Value recovery from Solid Confectionery Waste

Technical report

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EXECUTIVE SUMMARY

In South Africa, we produce in excess of 300 000 tpa of sugar-based confectionery products, in addition to further chocolate-based and starch-based confectionery products. This compares to some 43.2 x 10^6 tpa in the EU. It is estimated that, on average, 4.1% of the material is wasted in the process and 5.7% following packaging, totalling 12 300 tpa and 17 100 tpa respectively of waste material from sugar-based confectionery only in South Africa. Currently these solid wastes are land-filled; however, changing legislation, restricted landfill space and the environmental burden of this choice demands a new fate for these waste materials. Further to this, confectionery waste has potential to be considered as a resource and valorised, improving resource efficiency which is defined as the ratio of benefit accrued from a raw material to environmental burden generated from its use. As confectionery waste is organic in nature and rich in sugars, it is an ideal substrate for bioprocesses to yield a range of products from bioenergy products, through bio-based commodity products, platform chemicals and biopolymers to fine chemicals. In this study, we explore the waste biorefinery concept for re-purposing of confectionery waste with valorisation.

Through a review of the literature, we conclude that, while negligible valorisation of confectionery waste has been implemented in South Africa, this is growing in the EU. Some implementation is reported with several plants having installed biogas reactors to provide combined heat and power (CHP). Currently production of the bio-based biodegradable polymer PHA is in Technology Readiness Levels (TRL) 7 and 8 in the Netherlands towards repurposing this sugar-rich source.

Following the identification of a range of potential products and product classes for manufacture from confectionery waste, key aspects of and approaches to product selection are presented. We have explored example products including bioethanol and biogas as bioenergy products, the polyalkanoates (PHA) as bio-based, biodegradable polymers, the polymer polyglutamic acid as a soil enhancer through N supply and water retention, water treatment chemical, raw material for textiles and food additive, and the purple *Monascus*-like natural pigment as a colourant. For each compound, we have considered product uses, their relation to the confectionery industry, shown proof of concept for production on at least one category of confectionery waste (from hard candy, marshmallow, chocolate and starch-based confectionery products) and, in most cases, show kinetic studies comparing production on purified sugar sources and confectionery waste-based media.

Using PHA, we demonstrated that, while the use of waste resources as raw materials removes environmental burden with waste processing costs being offset against production costs, a balance is required between the improved economics of reduced raw material costs and maintaining ideal process performance. Waste materials that are sugar-rich such as confectionery waste, are particularly well suited for such valorisation. To further explore the techno-economics, we have selected the conversion of sugar-based confectionery waste to PGA product. For a high value food additive using conventional bioprocessing and downstream processing, it shows good performance with a 4.6 year payback time of 34% ROI, but is not feasible on production of a low grade, low value soil conditioner through the same process. It is suggested that simplifications to the process train and use of a lower cost reactor will improve the economic feasibility. We propose that intermediate value, high grade soil enhancers and water treatment chemical applications also be explored.

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1.1 A context for value recovery from confectionery waste

According to Stats SA, in the period September 2018 to February 2019, the Food and Beverages division of the South African manufacturing industry accounted for 23.1 % (StatsSA, 2019)of the total manufacturing revenue, which is over 3 % of the GDP (StatsSA, 2018). This was slightly above the contribution of the Petroleum, Chemical, Rubber and Plastic Products division and the Iron, Steel, and Non-Ferrous Metal Products, and Machinery division (StatsSA, 2019), and well ahead of the other seven reported manufacturing divisions. South Africa is said to be one of the largest and most well established confectionery markets in the African continent with consumption of 1.3 kg (in 2010) of chocolate per capita and 2.1 kg of sugar confectionery per year, as recorded in 2010 (Food Stuff SA, 2011). The confectionery industry is divided into three segments, namely chocolate, sugar and flour (starch) confectionery. Chocolate confectionery comprises mainly of chocolate bars, chocolate blocks, boxed chocolates and other chocolate products. Flour confectionery includes items made from the flour or starch, mainly as bakery products. Sugar confectionery includes the rest of the products in the confectionery industry. A fourth type, the sugar-free confectionery, containing no-sugar or sugar alternatives has also emerged (Ersahin et al., 2011).

A South African confectionery company produces approximately 625 tonnes/ year of confectionery waste (chocolate, candy and marshmallow) which is discarded in landfills. As an alternative, this waste can be used to produce biofuel, energy or chemicals. Information on the processing of confectionery waste in South Africa is limited. Over recent years, limited research on valorisation of these confectionery waste streams is emerging internationally (Lopez et al., 2011). The need to drive research on the valorisation of confectionery, and other food, wastes is driven by an increasing focus on the circular economy and the need to enhance resource efficiency i.e. gaining the maximum benefit from resources used while creating the minimum environmental burden. Importantly, many of these wastes currently report to landfill sites in which capacity is rapidly depleting and alternatives to avoid landfilling are imperative and urgent. The urgency implies that, in some countries, policy is now driving replacement of landfill disposal. Further, landfilling of readily degradable organics often leads to biodegradation, resulting in methane generation without effective capture, contributing to greenhouse gases, as well as acidic leachates mobilising metals from the landfill. Alternatives to landfill are clearly urgent from many perspectives. Alternatives to this include the improved design of landfills and their selective filling with organic materials to facilitate methane capture for bioenergy or their active treatment through anaerobic digestion processes.

1.2 The potential for valorising confectionery waste

Hacking (1986) presented a typical analysis of confectionery waste solids (%w/w) as 55% sucrose, 16 % glucose, 22% starch, 3.5% gelatine (protein), 2% caramel, 1% organic acids and 0.5% coconut. This translates to 76% sugar, 22% starch and 3.5% protein which makes it an ideal feedstock for manufacture of bio-based products of higher value than bioenergy such as alcohols, enzymes, organic acids, SCP, pigments and platform chemicals. The production of value-add products such as

these from confectionery waste is not yet commercialised but is under investigation; for example the production of polyhydroxyalkanoates from the Mars factory liquid effluent stream rich in chocolate has been demonstrated (Delta, 2013). A simpler alternative to this is its use as an energy source through its anaerobic digestion, combustion or pyrolysis or fermentation to liquid biofuels. As an example of the energy route, Nestlé (UK and Ireland) has commissioned on-site anaerobic digestion at their Newcastle upon Tyne Fawdon production site and is diverting their waste, called "chocolate soup", to this facility. This "soup" consists of four tonnes of solid waste and 200 000 litres of liquid effluent treated daily (Dutch Water Sector, 2014; Kane, 2015). Similarly, a selection of technology package offerings of Paques (Netherlands) for conversion of organic wastes to bioenergy products include the upflow anaerobic sludge blanket reactors and their improved designs (BIOPAQ®UASB, BIOPAQ®IC AND BIOPAQ®ICX) as well as their compact BIOPAQ®UBOX with both anaerobic and aerobic modules (Paques, n.d.).

Although not part of this project but still very important to South Africa is the value that can be recovered from the wastewater from the confectionery industries, in addition to their solid waste. The confectionery industry generates high amounts of wastewater that contain high concentrations of readily biodegradable organic materials characterized with high chemical oxygen demand (COD) and biological oxygen demand (BOD) (Beal and Raman, 2000; Diwani et al., 2000; Ersahin et al., 2011). The range of COD reported in confectionery wastewater is between 2840 – 19 900 mg/l COD and 1840 – 4910 mg/l BOD (Ersahin et al., 2011). Disposal of such high strength waste is expensive (Kim et al., 2006; Viñas et al., 1994) and represents a lost opportunity. These waste materials have high organic content, therefore must be treated before disposal. In place of disposal, the confectionery waste and its wastewater is proposed as a low-cost feedstock for developing novel biorefinery schemes, producing bioproducts or energy products or both and improving resource efficiency.

