Baskar, V. and Madhan, R. 2011. Production and isolation of polyhydroxyalkanoates from *Haloarcula marismortui* MTCC 1596 using cost effective osmotic lysis methodology. *Europan Journal of Experimental Biology*, 1 (3): 180-187.

Shah, K. 2014. Optimization and production of Polyhydroxybutarate (PHB) by *Bacillus subtilis* G1S1 from soil. *International Journal of Current Microbiology and Applied Science*, 3 (5): 377-387.

Shen, L., Hu, H., Ji, H., Zhang, C., He, N., Li, Q. and Wang, Y. 2015. Production of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) from excess activated sludge as a promising substitute of pure culture. *Bioresource Technology*, 189: 236-242.

Simon-Colin, C., Raguénès, G., Crassous, P., Moppert, X. and Guezennec, J. 2008. A novel mcl-PHA produced on coprah oil by *Pseudomonas guezennei* biovar. tikehau, isolated from a "kopara" mat of French Polynesia. *International Journal of Biological Macromolecules*, 43 (2): 176-181.

Singh, G., Kumari, A., Mittal, A., Yadav, A. and Aggarwal, N. K. 2013. Poly β-hydroxybutyrate production by *Bacillus subtilis* NG220 using sugar industry waste water. *BioMed Research International*, 2013: 10.

Siracusa, V., Rocculi, P., Romani, S. and Dalla Rosa, M. 2008. Biodegradable polymers for food packaging: a review. *Trends in Food Science & Technology*, 19 (12): 634-643.

Southern African Grain laboratory Grain Building (SAGLGB). 2010/2011. *South African Maize Crop*.Available:https://www.google.co.za/search?rlz=1C1CHFX_enZA823ZA823&ei=pCn5W_rEKYyCacPjtvAD&q=South+African+Maize+Crop+2010%2F2011&oq=South+African+Maize+Crop+2010%2F2011&gs_l=psy-ab.12...12390.18592..20707...0.0..0.1812.12123.5-2j2j3j3.....0....1..gws-wiz.....0i71j0i22i30j33i160j33i22i29i30.Idf8kej5fEc (Accessed 14/11/2017).

Sridewi, N., Bhubalan, K. and Sudesh, K. 2006. Degradation of commercially important polyhydroxyalkanoates in tropical mangrove ecosystem. *Polymer Degradation and Stability*, 91 (12): 2931-2940.

Steinbüchel, A., Hustede, E., Liebergesell, M., Pieper, U., Timm, A. and Valentin, H. 1992. Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. *FEMS Microbiology Letters*, 103 (2-4): 217-230.

Steinbüchel, A. and Lütke-Eversloh, T. 2003. Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. *Biochemical Engineering Journal*, 16 (2): 81-96.

Sudesh, K., Abe, H. and Doi, Y. 2000. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Progress in Polymer Science*, 25 (10): 1503-1555.

Sun, B., Liu, J., Liu, S. and Yang, Q. 2011. Application of FT-NIR-DR and FT-IR-ATR spectroscopy to estimate the chemical composition of bamboo (Neosinocalamus affinis Keng). *Holzforschung*, 65 (5): 689-696.

Suriyamongkol, P., Weselake, R., Narine, S., Moloney, M. and Shah, S. 2007. Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants—a review. *Biotechnology Advances*, 25 (2): 148-175.

Tan, G.-Y., Chen, C.-L., Li, L., Ge, L., Wang, L., Razaad, I., Li, Y., Zhao, L., Mo, Y. and Wang, J.-Y. 2014. Start a research on biopolymer polyhydroxyalkanoate (PHA): a review. *Polymers*, 6 (3): 706-754.

Tasman, J. and Berzins, V. 1957. The permanganate consumption of pulp materials. *Tappi*, 40 (9): 691.

Templeton, D. W., Sluiter, A. D., Hayward, T. K., Hames, B. R. and Thomas, S. R. 2009. Assessing corn stover composition and sources of variability via NIRS. *Cellulose*, 16 (4): 621-639.

Thakor, N., Trivedi, U. and Patel, K. 2005. Biosynthesis of medium chain length poly (3-hydroxyalkanoates)(mcl-PHAs) by *Comamonas testosteroni* during cultivation on vegetable oils. *Bioresource Technology*, 96 (17): 1843-1850.

Tian, J., Sinskey, A. J. and Stubbe, J. 2005a. Detection of intermediates from the polymerization reaction catalyzed by a D302A mutant of class III polyhydroxyalkanoate (PHA) synthase. *Biochemistry*, 44 (5): 1495-1503.

Tian, J., Sinskey, A. J. and Stubbe, J. 2005b. Kinetic studies of polyhydroxybutyrate granule formation in *Wautersia eutropha* H16 by transmission electron microscopy. *Journal of Bacteriology*, 187 (11): 3814-3824.

Tripathi, A. D., Yadav, A., Jha, A. and Srivastava, S. 2012. Utilizing of sugar refinery waste (cane molasses) for production of bio-plastic under submerged fermentation process. *Journal of Polymers and the Environment*, 20 (2): 446-453.

UNEP. 2009. Converting waste agricultural biomass into a resource. Division of Technology, Industry and Economics International Environmental Technology Centre, Osaka/Shiga, Japan. Availabe: http://wedocs.unep.org/handle/20.500.11822/7614 (Accessed 10/04/2017).

Valappil, S. P., Peiris, D., Langley, G., Herniman, J., Boccaccini, A. R., Bucke, C. and Roy, I. 2007. Polyhydroxyalkanoate (PHA) biosynthesis from structurally unrelated carbon sources by a newly characterized *Bacillus* spp. *Journal of Biotechnology*, 127 (3): 475-487.

Van de Vyver, W., Beukes, J. and Meeske, R. 2013. Maize silage as a finisher feed for Merino lambs. *South African Journal of Animal Science*, 43: 111-115.

Van Soest, P. J. 1963. Use of detergents in the analysis of fibrous feeds. 2. A rapid method for the determination of fiber and lignin. *Journal of the Association of Official Agricultural Chemists*, 46: 829-835.

Van Soest, P. J. 1967. Use of detergents in the analysis of fibrous feeds. IV. Determination of plant cell wall constituents. *Journal of the Association Official Analytical Chemistry*, 50: 50-55.

Varner, J. E. and Lin, L.-S. 1989. Plant cell wall architecture. *Cell*, 56 (2): 231-239.