One of the main objectives of the South African Research, Development and Innovation (RDI) programme on waste is to find alternative uses for waste currently disposed into landfills. Using confectionery waste in an alternative way is proposed in this project. South Africa currently has a high demand for energy and other "green" or bio-based products. Further the South African Bio-economy Strategy (DST SA, 2013) presents an intention to expand the bio-based product sector in South Africa with a concomitant focus on the green economy, circular economy and de-carbonisation. The concept of a waste biorefinery is employed, in which feedstock(s) is processed to yield multiple products. This is of interest in the quest for sustainable processes for production of bio-based chemicals and fuel. Greener alternatives of waste processing seek to use waste streams for value addition and to reduce the environmental burden of disposing of this waste, according to the principles of industrial ecology. The biorefinery approach provides opportunity to utilise all components of the waste stream while not constraining parameters defining process performance. The generation of bio-ethanol, electricity, hydrogen and chemicals are examples of value addition that can be achieved. The conversion of waste to value via a bioprocess in a biorefinery is centred on the conversion of the organic carbon, nitrogen, phosphorous and other contaminants in the waste stream to value added products. This concept is gathering interest and is increasingly recognised for its potential contribution to the bioeconomy or bio-based economy as well as augmenting industrial ecology and cleaner production (Verster et al., 2014). A biorefinery needs to

generate a product of sufficient value to make it economically viable, while simultaneously remediating the waste as an important environmental contribution. This concept contributes to valuing the waste conversion or treatment as an integrated component of a wider system rather than a unit process for "end-of-pipe treatment". This leads to the recovery of resources in increasingly 'more closed' loop cycles, and thus contributes to progress towards a circular economy.

1.3 Research Approach

The aims of the project, as laid out in the proposal are as follows:

- Examine the confectionery industry and assess the scale of confectionery waste in South Africa.
- Investigate the composition of the confectionery waste and compare it to that required for use in the bioprocess. Investigate variability and potential presence of contaminating compounds.
- Evaluate literature and assess possible products and processes that use these components as feedstocks.
- Examine the potential of using the biorefinery concept to add value to confectionery waste by its bioconversion to a range of products selected.
- Techno-economic evaluation of the production of value added products from the waste sources, according to example proposed process flowsheets compiled using project outcomes.

The project was approached through the following sub-studies:

- A literature review of the scope and scale of the confectionery industry in South Africa and its associated waste, with the emphasis on the sugar, chocolate and starch confectioneries, was conducted.
- Typical treatment approaches to confectionery waste were reviewed globally to ascertain approaches to value creation from similar wastes elsewhere.
- The waste biorefinery concept was explored to draw generic approaches from other wastes together for application to the confectionery waste.
- Appropriate products with potential for market demand were identified. Microbial production systems suited to the bioconversion of confectionery waste to these products were proposed.
- A detailed experimental programme was undertaken to collect productivity and yield data for the production of the selected products.
- Flowsheets were developed for one product for the integrated utilisation of waste components while maximising value creation. Material and energy inventories were compiled through material and energy balancing to allow process assessment. This was used to inform the techno-economic feasibility of the process of using solid waste confectionery as feedstock to the biorefinery.

2 An Overview of the Confectionery Industry

2.1 Ingredients and Processes in the Confectionery Industry

The general composition of confectionery is summarised in Table 2-1 sourced from Foods and Food Production Encyclopaedia (Considine and Considine, 1982), Edwards (2001), Šebečić and Vedrina-Dragojevic (2004) and Ashokkumar (2010). By focussing on the ingredients of these products, information is gained on the typical components of the waste products generated through incomplete conversion or use, equipment cleaning and components and products not meeting specification.

Table 2-1 *Sugar and chocolate confectionery compositions* (Ashokkumar, 2010; Considine and Considine, 1982; Edwards, 2001; Šebečić and Vedrina-Dragojevic, 2004)

Confectionery products	Raw materials	Product chemical composition	Nutritional content
Hard candy	Sucrose, Glucose syrup Cysteine*, Proline*, Honey*, Additives, Proline, Vegetable fat	Glucose, Maltose, Glucose saccharides, Sulphur*, Furans*, Nitrogen*, Organic acids, Salts, Free fatty acids	Energy 386 calories Water 1.4% Carbohydrates 97.2 g/100 g Protein 0 g/100g Fats 1.1 g/100g
Marshmallow	Sucrose, Glucose syrup, Invert sugars, Gelatine Starch, Milk	Glucose saccharides, Maltose Fructose, Glucose, Lactose Whey protein, Organic acids	Energy 319 calories Water 17.3% Carbohydrates 24g/100g Protein 2g/ 100g
Chocolate	Sucrose, Cocoa, Vegetable fat/ Cocoa butter, Milk, Lecithin	Free fatty acids, Polyphenols, Lactose, Protein, Potassium, Magnesium, Copper, Iron	Energy 507 calories Water 1.1% Carbohydrates 57 g/100 g Protein 4.2 g/ 100 g Fats 35.7 g/100 g
Biscuits	Flour, Fat, Sugar	Starch, Proteins, Vitamins, Zinc, Copper, Phosphorous Magnesium, Potassium, Iron	Energy 1880 kJ Protein 7.8g/ 100 g Glycaemic carbohydrate 19.7 g/100 g Cholesterol 39 mg/100 g Dietary fibre 22 g Total sodium 100-338 mg/ 110 g

*selected products

2.1.1 Sugar confectionery

Sugar confectionery includes most of the products in the confectionery industry. It includes product categories such as boiled candy (hard candy), aerated products (marshmallows), grained sugar products (fondant and fudge) and gelled products (jelly sweets). This also includes the sugar-free confectionery, containing no-sugar or sugar alternatives (Ersahin et al., 2011). A wide range of sugars are used in the manufacturing of candy products.

The most commonly used type of sugar is cane sugar or beet sugar, a source of sucrose. Sugar derivatives or substitutes can also be used in place of sucrose i.e. glucose syrup, invert sugar syrup and fructose syrup, the latter carrying a much higher 'sweetness' per gram sugar. Glucose syrup is a purified, concentrated aqueous solution of glucose produced from starch hydrolysis, whereas invert sugar is a mixture of fructose and glucose produced from sucrose hydrolysis (Wolf, 2016).

Through isomerisation, invert sugars can be converted to be dominantly glucose or fructose. Hard candy confectionery is typically manufactured at high temperatures (150˚C) using sucrose. Under high temperature evaporative conditions, the sucrose undergoes crystallisation. This is desirable for boiled sweets and undesirable for aerated, grained and gelled sugar products. Therefore, in the latter products, crystallisation at high temperatures is avoided and monomeric sugars are typically used. For gelled products, the sucrose levels are kept below 75%; however, care must be taken to avoid crystallization which may result into products not conforming to specification. Glucose syrup and invert sugars limit crystallisation in this product but also increase the sugar levels in the solution (Lees, 1973).

Other sugar substituents and derivatives used in sweet confectioneries are honey and starch. Honey is mostly used for its sugar, organic acid composition and flavouring components which is preferred in certain speciality of sweet products. The organic acids present in honey include gluconic, acetic, citric, formic, lactic, and malic acid. The processing of honey during the sweet production may yield levulinic acid and polymers of hydroxymethyl furfuraldehyde. These products have an adverse impact on quality and yield of the product. To counter the effect of these products, the concentration of organic acid is increased. Furthermore, starch may also be used at high concentration in honey-based confectionery to balance the levels of the honey degradation products (Lees, 1973). Starch is also used in jelly gum production as a gelling agent and for moulding purposes (Autio et al., 1992). Contrary to honey, starch degradation products are found to be desirable in confectionery production i.e. amylopectin and amylose, obtained by enzymic or acid hydrolysis, result in gel formation. Desirable properties of starch and starch derivatives in the confectionery industry include low-sweetness, low hygroscopicity, viscosity and control of sugar crystallisation.

Non-sugar ingredients used in sugar confectioneries include organic acids, buffers of sodium salts, pectin, gelling agents and amino acids. Organic acids, such as citric, malic, tartaric, lactic and acetic acid, are used as additives in sugar confectioners to add flavour; for example, in fruit flavoured products to complement the fruity flavour (Edwards, 2001). The organic acid content of hard candy with a fruity and mild fruity flavour is 1% and 0.5% respectively (Lees, 1973). To maintain the appropriate pH levels of the products, buffers containing sodium salts are used (Edwards, 2001). For the production of pectin-containing jellies, sodium citrate is used to aid in the gelling of pectin at more acidic levels (Lees, 1973). Interestingly, amino acids, such as cysteine and proline are added into selected sugar confectioner's e.g. toffees to aid in the browning of sugars (Maillard reaction) and to enhance flavour. However, the amino acids are not detected in the final product since they undergo various complex degradations.

2.1.2 Flour-rich confectionery

The flour confectionery consists mainly of baked goods (biscuits, cakes and doughnuts), fermented products (bread) and pastries. These include both long shelf life and short shelf life products. There are three main ingredients used in flour confectionery, namely flour, fat and sugar. These essential ingredients may be supplemented with other optional ingredients are used to enhance flavour, appearance, texture and end-product conformation. Flour is considered as the main ingredient in this confectionery industry due to its versatile nature (Ashokkumar, 2010).

Flour in confectionery production is used as the central component as well as a binding and absorbing agent of other ingredients to ensuring equal distribution of ingredients in the mixture. Additionally, it enhances flavour and adds to the nutritional values of the product. Different sources of flour are preferred based on the desired confectionery end-product and cost-effectiveness. The most commonly used flour is wheat flour. Its quality is determined by the quality of the wheat grain. The wheat grain consists of vital components such as bran, endosperm and germ. These contribute to the nutritional value of the end products and the properties of the flour. The bran makes up 15% of the wheat and is made up of several layers that include the aleurone layer. The aleurone layer consists of cells that contain α-amylase and β-amylase that hydrolyse starch conserved in the endosperm. The endosperm contains more nutritional value including, proteins, pantothenic acid, riboflavin and thiamine (Ashokkumar, 2010). Moreover, in confectionery production wheat flour is reported to be a source of inorganic nutrients such as zinc and copper detected in hard biscuits (Šebečić and Vedrina-Dragojevic, 2004). Maize is the source of cornflour and contains high amounts of starch. Cornflour is used as a thickening agent for confectionery products such as puddings and custard (Ashokkumar, 2010).

Recent studies report flour and starch derived from sweet potato as a cost-effective nutritious substitute to wheat in confectionery production. The dry matter of sweet potato consists of 80-90% starch and sugar including essential elements such as Zn, P, Mg, K and Fe. The sweet potato roots are processed into flour and starch using standard methods. The sweet potato starch and flour may be blended with wheat flour to improve the flour functional properties desired for confectionery production (Hussein et al., 2015) or used alone. Soya flour derived from soybeans has also shown great potential in the confectionery industry. It contains higher amounts of fats, protein and non-starch carbohydrates than wheat (Ndife et al., 2011). The use of soya flour in flour confectionery increases the shelf life of bakery confections. The protein component of the soya flour may improve quality and appearance owing to the aerating and emulsifying properties of the protein. Soya flour has also been employed in sugar confections for the production of caramels, fudge, toffee and chewable candies (Jideani, 2011). Almond flour may also be used. The use of these alternative flours is also of interest where wheat allergens are to be avoided.

2.1.3 Chocolate-based confectionery

The chocolate confectionery industry is very broad, including chocolate bars, chocolate blocks, boxed chocolates and other chocolate products. Chocolate itself includes dark, white and milky chocolate, but it is frequently combined with other components such as nuts, dried or candied fruit, or other confectionery. The taste and texture of chocolate depends on the raw material and particle properties. Chocolate is a dense solid made up of 60-70% particulate solids (Afoakwa, 2008), with cocoa, sugar, vegetable fats or cocoa butter or both as main ingredients.

The flavour of the chocolate is determined by the cocoa beans and can be modified by removing organic acids contained in the cocoa (Afoakwa, 2008). Cocoa is used in the form of liquid (liquor) or powder which is the non-fat component of cocoa while cocoa butter is used as a fat substitute and contains triglyceride fatty acids dominated by saturated stearic, palmitic and monosaturated oleic acids (Afoakwa, 2008). Furthermore, chocolate also contains minerals such as potassium, magnesium, copper and iron (Afoakwa, 2008).

Sucrose is usually used as the sugar source in chocolate confectionery. In production of milky chocolate, milk-based ingredients are added. The milk components of interest are lactose sugar, whey protein and milk fats which enhance the texture and chocolate taste. However, lactose can also be used to reduce the sweetness of the chocolate products. Moreover, to improve the texture of chocolate, lecithin is added in low quantities as an emulsifier (Talbot, 2009).

2.2 The Confectionery Industry in South Africa

South Africa has one of the most established confectionery markets in Africa, including a sizeable market for chocolate confectioneries. In terms of production of non-starch-based confectionaries, the sugar confectioneries dominate at 56% of the total market with chocolate confectioneries accounting for 34%, followed by the chewing gum with 10%. The sales of sugar confectionery by volume and type in 2016 is presented in Table 2-2. However, in total sales value, chocolate confectionery leads at 50.5%, followed by the sugar confectioneries at 41% and chewing gum making up the remaining 8%. Some 95% of the chewing gums sold are sugar-free gums (Food Stuff SA, 2011).

Table 2-2 Sales volumes of confectionery in South Africa in 2016, given by confectionery type in unit of a thousand tonnes (Euromonitor International, 2016)

Sugar confectionery type	2011	2012	2013	2014	2015	2016
Boiled sweets	16.1	15.7	15.4	15.0	15.2	15.3
Liquorice	1.6	1.7	1.7	1.7	1.7	1.8
Lollipops	4.0	4.1	4.3	4.5	4.8	5.1
Medicated confectionery	0.8	0.8	0.8	0.8	0.9	0.9
Mints	0.4	0.4	0.4	0.4	0.4	0.4
Pastilles, gums, jellies, chews	17.2	18.0	19.0	20.0	21.0	21.8
Toffees, caramels, nougat	6.8	7.1	7.5	8.0	8.5	8.8
Other	4.5	4.7	5.0	5.3	5.5	5.9
Total	51.4	52.5	54.1	55.7	58.0	60.0

According to the reports of the "Who Owns Who African Business Information" group (https://www.whoownswhom.co.za/store/info/3296), South Africa's confectionery industry was valued at R11 – 12 bn in 2015 with a 10% compound annual growth rate, R12.4 – 13.5 bn in 2016 with a 2.4% growth rate and at R12.5 – 13.5 bn in 2019, following a period of limited growth caused by the economic downturn. The number of key companies operational in South Africa is largely unchanged at 21 in 2015 and 23 in 2019. The chocolate component of this sector is dominated by three major multinational companies and has grown from R6 bn in 2016 to R 6.4 bn in 2019. Mondelez SA's Cadburys brand (previously Kraft Foods), Tiger Brands Beacon chocolate and Nestle make up some two thirds of the chocolate supply, although this has declined from 73% in 2013 to 68% in 2015, owing to an increasing market share to imported chocolates and artisanal chocolates. The largest national chocolate manufacturer is Kees Beyers Chocolate, acquired by Sweetie Pie in 2013. Other local companies of significance are DV Chocolate supplying artisanal chocolates and

Shooga Shooga. In the sugar confectionery area Lodestone Brands (Candy Tops), Zambian-owned Trade Kings and Manhattan (acquired by Premier in 2013) are the main players. Shifts in product portfolios in confectionery industries result from trying to keep up with innovative trends and move into new market areas e.g. the artisanal chocolate space and 'healthy' candies. Additionally, price affects product market distribution in the tight economic climate (BMi Research, 2013). A list of key confectionery producers in South Africa is given in Table 2-3.