Varsha, Y. and Savitha, R. 2011. Overview on polyhydroxyalkanoates: A promising biopol. *Journal of Microbial and Biochemical Technology*, 3: 99-105.

Verlinden, R. A., Hill, D. J., Kenward, M., Williams, C. D. and Radecka, I. 2007. Bacterial synthesis of biodegradable polyhydroxyalkanoates. *Journal of Applied Microbiology*, 102 (6): 1437-1449.

Verlinden, R. A., Hill, D. J., Kenward, M. A., Williams, C. D., Piotrowska-Seget, Z. and Radecka, I. K. 2011. Production of polyhydroxyalkanoates from waste frying oil by *Cupriavidus necator*. *AMB express*, 1 (1): 11.

Vieira, M. G. A., da Silva, M. A., dos Santos, L. O. and Beppu, M. M. 2011. Natural-based plasticizers and biopolymer films: A review. *European Polymer Journal*, 47 (3): 254-263.

Wallen, L. L. and Rohwedder, W. K. 1974. Poly-. beta.-hydroxyalkanoate from activated sludge. *Environmental Science & Technology*, 8 (6): 576-579.

Wang, K., Yang, H. Y., Xu, F. and Sun, R. C. 2011. Structural comparison and enhanced enzymatic hydrolysis of the cellulosic preparation from Populus tomentosa Carr., by different cellulose-soluble solvent systems. *Bioresource Technology*, 102 (6): 4524-4529.

Wilkinson, J. M. and Toivonen, M. I. 2003. World silage: a survey of forage conservation around the world. Chalcombe Publ.

William, H. 1980. Official methods of analysis of the Association of Official Analytical Chemists. 13th edition. Association of Official Agricultural Chemists, USA.

Williamson, D. and Wilkinson, J. 1958. The isolation and estimation of the poly-β-hydroxy-butyrate inclusions of *Bacillus* species. *Microbiology*, 19 (1): 198-209.

Wójciak, A., Kasprzyk, H., Sikorska, E., Krawczyk, A., Sikorski, M. and Wesełucha-Birczyńska, A. 2014. FT-Raman, FT-infrared and NIR spectroscopic characterization of oxygen-delignified kraft pulp treated with hydrogen peroxide under acidic and alkaline conditions. *Vibrational Spectroscopy*, 71: 62-69.

Woolford, M. K. and Pahlow, G. 1998. The silage fermentation. In: *Microbiology of Fermented Foods*. Springer, 73-102.

Xu, Y., Wang, R.-H., Koutinas, A. and Webb, C. 2010. Microbial biodegradable plastic production from a wheat-based biorefining strategy. *Process Biochemistry*, 45 (2): 153-163.

Yamane, T. 1993. Yield of poly-D (-)-3-hydroxybutyrate from various carbon sources: A theoretical study. *Biotechnology and Bioengineering*, 41 (1): 165-170.

Yu, J. and Stahl, H. 2008. Microbial utilization and biopolyester synthesis of bagasse hydrolysates. *Bioresource Technology*, 99 (17): 8042-8048.

Zhao, X., Cornish, K. and Vodovotz, Y. 2019. Synergistic mechanisms underlie the peroxide and coagent improvement of natural-rubber-toughened pol(3-hydroxybutyrate-co-3-hydroxyvalerate) mechanical performance. *Polymers*, 11 (3): 565.

Zhao, X., Venoor, V., Koelling, K., Cornish, K. and Vodovotz, Y. 2019. Bio-based blends from poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and natural rubber for packaging applications. *Journal of Applied Polymer Science*, 136 (15): 47334.

APPENDIX

Production and characterization of PHA from an agricultural by-product using *Bacillus thuringiensis* CICIM (B2031)

Production and characterization of PHA from an agricultural by-product using *Bacillus thuringiensis* CICIM (B2031)

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Abstract:

Polyhydroxybutyrate (PHB) is a potential substitute candidate for the production of biodegradable plastics. In this study, *Bacillus thuringiensis* CICIM (B2031) was utilized for the production of PHB using agricultural by-product corn silage as a carbon source. 12% and 24% corn silage concentrations in media were used for the production, yielding maximum PHA of 40.48% and 33.33%, respectively after 48 hrs. Extracted PHA was characterized using FT-IR, UV spectroscopy, and GC-FID. The study revealed that corn silage can be utilized as a renewable carbon source for PHA production.

Introduction

In contemporary times, synthetic polymers from petrochemical materials have emerge as a necessary part of life. These polymers are resistance to biodegradation, which poses a grave risk to the environment (Charen *et al*., 2014). Tenacity in the surroundings leads to harmful outcomes on wild existence and significantly affects aesthetics of the cities. Increased cost of stable waste disposal as properly as the potential risks from waste incineration such as dioxin emission structure poly-vinyl chloride (PVC) makes synthetic plastic a waste administration problem (Gowda and Shivakumar, 2014). The search for options is vital in reducing mankind's dependency on non-renewable resources (Getachew and Woldesenbet, 2016). The most suitable method of decomposing plastic is biodegradation or microbial degradation. Degradation of plastics by microorganisms can also be a result of enzymatic pastime that can lead to chain cleavage of polymers into monomers. Microorganisms ought to be in a position make use of the polythene film as a sole source of carbon ensuing in partial degradation of plastics. Many solid waste administration packages have been regulated with the aid of various international locations to limit plastic waste and have come up with novel ways to produce biodegradable plastic materials such as those from poly-hydroxyalkanoate (PHA) (Alarfaj *et al*., 2015). PHB are natural biopolymers, that are synthesized and catabolized via microorganisms especially microorganism (Akar *et al*., 2006, Berlanga *et al*., 2006, de Roo *et al*., 2002). Wide vary of one-of-a-kind Gram-positive and Gram-negative bacteria are known

to synthesize these bio-polyesters and they are deposited as insoluble cytoplasmic inclusions. *Bacillus thuringiensis* is a Gram-positive bacterium which has a capacity to accumulate PHB and it is an active PHB producer. However, the excessive production cost of biopolymers and the availability of low-cost petrochemical make PHAs economically unattractive (Verlinden *et al*., 2011). Most reviews regarding the production of PHA promote that the essential contributor to the ordinary production value is the carbon substrate source. As such the decision of efficient carbon substrate is a key aspect. The choice method is to pick renewable, economically feasible and most quite simply handy carbon substrates for each microbial growth and environment friendly PHA production (Getachew and Woldesenbet, 2016). Therefore, the objective of this study was to produce and characterize PHAs from agriculture corn silage (CS) using *Bacillus thuringiensis* CICIM (B2031) as the PHB producer.