The main channels of product distribution are through general retailer and wholesaler sectors. Almost 42.3% of industrial output is distributed to densely populated regions in South Africa such as the Kwazulu-Natal, Gauteng and the Western Cape. In the less populated and less urbanised areas of South Africa, these products are distributed in lesser quantities (Fastmoving, 2016). The production quantities of some confectionery products i.e. boiled candy in South Africa has decreased in the past years, hence a decrease in product distribution. However, distribution volumes of these products were stable in less populated regions such as the Northern Cape to 2012. Since the volumes distributed in these regions are lower than those distributed in high populated region, this has minimal impact on overall product distribution. Grained sugar and gelled products showed significant increase in both production and market distribution up to 2012. The distribution of these products increased in all South African regions (BMi Research, 2013). Further to national consumption, South Africa has exported some US$ 80 million of sugar confectionery and US$ 60 million of chocolate per annum over the period 2011 – 2016 (UN Comtrade, 2017). Over the same period, export of baked goods decreased from US$ 80 million to US$ 60 million.

The confectionery manufacturing industry in South Africa is clustered and are located along the coast, in proximity to sugar mills or in proximity to the customer. The main hubs are situated in Gauteng and KwaZulu Natal, although two of the largest chocolate producers (Nestlé and Cadbury) are situated in the Eastern Cape.

Table 2-3 Confectionery manufacturers in South Africa

Name	Products	Location	Website
Western Cape			
Amajoya	Hard candy	Atlantis Industrial	http://www.amajoya.com/
Broadway sweets	Bubblegum, lollipops, hard candy, soft gums	Parow, Cape Town	https://www.broadwaysweets.co.za/
Caring Candies (Banting friendly)	Chocolate, hard candy	Montague Gardens	https://www.caringcandies.co.za/
Eurochoc Confectionery		Lansdowne	https://www.gmdu.net/corp-810085.html
Ezulwini chocolat	Chocolates	Airport City, Cape Town, 7490	
Joya Brands		Cape Town	
KandyLand: Novelty Candies	Marshmallows, jellies, hard candy	Durbanville	http://www.kandyland.co.za/?Task=system&CategoryID=7838&HeadingText=Catalogue
L & C Messaris brothers manufacturing	Almonds, cashew nuts, walnuts, potato chips, bubbles, peanuts and raisins	Elsies Rivier 7490	http://www.messaris.co.za/
Orbit Confectioners	Sweets, chocolates	Lansdowne	
Shooga shooga	Hard candy, peanut brittle, lollies	Maitland	http://www.shoogashooga.co.za/candycontactcapetown.html
Sally Williams		Cape Town	
Simba (PTY) Ltd	Chips	Parow Industria	https://www.simba.co.za/
Kwazulu Natal			
Tiger Brands (Ltd)	Beacon confectionery products	Jacobs, Durban	
Gauteng			
Premier FMCG		Waterfall City, 2090	
Mondelez SA (prev. Kraft Foods)		Johannesburg,1709	
Broadway Sweets (Stumbo Pops)		Cleveland,1401	
Pioneer Foods		Johannesburg 2001	
Dicks Sweets		Johannesburg, 2091	
Lodestone Brands (Mister Sweet)		Germiston 1428	
Tiger Brands			
National Brands AVI			
Kees Beyers Chocolates		Kempton Park 1619	
North West Province			
T&J Products (Pty) Ltd			
Eastern Cape			
Nestle Chocolate	Smarties, Quality Street, Kitkat		

2.3 Waste Generation from the Confectionery Industry

The waste stream(s) produced by major commercial confectionery plants consists of un-used raw materials, wastes from the pre-processing stages (e.g. forming chocolate and milk crumb) as well as production wastes in the form of dough, chocolate mass, fatty flavourings and starch from jelly production (Rusín et al., 2015; Miah et al. 2018). It also includes products that are misshapen or do not meet specifications as well as waste generated when the high-speed production lines are interrupted or fail and the material cannot be re-worked into product. While increasingly routes are being considered to re-process such material (Silverson, 2019), this is frequently not possible. Typically, during the processing stage 4.1% of sugar-based products, 2% of chocolate products and 5.7% of biscuit products enter the waste stream (Miah et al.,2018). Subsequent to packaging a further 5.7% of confectionery products become waste products. Typically these wastes are disposed of into landfill as food waste (Duygu Ozsoy and van Leeuwen, 2010). While some move towards processing the waste into energy is found globally, this is not yet the case in South Africa. Further to this, the energy footprint of the confectionery factory represents its second highest contribution to global warming potential (Miah et al., 2018). Owing to the high organic content and simple organic content of these wastes, they represent a good source for further processing, especially bioprocessing, to improve resource efficiency and expand the circular economy.

3 The Waste Biorefinery: An Overview

3.1 The Concept of a Waste Biorefinery

The biorefinery has been defined as the sustainable processing of biomass into a range of products and energy, including heat, power and fuels (IEA Bioenergy Task 42, n.d.). In recent years, this has been expanded beyond energy products to include products of increasing value such as commodity products, platform chemicals and fine chemicals. Products of the biorefinery have several outlets or markets, including final products for sale, products for use on site, and intermediates for final processing elsewhere. Biorefineries can operate at various scales or levels of complexity, ranging from conversion of a single feedstock to a single product, a single feedstock to multiple products, or multiple feedstocks to multiple products. While the term 'biorefinery' was originally used for the processing of woody biomass and plant material, the biorefinery concept is uniquely suited to application in valorising waste through the 'waste biorefinery'. Integration of waste valorisation processes into existing industries aids the move from a linear economy to a sustainable circular economy, with wastes from one process forming feedstock for another (Dahiya et al., 2018) in line with in the industrial ecology model. Biorefinery feedstocks can be mixed depending on the conversion technologies and the feed composition necessary for ideal productivity.

A typical schematic diagram of a biorefinery process is shown in Figure 3-1 where biomass feedstocks are used to produce a variety of products using different conversion processes (Fernando et al., 2006). The feedstocks that can be used in a waste biorefinery are varied and copious. Typically they do not compete with food production as these are the waste from several industries such as agri-processing, food manufacturing, and the pulp and paper industry, amongst others. The availability of feedstock can be affected by climatic and weather conditions, location, socio-economic issues and government policies (Ghatak, 2011).

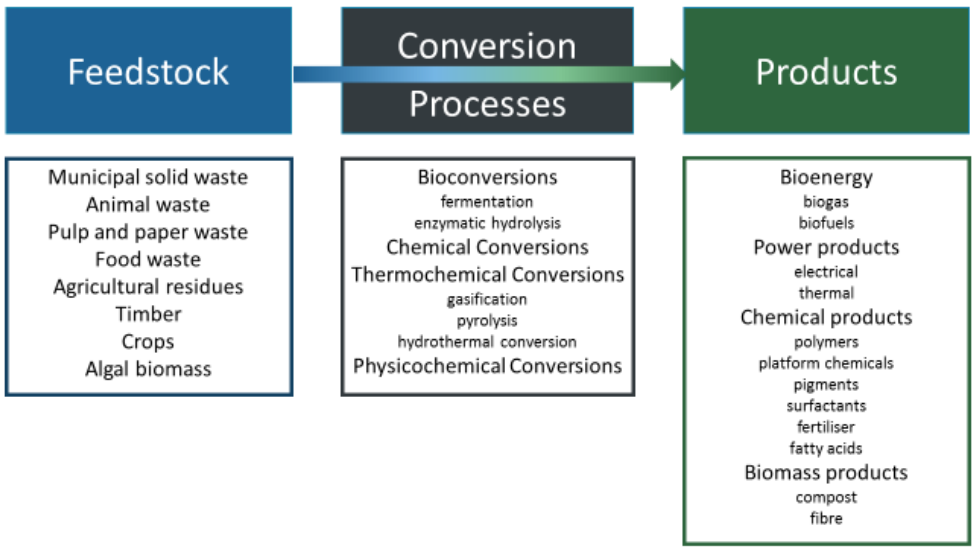

Figure 3-1 Schematic diagram of a biorefinery process (based on Fernando et al. (2006))