Materials and methods

Cultivation of *Bacillus thuringiensis* CICI (B2031) strain

Bacillus thuringiensis CICIM (B2031), was obtained from the Culture and Information Centre of Industrial Microorganisms of China Universities (CICIM) and was grown at 37°C in a medium containing 4 g/l yeast extract, 8 g/l tryptone and 2 g/l NaCl. Production studies were carried out in 250 ml flasks containing 50 ml culture medium and incubated at 37°C for 48 h on a shaker at 200 rpm. The pH of the medium was adjusted to 6.5, 7 and 7.5 with HCl and NaOH for optimization purposes. Growth profile was monitored by eliminating 2 ml aliquots of the culture at 6 h intervals and the absorbance was measured at 600 nm for a period of 48 h using Perkin-Elmer spectrophotometer. The bacteria were harvested at the end of the logarithmic phase by centrifugation at 6000 x g and cells were washed three times with deionized water (Charen *et al*., 2014). All cultures and measurement were performed in triplicate.

Screening of PHA-producing bacteria.

B. thuringiensis cells were screened using the lipophilic stain Sudan black B for PHA production. A loop full of culture was taken from the media and mixed with 20% brine and smeared on a clean glass slide. The smear was allowed to dry through air drying and then fixed by immersing in 2% acetic acid for 5 min. then the slide was removed, dried, flooded with Sudan black B solution (0.3 g of Sudan black B in 100 ml of ethanol) at room temperature for 15 min. The remaining stain was drained off and the slide was dried carefully then cleared by adding xylene. The slide was blotted and 0.5 % aqueous Safranin was utilized as a counter stain for 10 sec. the slide was then washed with distilled water, dried and observed under light microscope (Selvakumar *et al*., 2011).

Biomass determination

50 ml sterilized Falcon tube was utilized to determine cell concentration defined as dry weight of cells per litre of culture broth. Cells were harvested by centrifugation at 4000 x g for 15 min. Supernatant was discarded and the collected cell pallet was washed twice with deionized water and freeze-dryer with the ice condenser set at -84°C and vacuum set at 1.0 mbar (Rohini *et al*., 2006). The dried cells were then weighed for quantification and then stored at -80°C in the bio-freezer until PHA extraction was conducted.

PHA extraction and production

Mixture of sodium hypochlorite and chloroform was used in the recovery of microbial PHA. 1g of lyophilized cell pallet was shaken for 90 min at 37°C with chloroform and 15% sodium hypochlorite (1:1). The dispersion was centrifuged at 4000 x g at room temperature for 10 min. The lower chloroform phase that contained the solubilised polymer was recovered and the polymer was precipitated by addition of 4 volumes methanol (Rohini *et al*., 2006). Acetone was utilized to wash the precipitated polymer and air dried in the desiccator and later used for physical characterization.

UV-Vis characterization

10 mg of the extracted polymer was dissolved in 2 ml of chloroform and subjected to scanning in UV-Vis range against chloroform blank (Selvakumar *et al*., 2011, Alarfaj *et al*., 2015).

FT-IR spectroscopy

The spectrum of the extracted polymer from *B. thuringiensis* CICIM (B2031) poly(3HB) was compared with poly(3HB) pure standard obtained from Sigma-Aldrich. Structural analysis of the functional groups was performed utilizing Fourier-transform infrared Perkin Elmer 537 spectrophotometer. The sample was placed on the sample holder and scanned from 4000 – 400 cm^{-1}. The spectra were recorded using an attenuated total reflectance (ATR) disc and absorption frequencies were expressed as $vmax.cm^{-1}$.

GC-FID characterization

Gas chromatography (GC) was utilized to characterize the monomer composition of the extracted polymer. 5 mg of the obtained polymer was subjected to transesterification using 1ml chloroform, 0.85 ml methanol and 0.15 ml sulphuric acid mixture in a small screw-cap test tube for 60 min at 100°C in a thermostat-equipped oil bath. The mixture was allowed to cool down to room temperature. After cooling, 0.5 ml of distilled water was added into the test tube and mixed for 1 min using vortexed. The upper phase was removed using plastic pipettes, then the lower chloroform phase containing the methyl ester of β-hydroxyl acid was recovered and transferred to a small screw cap vial. 2µl of sample was injected into the injector pot and the inlet pressure of the carrier gas (nitrogen) was set at 109.2 kPa. For effective separation of esters, the temperature program was used [60°C for 1 min, temperature increase 25 °C/min to 230°C (230°C for 1 min)]; Flow controller: split, total flow 301.3 ml/min, column flow 1.48 ml/min; the temperature of the injector and detector were 200 and 230°C, respectively (Pantazaki *et al*., 2009). Different β-hydroxyalkanoatic acid methyl ester standards were utilized as references.

Results and Discussion

Previous studies have shown that both Gram-positive and Gram-negative bacterial species produce PHAs (Gomaa, 2014). The sheer diversity of the microbial world calls for the identification of bacteria and cheap substrates capable of producing large amounts of PHAs (da Cruz Pradella *et al*., 2012). The most studied bio-polyhydroxy-ester is PHB which belongs to PHA family. In this study, PHA was produced in broth supplemented with corn silage (cs) as a cheap carbon source collected from the Department of Agriculture, KwaZulu Natal. *B. thuringiensis* is known for both the production of an insecticidal δ-endotoxin and accumulation of PHA. However, no study has been done to evaluate the capability of the strain to utilize agro byproducts and the influence of different carbon sources on the accumulation of PHAs by *B. thuringiensis*. It was also noted that maximum biomass production at stationary phase (max ODs) coincided with the accumulation of the polymer and the biomass was harvested after 48 hrs (Rohini *et al*., 2006). The amount of accumulated PHA declines gradually after stationary phase if cell mass is not harvested due to PHA metabolism (Rohini *et al*., 2006).