The processes of a biorefinery can be categorized into primary and secondary stages. Primary biorefinery encompasses mostly of the preparation and pre-treatment of the feedstock and its requirement is dependent on the nature of the feedstock used. The pre-treatment stage includes technologies such as dilute acid, alkaline pre-treatment, enzyme addition and auto hydrolysis to ensure readily accessible simple molecules for further processing. It is of lesser importance in a waste biorefinery utilising readily accessible feedstocks from the food industry. The secondary biorefinery converts the primary by-products by biological, biochemical, physical, thermal or chemical processing and separation. This conversion process is carried out in a variety of reactor configurations and conditions and finally the products are further recovered and purified during the downstream processing for their specific applications.

Currently biorefineries make use of four main types of conversion processes, namely biochemical, thermochemical, chemical and physical methods. Biochemical methods use biological organisms or systems to convert the selected feedstock into energy and bio-based products. Thermochemical processes make use of high temperatures and physicochemical processes use mechanical or chemical means for conversion of the feedstock. Selection of a biorefinery technology is dependent on various factors including type of feedstock, desired products, associated costs and biorefinery location (Nizami et al., 2017).

The waste biorefinery expands on the concept, with various waste streams viewed as renewable feedstocks for the production of energy or value-added products through sustainable processes (Mohan DevTavva et al., 2016; Harrison et al., 2017). For example, a wastewater biorefinery can be defined as a process design that uses wastewater streams as raw materials for biological processes to produce potential products while simultaneously purifying the water (Harrison et al., 2017b). A waste biorefinery has the benefit of addressing waste disposal issues (Nizami et al., 2017), thereby reducing environmental burden and associated impact, and improving biorefinery competitiveness and acceptance (Mohan et al. 2016). Further improvement comes through the integration of the various processes used to valorise the waste, with enhanced process efficiency and a greater possible product range (Dahiya et al., 2018), with diversity of product value as shown in the pyramid structure in Figure 4-2. Ideally, products of high value are prioritised and lower value products used further down the process train to ensure full conversion of all organic compounds and nutrients available. The valorisation may incentivise efficient waste treatment.

Waste remediation and valorisation have environmental benefits while also addressing economic and social factors, thereby satisfying the three requirements of sustainability. Harrison et al. (2017) have shown that the waste biorefinery approach has potential for using confectionery waste to produce products of value. Case studies have already shown the use of municipal wastewater to produce biogas (Naidoo, 2013), fertiliser from vinasse and PHA from chocolate wastewater (Tamis et al., 2014).

3.2 Classification of biorefineries

Based on the International Energy Agency Bioenergy Task 42 (IEA Bioenergy, n.d), biorefineries can be classified using the following main features (listed in order of importance)

1. Platforms
2. Products
3. Feedstock
4. Processes

A biorefinery system can be described as a conversion pathway from feedstock to products, via platforms and processes. The platform products, on which the platforms are based, are intermediates from which the final products are derived (Cherubini, 2010; IEA Bioenergy Task 42, n.d.).

3.2.1 Platforms

The platform concept is similar to that in the petrochemical industry, where the crude oil (feedstock) is fractionated (processed) into a large number of intermediates that are further processed to final products and chemical products. Therefore, platform products or platforms are intermediates which link feedstocks and final products. These platforms are recognised as the main "pillars" of the biorefinery classification, since they might be reached via different conversion processes applied to various raw materials (Cherubini, 2010).

The most important platforms listed in energy-driven biorefineries are (Cherubini, 2010):

- Biogas (a mixture of CH_4 and CO_2) from AD
- Syngas (a mixture of H_2 and CO_2) from gasification
- Hydrogen (H_2) from water-gas shift reaction, steam reforming, water electrolysis and fermentation
- C6 sugars (glucose, fructose, galactose) from hydrolysis of sucrose, starch, cellulose and hemicellulose
- C5 sugars (xylose, arabinose) from hydrolysis of hemicellulose and food and feed side streams
- Lignin ($C_9H_{10}O_2(OCH_3)_n$) from the processing of lignocellulosic biomass
- Pyrolysis liquid (a multicomponent mixture of different size molecules) from pyrolysis
- Oil (triglycerides) from oilseed crops, algae and oil based residues
- Organic juice from the liquid phase extracted after pressing of wet biomass (e.g. grass)
- Electricity and heat that can be internally used to meet the energy needs of the biorefinery or sold to the grid

3.2.2 Products

Biorefineries can produce both energy and non-energy products. These can be grouped into two main classes (Cherubini, 2010)

- Energy products where the biomass is primarily used for the production of bioethanol, biodiesel, synthetic biofuels, biogas, heat and electricity
- Material products where the biomass is converted to products such as biomaterials, platform chemicals, bio-lubricants, chemicals, food and feed

The diverse range of material bioproducts show potential. This is well represented in Figure 3-2 in which bioproducts are linked to possible raw material feedstocks.

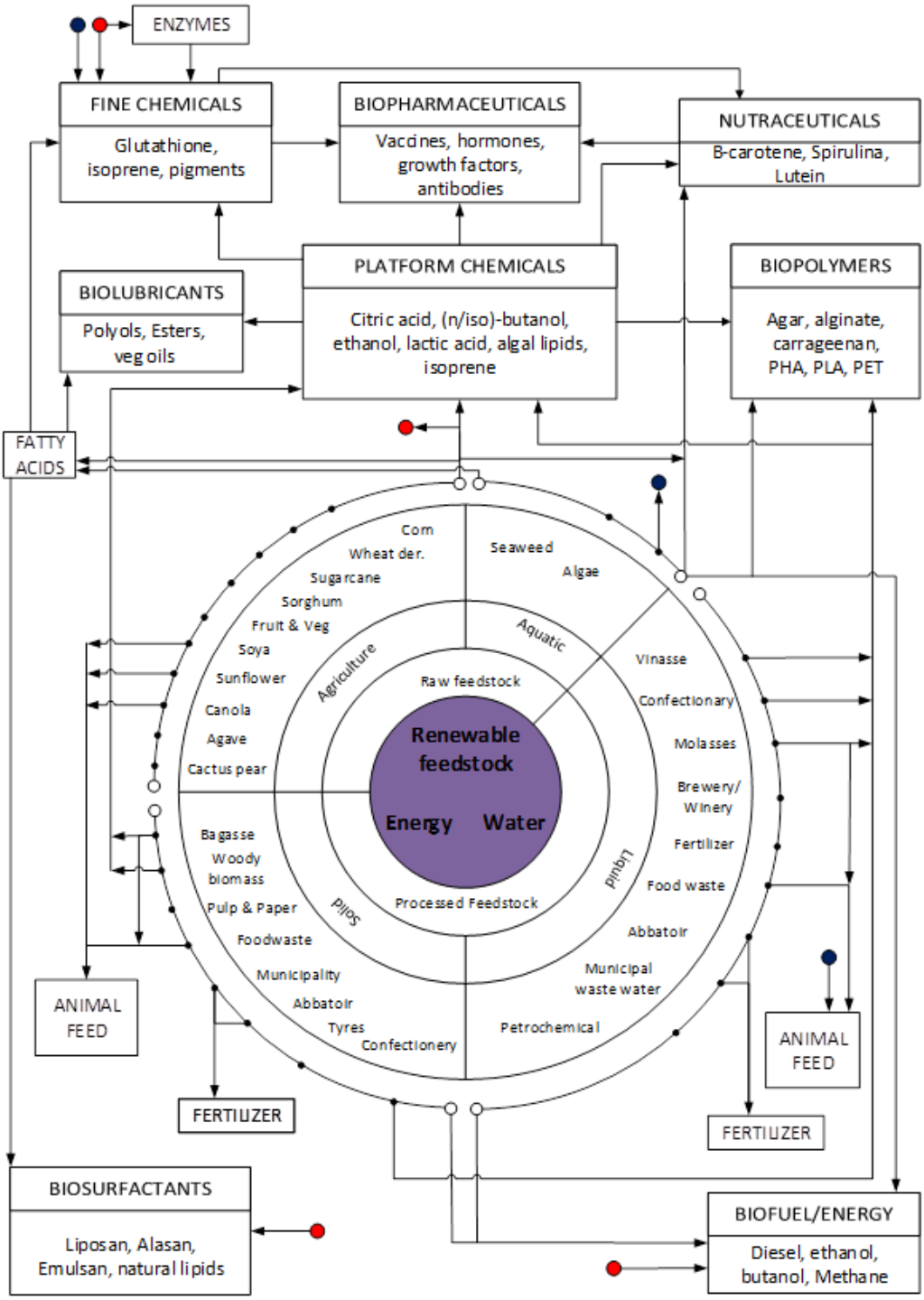

Figure 3-2 Linking potential bioproducts with raw materials from which they may be manufactured (Harrison et al., 2016)*, thereby providing insight into the potential diversity of biorefinery products*

3.2.3 Feedstocks

The biomass feedstocks can be derived mainly from the following sectors (Cherubini, 2010):

- Agriculture (dedicated crops and crop residues)
    - sugar crops (sugarbeet, sugarcane)

    - starch crops (wheat, corn, sweet sorghum)
    - grasses crops (green plant material, grass silage, cereals, switchgrass)
    - oil-based crops (rapeseed, soya, palm oil, Jatropha)
- Forestry (wood and logging residues)
- Industry (process residues and wastes)
    - Oil based residues (animal fat from food industries, used cooking oil)
    - Organic residues (organic food waste from food processing, retail and hospitality sectors, organic urban waste, manure, wild fruit and crops)
- Municipal solid waste and domestic waste (organic residues)
- Wastewater and wastewater-associated sludges as a feedstock was recognised as a key opportunity (Susan T. L. Harrison et al., 2017)
- Aquaculture (algae, seaweed)

These biomass feedstocks vary in composition (cellulose, hemicellulose, lignin, starch, triglycerides, proteins and sugars), concentration and volume. They may be seasonal and vary in complexity, water content and heating values.

3.2.4 Conversion processes

There are four main groups of conversion processes, namely (Cherubini, 2010):

- Mechanical or physical processing (pressing, pre-treatment, milling, separation, distillation): these processes do not change the chemical structure of the biomass but does perform size reduction or a separation of the feedstock components
- Chemical processes (hydrolysis, transesterification, hydrogenation, oxidation, pulping), where a chemical change or reaction in the feedstock occurs
- Thermochemical processes (pyrolysis, gasification, hydrothermal upgrading, combustion), where feedstock undergoes extreme temperature and pressure conditions resulting in chemical change
- Biochemical processes (anaerobic digestion, aerobic cultivation and anaerobic fermentation, enzymatic conversion), which occurs at milder temperature and pressure, catalysed by micro-organisms and enzymes, resulting in a chemical change.

3.2.5 Generations of biorefinery

Biorefineries can be classified according to the generation of feedstock that is processed, namely first, second or third. A *first generation biorefinery* processes valuable feedstocks such as edible agricultural crops (corn and sugar) to produce biofuel (1st generation biofuel). This approach is no longer considered acceptable, owing to the 'food-fuel' debate. The 1st generation biorefinery processes were operated on the basis of a "single raw material, a single major product". A *second generation biorefinery* processes non-food crops such as wheat straw, corn cobs and wood. These typically operate on the basis of a single feed material, and several major products and co-products. The feedstocks may also be termed first, second or third generation: arable crops, non-food biomass (such as agricultural residue, woody biomass and lignocellulose), and algae or waste materials respectively.

The *third generation biorefinery* is an emerging concept. In this biorefinery, a combination of any of the six bio-platforms (biogas, H_2, C6 sugars, C5 sugars, oils, and organic juice from pressing wet biomass) can be incorporated to process different types of feedstocks, typically sources from waste streams or from algal biomass, with various technologies, i.e. multiple feedstocks, multiple products and multiple processes (Susan T. L. Harrison et al., 2017).

3.3 The Waste Biorefinery in Context

Typically, the biorefinery concept has been applied to woody biomass or lignocellulosic feedstocks (Fernando et al., 2006; Kamm et al., 2010) but interest is developing in the "waste biorefinery" (Susan T. L. Harrison et al., 2017; Verster et al., 2014; Verster, 2019). Burton et al. (2009) studied the potential for generating energy from waste by identifying the potential for bioconversion of components within wastewater streams to biogas via anaerobic digestion, fermentation to bioethanol and biobutanol, production of biomass, including algal biomass, for combustion and gasification, algal lipid for biodiesel, and electricity via microbial fuel cells. As an example of its potential, it was shown that conversion of the organic fraction of South African wastewaters to electricity via biogas could contribute some 7% of South Africa's energy yields.

In a study by Harrison et al. (2017), the wastewater biorefinery (WWBR) concept was developed and its potential for value addition from wastewater was explored using a variety of case studies (biogas production from municipal wastewater and associated sludges, polyhydroxyalkanoates (PHA) production, biogas and fertiliser from vinasse, polyglutamic acid (PGA) production). Using the wastewater biorefinery framework developed, preliminary case studies of the abattoir, sugar, and pulp and paper industry wastewaters were explored to demonstrate both the potential and shortcomings of this approach.

3.3.1 An overview of a waste(water) biorefinery

The overview flowsheet of the WWBR is shown in Figure 3-3. This can be used to demonstrate the nature of a waste biorefinery, albeit being specific to water streams. The WWBR can produce multiple products of value, coupled with treating the water. In the solid waste biorefinery, the production of multiple products of value is coupled instead with the minimisation of residual waste and of environmental burden. In the WWBR, multiple wastewater streams and unit operations were considered when the flowsheet was developed. The WWBR uses a single stream or multiple streams of wastewater depending on the complexity and load of the streams, as well as the nutrient balance. The (combined) wastewater stream enters the biorefinery. Solids are separated from the supernatant and sent to the solids bioreactor (Unit 4 in Figure 3-3). The wastewater is then transferred to Unit 1, the bacterial bioreactor, followed by Unit 2, the algal bioreactor and Unit 3, the macrophyte bioreactor for sequential remediation, as required. From each reactor, solids, products and wastewater streams are separated and, respectively, sent to the solids bioreactor, further downstream processing as required and the following wastewater bioreactor. The bioreactor systems can produce an array of valuable product streams (V, W, X, Y) along with the (essential) compliant water stream (Z).