PHA production

B. thuringiensis CICIM (B2031) was screened for PHA accumulation in the cytoplasm using Sudan Black B stain (Selvakumar *et al*., 2011). The lipophilic staining with Sudan Black B reportedly has a high sensitivity in PHA screening. Following Sudan Black B staining, *B. thuringiensis* CICIM (B2031) showed accumulated PHAs which was evidenced by blue-black inclusions in cytoplasm. The existence of blue-black granules showed the produced PHA and capability of *B. thuringiensis* to produce these polymers (Figure 1). The findings supported the findings of Charen *et al*. (2014), Sawant *et al*. (2014).

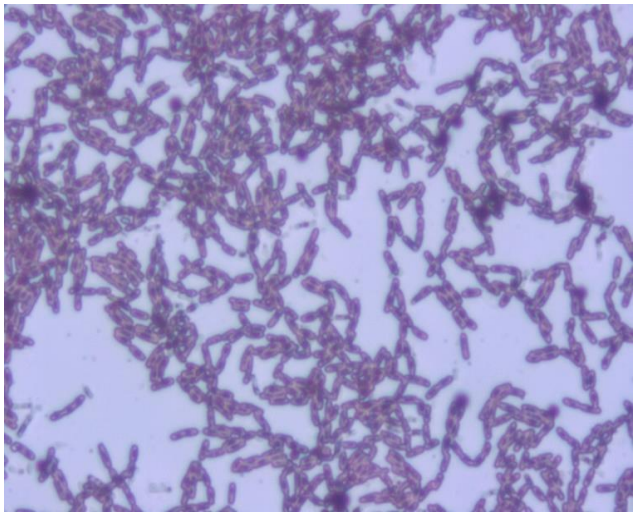

Figure 1 Micrograph of *B. thuringiensis* CICIM (B2031) with Sudan Black B stain showing PHA accumulation when counter strained with safranin using light microscope; magnification 1000×.

As an agricultural by-product, corn silage is a cheap and easily available substrate and it may help to reduce the cost of PHA production. Corn silage has a very high starch content (>28%, w/w) and sugar (8%) (Chahine, 2009). It also contains trace elements and vitamins (0.09 %) (Erdman *et al*., 2011). In view of all these compounds and minerals, corn silage as an agricultural residue was used in this present study as less expensive substrate to produce PHAs by *B. thuringiensis* CICIM (B2031). In all growth experiments performed, the cultures were harvested when the cells reached the early stationary phase and followed by determination of polymer composition. During bacterial growth, the substrate composition of the medium changes because of buildup and subsequent utilization of metabolic intermediates, henceforth change in the monomer composition of the PHA as a function of the age of the culture might be expected (Brandl *et al*., 1988).

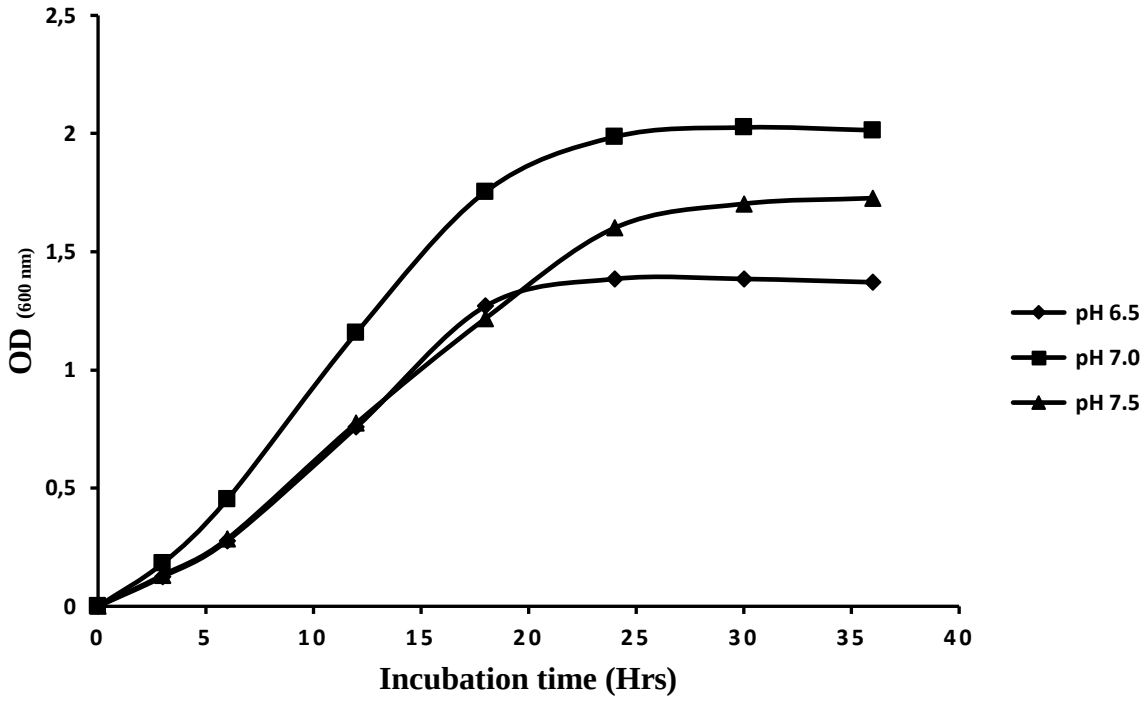

Figure 2 Growth of *B. thuringiensis* at different pH 6.5, 7.0, and 7.5.

After 48hrs it was noticed that pH 7.0 yielded the highest biomass as compared to pH 6.5 and pH 7.5 (**Fig. 2**). *B. thuringiensis* and some other bacteria are known to produce PHB naturally (Charen *et al*., 2014). After 20 hrs. of incubation it was seen that the highest cell density was achieved (Chen *et al*., 2012).