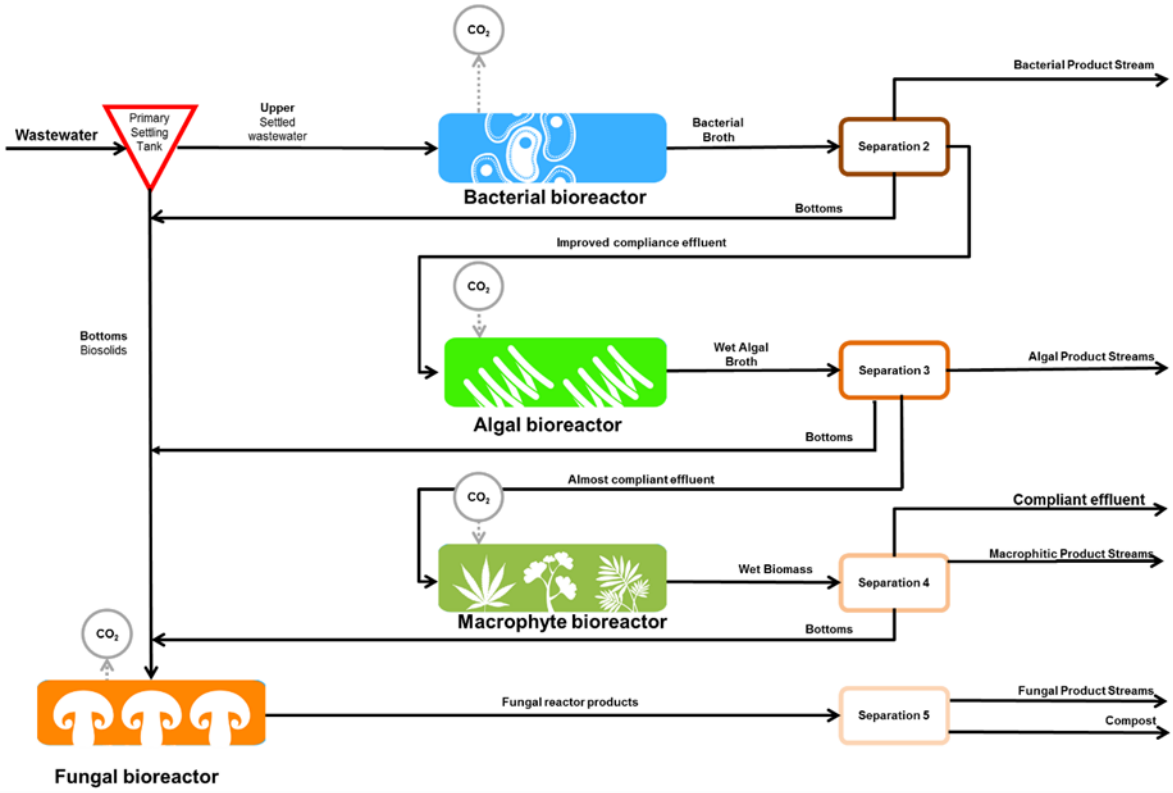

Figure 3-3 *Overview of a wastewater biorefinery* (Susan T. L. Harrison et al., 2017) *Current South African research on "Waste to Energy" (WtE)*

Energy products are the lowest-hanging fruit in the waste biorefinery, albeit not the most beneficial for valorisation. From the SANEDI report on the waste to energy (WtE) research using the different WtE conversion technologies at South African universities, universities of technology (UoT) and other research institutes (SANEDI, 2015), it was found that South African research is mainly focused on biochemical (42%) and thermochemical (40%) conversion. "Of the biochemical conversion technologies, 54% of research is done on anaerobic digestion while pyrolysis (22%) and co-gasification (20%) form the dominant thermochemical technologies researched. However, when taking all conversion technologies into consideration, anaerobic digestion (22%), fermentation (9%), pyrolysis (9%), transesterification (8%), co-gasification (8%), gasification (7%) and microbial fuel cells (6%) are the present priority research areas in South Africa" (van der Merwe, 2014).

Through these conversion technologies, a wide array of energy products can be produced. It is evident that biogas (29%) production dominates the research sector in South Africa; however, syngas (16%), bio-oil (10%), biohydrogen (10%), bioethanol (8%) and biodiesel (8%) are also energy products currently being researched. Some energy products can be further processed to produce higher value products with various applications (ASSAF, 2014).

3.3.2 Confectionery waste as feedstock

Using confectionery waste as a potential feedstock for the production of bio-based chemicals was highlighted in the feasibility report on the bio-based chemical landscape of South Africa with focus on establishing a low carbon, circular economy in South Africa (Harrison et al., 2017; Harrison et al.,

2016) . The confectionery industry generates solid and liquid waste streams rich in mono- and disaccharide sugars when manufacturing hard candy, lipids and sugars when manufacturing chocolate, and starch and sugars when the manufacturing flour-based confectionery products. These streams may be generated as mixed waste streams or as a stream rich in one type of waste dependent on whether the factory in question predominantly produces one confectionery product or where the waste streams are not mixed.

In many literature examples of waste biorefineries, the feedstock is from agricultural residues where complex molecules need to be converted to fermentable sugars prior to use in the various bioprocesses. Food waste, including confectionery waste, can be characterised in terms of complexity, nutrient content, as well as the scale at which it is generated. Key issues to be considered for food waste (including confectionery waste) is the volume of the streams, their organic loading, their regional proximity, and their potential seasonality. Complexity of the waste material must also be considered. In this case, the questions are typically around fat content, whether complex molecules are present needing pretreatment and whether inhibitory compounds such as acidulants, stabilisers and preservatives are present. Further, the provision of balanced nutrients for the biorefinery process may require supplementation of a specific waste stream with either a second waste stream to balance the nutrient profile or the addition of specific nutrients. Most typically, the C:N ratio must be considered.

For a confectionery waste biorefinery, the feedstock is already in a form that does not require the same pre-treatment steps as agricultural waste to liberate the sugars for use in a sugar platform. According to Hacking (1986), a typical analysis of confectionery waste solids (% w/w) is: sucrose 55%, glucose 16%, starch 22%, gelatine (protein) 3.5%, caramel 2%, organic acids 1% and coconut 0.5%. This translates to 76% sugar, 22% starch and 3.5% protein; hence, it is an ideal feedstock for bio-based products such as alcohols, enzymes, organic acids, single cell proteins (SCP), pigments and biofuels.

An example of a process option matrix for a sugar-based biorefinery to produce multiple bio-based chemicals using aerobic and anaerobic fermentations is shown in Figure 3-4. This, again, indicates the broad selection of potential products from the C6-based biorefinery. Starch in the feedstock can be relatively easily hydrolysed to sugar monomers as a pretreatment (not shown). For any aerobic or anaerobic bioprocess, the nutrient requirement in terms of the carbon to nitrogen ratio must be balanced; if the nitrogen content is insufficient, it must be supplemented, or vice versa. Similarly, supplementation of other nutrients may be necessary.