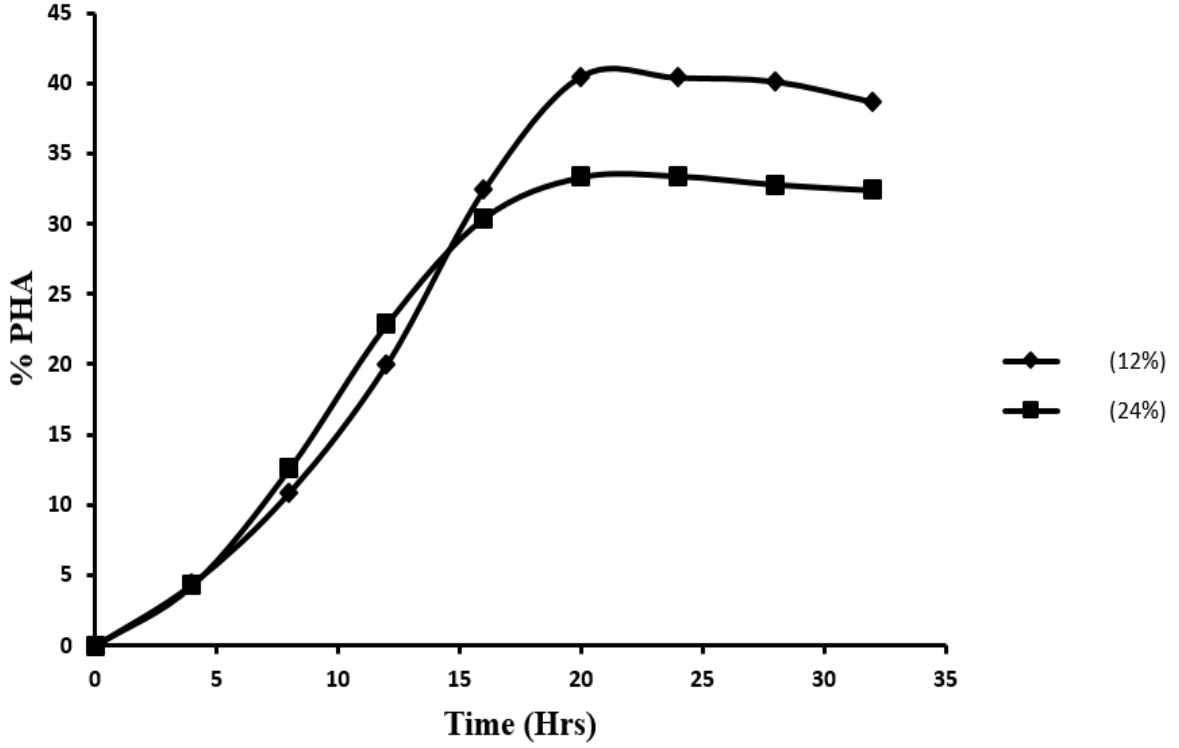

Figure 3 Production of PHB accumulation in production medium containing 12% and 24% CS per liter.

Maximum PHA synthesis in this study coincided with the beginning of the stationary phase (Figure 3). It was observed that as the cultures reached plateau, greater concentration of PHA is accumulated by *B. thuringiensis*. After 24 hrs. of accumulation the amount of accumulated PHB started to decline possibly due to the fact that they are synthesized and used as important energy reserves for bacteria hence PHB metabolism.

CS supported PHB growth, 12% substrate yielded maximum cell growth as well as maximum PHB accumulated. PHB accumulation of 40.48 % in the growth cycle of *B. thuringiensis* was recorded (**Table 1**). This indicates that *B. thuringiensis* strain utilizes the carbon source supplied essentially for the accumulation of PHB. Therefore, this less expensive substrate agricultural by-product showed ability of being utilized in reducing the production cost of PHAs.

Table 1 PHA production by *B. thuringiensis* using CS as a carbon source

% CS in media	Biomass (g/L)	Polymer (g/L)	% PHA content (w/w)
12%	1.03 ± 0.20	0.42 ± 0.31	40.48 ± 2.25
24%	1.14 ± 0.11	0.38 ± 0.28	33.33 ± 1.20

*Data represent the mean of 3 different readings ± standard deviation

B. thuringiensis showed a reasonably high yield of PHB in a medium containing two concentrations (Table 1) of corn silage 12% and 24% producing 40.48% and 33.33% PHB, respectively. As shown in Table 1, the most efficient concentration of corn silage supernatant for *B. thuringiensis* biomass production was that of 24% (w/v), though biomass reached the maximum value of 1.14 g/l after 20 h of incubation, however, biomass derived from the other culture was lower. Additionally, the PHA maximum concentration (0.42 g/l) and PHA content (40.48%) were obtained from the culture containing 12% (w/v) corn silage supernatant. PHA yield is not influenced by biomass yield. Chen *et al*. (1991) studied the production PHB by a range of *Bacillus* spp, and 50 % (w/w) of PHB on dry cell weight basis was produced by these organisms. In another study, 29 *Bacillus* strains were investigated for PHB production of which *B. megaterium* indicated maximum production of 0.207 g/L with a yield of 48.13 %. *B. subtilis* K1 reported the lowest production of 6.53 % (Aslim *et al*., 2002).

PHA characterization with UV-Visible spectroscopy.

UV-Vis scanning of synthesized PHB revealed a similar spectrum with commercial PHB (Fig. 4). This scan range indicates the occurrence of the PHAs and confirms corn silage as an active PHA producer.

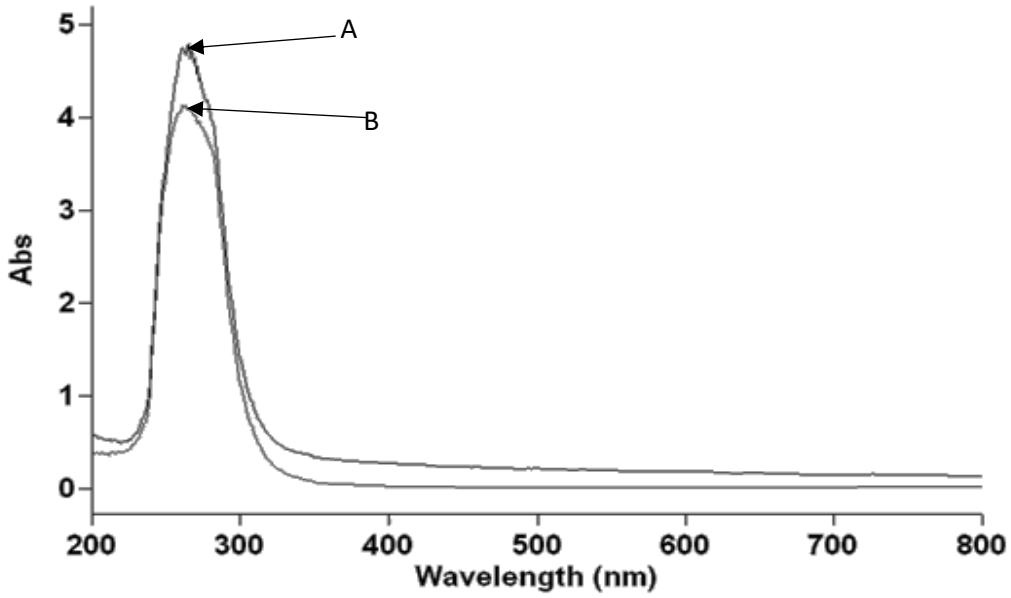

Figure 4 UV-Vis absorption spectra of standard PHB (Sigma-Aldrich) (A) and the PHB polymer obtained from *B. thuringiensis* CICIM (B2031) (B).

UV-Vis spectra of PHB isolated from *B. thuringiensis* was measured between 200 to 800 nm against the commercial standard using $CHCl_3$ as blank. Both spectra of the commercial PHB standard and extracted PHB indicated a sharp peak around the same range. Similarities on both spectra indicated the presence of PHB in the isolated sample. The results simulated the findings of Shah, 2014.

FT-IR spectroscopy analysis

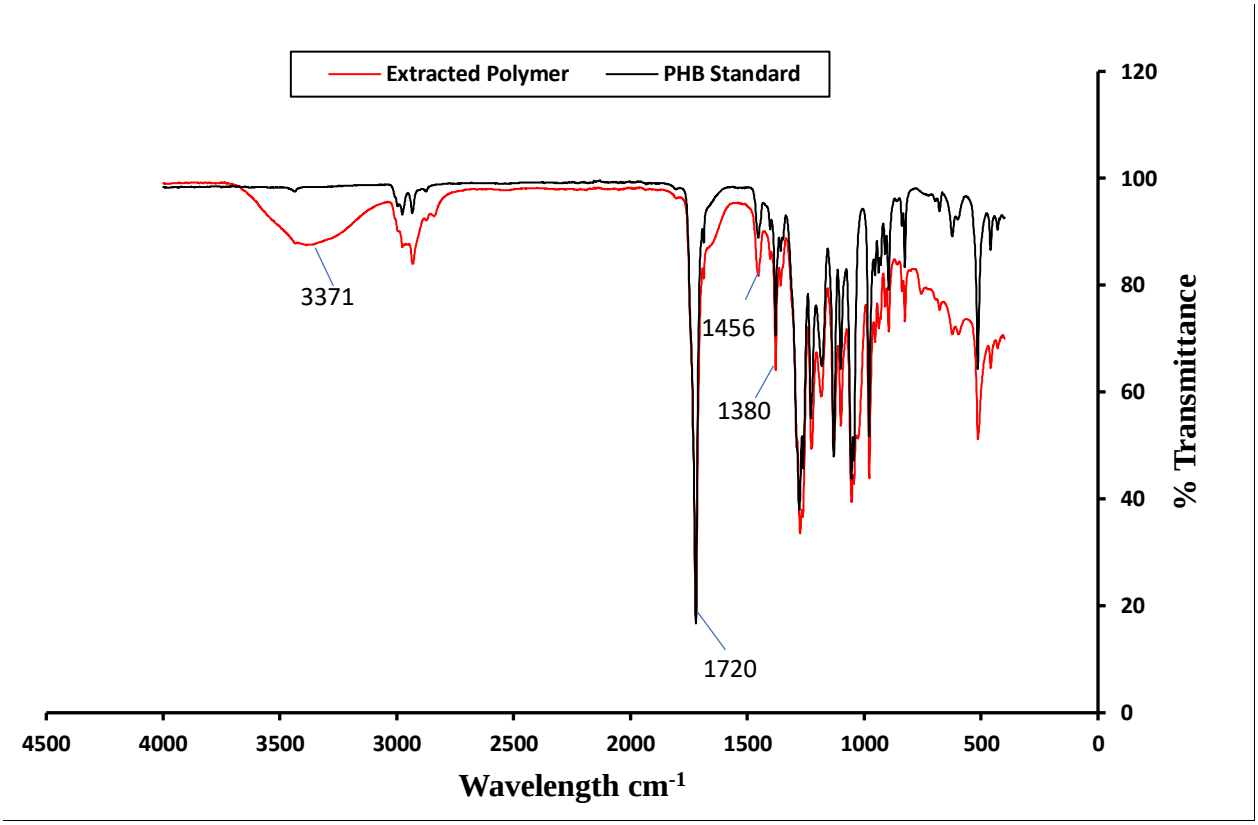

Figure 5 - FTIR analysis of polyhydroxybutyrate polymer commercial standard (Sigma - Aldrich) and polymer isolated from *B. thuringiensis* CICIM (B2031). The spectra indicate that the signal peak (1720 cm^{-1}) of the extracted polymer is identical to the C=O vibration absorption peak of the PHB standard.

From the FT-IR spectrum obtained (Figure 5) it can be concluded that the 3375 cm^{-1} band indicated stretching of the strong H bond formed by the terminal OH groups found in corn cobs. Comparable results have been reported in other studies (Getachew and Woldesenbet, 2016, Gumel *et al*., 2012). A close similarity in absorption bands obtained from the commercial poly-(3HB) and the bacterial poly (3HB) was seen in the region of 4000 – 300 cm^{-1}. According to Misra *et al*. (2000), Getachew and Woldesenbet (2016) the absorption band at 1719 cm^{-1} is a PHA marker band allocated to C=O (carbonyl) stretches of the (COO) ester group located in the chain of exceedingly ordered crystalline structures. The stretch of bands ranging from 1060 – 1281 cm^{-1} characterizes the valence vibration of the carboxyl (-C-O-) polymeric group (Getachew and Woldesenbet, 2016). These are characteristics of polyhydroxybutyrate. The band at 1456 cm^{-1} corresponds to CH showing asymmetric

stretching and bending vibration in the CH_3 group, whereas the band at 1380 represents the COH bond (Alarfaj *et al*., 2015). The analyzed results simulate the report of Alarfaj *et al*., (2015) verifying that PHB was the isolated compound.

GC-FID chromatography analysis

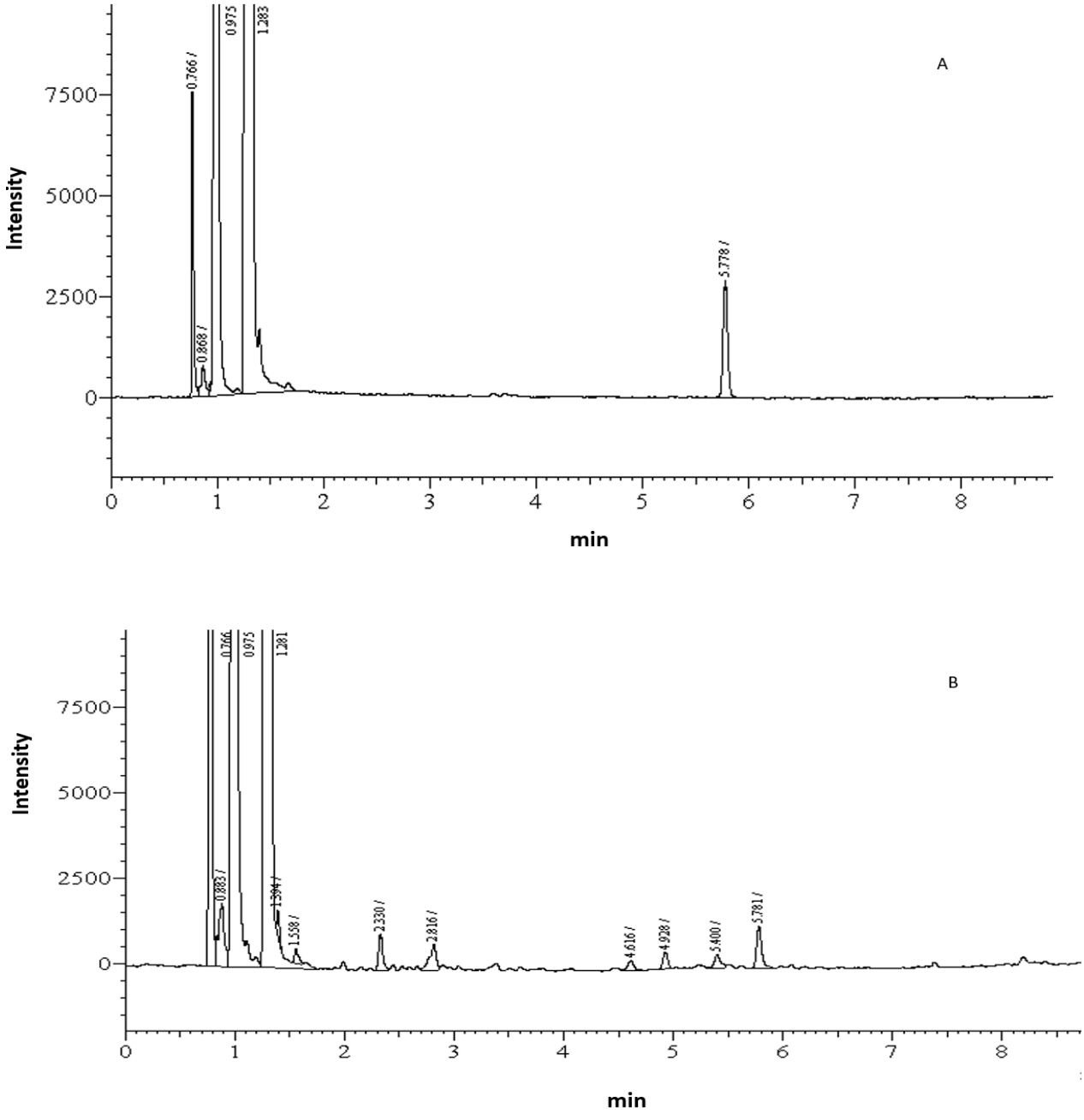

Figure 6 GC-FID analysis of PHB produced by *B. thuringiensis*: chromatography of standard (A) and chromatography of sample (B).

PHA quantification by GC is largely used in microbial PHA research and this is the standard method for PHB quantification which involves methanolysis followed by GC analysis (Alves *et al.*, 2017). The key compounds of concern in this study were identified based on their retention peak. GC-FID analysis of the commercial PHB and extracted polymer eluted a similar peak at 5.7 min [Figure 6 (a) (b)]. This peak signifies that the compound extracted was of a biodegradable polyester family and therefore GC confirmed that the polymer produced in this study is a short-chain length PHB consisting of 4 carbons (Chee *et al*., 2010). Improved results for the transesterification of PHB were obtained with sulphuric acidic-methanolysis as the derivatization reagent but it was noted that this method resulted in the formation of side products. *B. thuringiensis* is capable of producing PHB using simple sugar polysaccharides like starch. The nutritional versatility of this strain can be explored for economic and ecofriendly PHB production using hemicelluloses, starch and other bio-waste products.

Conclusion

The present study offers the challenge of finding industrial applications for these polyesters as a possible alternative to traditional, petrochemically based plastics and expand the field of microbial produced PHAs. PHB production using agricultural by-product may be used as strategy for increased PHB production on the commercial scale. PHB yields could also be increased by media optimization and not necessarily cell densities. This strain promises to produce biopolymers from numerous agricultural by-products. This study however, revealed the need for novel derivatization methods for the analysis of scl-PHA to avoid excessive formation of side-products. A common transesterification method using suphuric acid-methanol was shown to be inadequate for the quantitative analysis of scl-PHA due to a serious underestimation of the PHA content. The PHA produced by the bacterium was indicated to be PHB by UV-VIS, FTIR and GC-FID and also this study opens an alternative way to achieve production of PHA by using animal feed carbon source such as corn silage.

AKAR, A., AKKAYA, E. U., YESILADALI, S. K., ÇELIKYILMAZ, G., ÇOKGOR, E. U., TAMERLER, C., ORHON, D. & ÇAKAR, Z. P. 2006. Accumulation of polyhydroxyalkanoates by *Microlunatus phosphovorus* under various growth conditions. *Journal of Industrial Microbiology and Biotechnology,* 33**,** 215-220.

ALARFAJ, A. A., ARSHAD, M., SHOLKAMY, E. N. & MUNUSAMY, M. A. 2015. Extraction and Characterization of Polyhydroxybutyrates (PHB) from *Bacillus thuringiensis* KSADL127 Isolated from Mangrove Environments of Saudi Arabia. *Brazilian Archives of Biology and Technology,* 58**,** 781-788.

ASLIM, B., YUKSEKDAG, Z. & BEYATLI, Y. 2002. Determination of PHB growth quantities of certain *Bacillus species* isolated from soil. *Turkish Electronic Journal of Biotechnology***,** 24-30.

BERLANGA, M., MONTERO, M. T., FERNÁNDEZ-BORRELL, J. & GUERRERO, R. 2006. Rapid spectrofluorometric screening of poly-hydroxyalkanoate-producing bacteria from microbial mats. *International Microbiology,* 9**,** 95.

BRANDL, H., GROSS, R. A., LENZ, R. W. & FULLER, R. C. 1988. *Pseudomonas oleovorans* as a source of poly (β-hydroxyalkanoates) for potential applications as biodegradable polyesters. *Applied and Environmental Microbiology,* 54**,** 1977-1982.

CHAHINE, M. F., T; SEWMAKER, G E. Target value for corn silage. *In:* UNIVERSITY OF IDAHO EXTENSION, ed. Idaho Alfalfa and Forage Conference Proceedings, 2009 University of Adaho Extension.

CHAREN, T., VAISHALI, P., KAUSHALYA, M., AMUTHA, K., PONNUSAMI, V. & GOWDHAMAN, D. 2014. Isolation and identification of Polyhydroxybutyrate producing bacterial strain (*Bacillus thuringiensis* GVP) from chlorine contaminated soil. *International Journal of ChemTech Research,* 5**,** 3197-3202.

CHEE, J.-Y., TAN, Y., SAMIAN, M.-R. & SUDESH, K. 2010. Isolation and characterization of a *Burkholderia* sp. USM (JCM15050) capable of producing polyhydroxyalkanoate (PHA) from triglycerides, fatty acids and glycerols. *Journal of Polymers and the Environment,* 18**,** 584-592.

CHEN, D., XU, D., LI, M., HE, J., GONG, Y., WU, D., SUN, M. & YU, Z. 2012. Proteomic analysis of *Bacillus thuringiensis* ΔphaC mutant BMB171/PHB− 1 reveals that the PHB synthetic pathway warrants normal carbon metabolism. *Journal of Proteomics,* 75**,** 5176-5188.

CHEN, G.-Q., KÖNIG, K.-H. & LAFFERTY, R. M. 1991. Occurrence of poly-d (−)-3-hydroxyalkanoates in the genus *Bacillus*. *FEMS Microbiology Letters,* 84**,** 173-176.

DA CRUZ PRADELLA, J. G., IENCZAK, J. L., DELGADO, C. R. & TACIRO, M. K. 2012. Carbon source pulsed feeding to attain high yield and high productivity in poly (3-hydroxybutyrate)(PHB) production from soybean oil using *Cupriavidus necator*. *Biotechnology Letters,* 34**,** 1003-1007.

DE ROO, G., KELLERHALS, M. B., REN, Q., WITHOLT, B. & KESSLER, B. 2002. Production of chiral R-3-hydroxyalkanoic acids and R-3-hydroxyalkanoic acid methylesters via hydrolytic degradation of polyhydroxyalkanoate synthesized by *Pseudomonads*. *Biotechnology and Bioengineering,* 77**,** 717-722.

ERDMAN, R., PIPEROVA, L. & KOHN, R. 2011. Corn silage versus corn silage: alfalfa hay mixtures for dairy cows: Effects of dietary potassium, calcium, and cation-anion difference. *Journal of Dairy Science,* 94**,** 5105-5110.

GETACHEW, A. & WOLDESENBET, F. 2016. Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low cost agricultural waste material. *BMC Research Notes,* 9**,** 509.

GOMAA, E. Z. 2014. Production of polyhydroxyalkanoates (PHAs) by *Bacillus subtilis* and *Escherichia coli* grown on cane molasses fortified with ethanol. *Brazilian Archives of Biology and Technology,* 57**,** 145-154.

GOWDA, V. & SHIVAKUMAR, S. 2014. Agrowaste-based Polyhydroxyalkanoate (PHA) production using hydrolytic potential of *Bacillus thuringiensis* IAM 12077. *Brazilian Archives of Biology and Technology,* 57**,** 55-61.

GUMEL, A. M., ANNUAR, M. S. M. & HEIDELBERG, T. 2012. Biosynthesis and characterization of polyhydroxyalkanoates copolymers produced by *Pseudomonas putida* Bet001 isolated from palm oil mill effluent. *PLoS One,* 7**,** e45214.

MISRA, A., THAKUR, M., SRINIVAS, P. & KARANTH, N. 2000. Screening of poly-β-hydroxybutyrate-producing microorganisms using Fourier transform infrared spectroscopy. *Biotechnology Letters,* 22**,** 1217-1219.

PANTAZAKI, A. A., PAPANEOPHYTOU, C. P., PRITSA, A. G., LIAKOPOULOU-KYRIAKIDES, M. & KYRIAKIDIS, D. A. 2009. Production of polyhydroxyalkanoates from whey by *Thermus thermophilus* HB8. *Process Biochemistry,* 44**,** 847-853.

ROHINI, D., PHADNIS, S. & RAWAL, S. 2006. Synthesis and characterization of poly-β-hydroxybutyrate from *Bacillus thuringiensis* R1. *Indian Journal of Biotechnology*. 5: 276-283.

SAWANT, S. S., SALUNKE, B. K. & KIM, B. S. 2014. A laboratory case study of efficient polyhydoxyalkonates production by *Bacillus cereus*, a contaminant in *Saccharophagus degradans* ATCC 43961 in minimal sea salt media. *Current Microbiology,* 69**,** 832-838.

SELVAKUMAR, K., SRINIVASAN, G., BASKAR, V. & MADHAN, R. 2011. Production and isolation of polyhydroxyalkanoates from *Haloarcula marismortui* MTCC 1596 using cost effective osmotic lysis methodology. *Eur J Exp Biol,* 1**,** 180-187.

SHAH, K. 2014. Optimization and production of polyhydroxybutarate (PHB) by *Bacillus subtilis* G1S1 from soil. *Intern J Curr Microbiol App Sci,* 3**,** 377-387.

VERLINDEN, R. A., HILL, D. J., KENWARD, M. A., WILLIAMS, C. D., PIOTROWSKA-SEGET, Z. & RADECKA, I. K. 2011. Production of polyhydroxyalkanoates from waste frying oil by *Cupriavidus necator*. *AMB Express,* 1**,** 11.