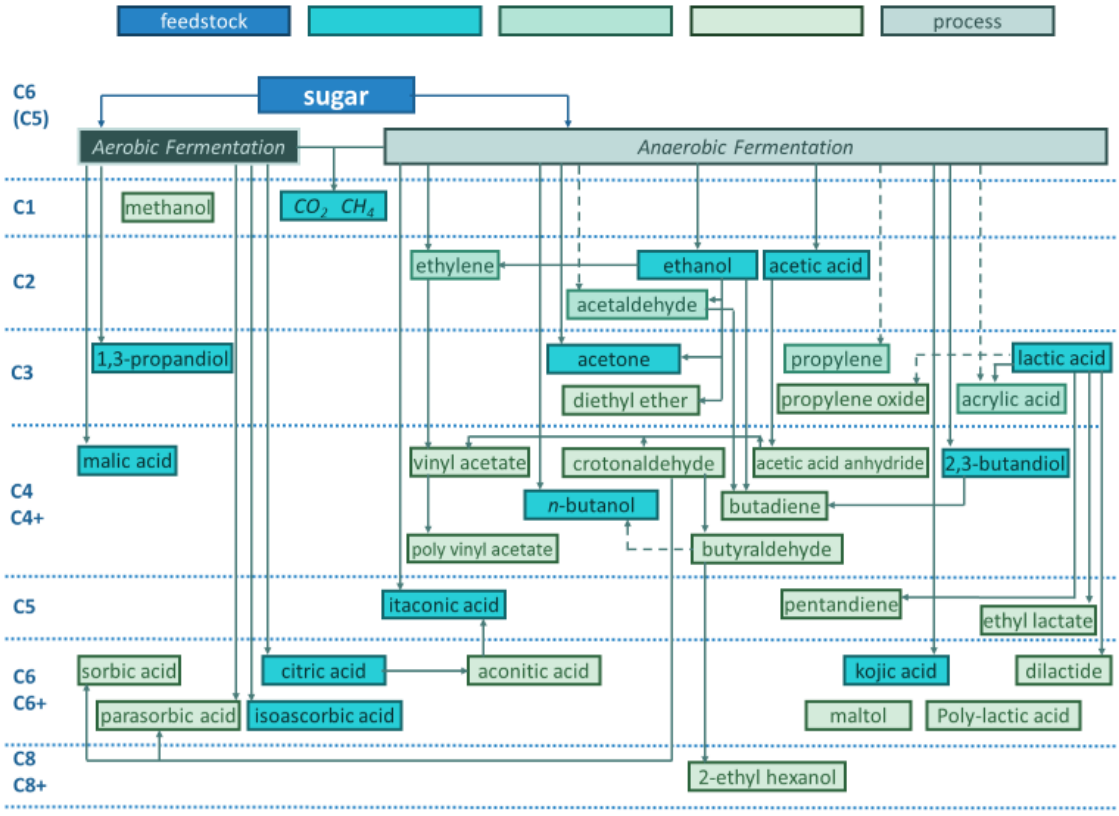

Figure 3-4 Various biobased chemicals that can be produced from the aerobic and anaerobic fermentation of sugar: after Kamm et al. (2010)

3.4 The relevance of the Waste Biorefinery

By integrating several bioreactor processes, the biorefinery approach provides opportunity to utilise all components of the waste stream by prioritising depletion of a specific component and formation of a particular product in each sub-process. Through multiple unit operations, both performance of individual processes and the overall biorefinery process can be optimised. The conversion of waste to value via the component bioprocesses in a biorefinery is centred on the conversion of the organic carbon, nitrogen, phosphorous and other contaminants in the feed stream to value-added products including bioenergy, bio-based chemicals and, when treating wastewaters, 'fit-for-purpose', compliant water. This approach is increasingly recognised for its potential contribution to the bioeconomy while augmenting industrial ecology and cleaner production (Susan T. L. Harrison et al., 2017). A biorefinery needs to generate products of sufficient value to make it economically viable, while simultaneously remediating the waste as a non-negotiable environmental contribution. This concept contributes to valuing the waste conversion as an integrated component of a wider system rather than a unit process for "end-of-pipe treatment".

Globally, a paradigm shift is required with respect to our perspective on waste generated by processes. This waste needs to be seen as a resource that can be valorised by another process, thereby improving resource efficiency. The concept “toward zero waste”proposes that as much of the waste generated in the manufacturing process as possible is treated, valorised and recycled into the process such that the waste sent to landfill, or otherwise disposed of, is minimised (Matete and Trois, 2008; Miah et al., 2015). This concept needs to respect the laws of entropy by which some waste will occur, hence the concept “towards zero waste” and not “zero waste”.

One of the goals of the Waste RDI Roadmap (DST SA, 2014) is the diversion of waste from landfill towards value-adding opportunities, consistent with the “towards zero waste” concept. This includes prevention of waste formation and the optimised extraction of value through re-use, recycling and recovery, as a means of creating significant social, economic and environmental benefit. This approach is aligned with the principles of cleaner production, industrial ecology and maximising resource productivity. In place of disposal to landfill, confectionery solid waste has potential to be used as alternative biomass for bioconversion in a biorefinery system. It is proposed that these streams which are rich in sugars, proteins and fats can be used as the primary feedstock in a waste biorefinery, using bioprocesses to create valuable products and therefore contributing to the circular economy. This can be used in formulation of generic fermentation media for production of valuable bioproducts. This is of interest in the sustainable production of bio-based chemicals and bioenergy. In a sustainable economy, processing waste streams to products serves the dual function of reducing the environmental burden of waste disposal and reducing the demand for primary feedstocks, in particular fossil fuels.

A biorefinery should produce bio-based products with either market value sold for profit or on site use to reduce costs or both. The products produced may be intermediate products such as platform chemicals; ultimately the products should be marketable and their pricing competitive. The biorefinery rests on three pillars: it provides a solution to environmental issues and contributes to social and economic growth, as well as to rural development and employment. The IEA Bioenergy Task 42 (n.d.) proposed that the process can only be economically viable if the feedstock and processing costs are relatively low; alternatively, the relative costs must be balanced by a combination of product market value and reduction in disposal costs of the waste.

Existing waste biorefineries approaches are typically limited to a single process, using anaerobic digestion (AD) to produce power and heat which can be fed directly back into the manufacturing process to reduce overheads costs. This has been commercially demonstrated by companies such as Nestlé United Kingdom (UK) and Ireland’s Fawdon factory in Newcastle upon Tyne who achieved “zero waste to landfill” by constructing an AD plant on site. This AD facility treats over 200 000 L/day of effluent and wash waters and 1 200 tonnes of residual products per year from the factory and produces biogas. The biogas produced by this process can generate approximately 200 kW of electricity by feeding the gas to a combined heat and power engine (CHP) (Kane, 2015), resulting in a 10% reduction in the sites carbon footprint, as well as substantial energy savings and reduction in disposal costs. The Mars chocolate factory in Veghel in the Netherlands, constructed a Memthane® wastewater treatment plant to treat the chocolate wastewater effluent generated by the factory. This plant is able reduce the COD from 10 000 to 50 mg/L without having to pre-treat the effluent. In addition, the treated water does not require a polishing step for the discharge before it is

transported to a nearby municipal wastewater treatment plant. The plant has a 1 000 000 m^3 capacity that is recirculated to the boiler house which contributes 10% of energy used to run the factory (Dutch Water Sector, 2014). Mondelēz International has managed to achieve its 2010 goals of "zero waste to landfill" at its Banbury manufacturing site in the U.K. by installing a biogas engine with the capacity to power 400 homes with electricity (Food and Drink Federation, 2012).

Based on the successes being implemented with AD on confectionery plants in Europe, we both explore this for South Africa and explore whether there is potential for further value creation through bioproduct manufacture. For such an initiative, product selection is key and is the focus of Chapter 4.

3.5 The Confectionery Waste Biorefinery

3.5.1 The confectionery waste biorefinery concept

In this study, all the waste streams (solid and liquid) leaving the confectionery manufacturing site are considered, as are the raw materials needed to produce the end product. Figure 3-5 provides an overview of the confectionery manufacturing process, its waste streams and the potential confectionery waste biorefinery. Ideally, the products of the confectionery waste biorefinery should contribute to the value chain of confectionery manufacture, providing a secure market. Where this is not possible, market demand for these products must be secured. For example, there may be opportunity to produce high-value products such as bioplastics that would contribute to the production of packaging material (García et al. 2011); bioenergy that can potentially be used for electricity production that can be used to run the plant (Kane, 2015); and PGA which can be used as a soil conditioner for the sugarcane crops needed to produce the sucrose required for confectionery manufacture (Zhu et al., 2014). The waste streams generated by the biorefinery consist mainly of spent biomass from fermentation as well as digestate from AD. The spent biomass can be used as feedstock to the AD process. Anaerobic digestate, a byproduct of anaerobic digestion, contains nutrients such as nitrogen, phosphate, potassium and trace elements. Depending on the anaerobic process configuration used, the digestate may be present as a slurry containing approximately 5% dry matter or a liquor from which most of the solid content has been removed together with a de-watered sludge as a dry solid material. The former two are favoured for agricultural irrigation and the latter as a soil conditioner (where geographical location permits). The advantage of using digestate, or concentrated digestate, in the agricultural sector is that no waste or wastewater is generated. Thus, there is potential to increasingly close the waste loop, working towards the "zero waste to landfill" model.

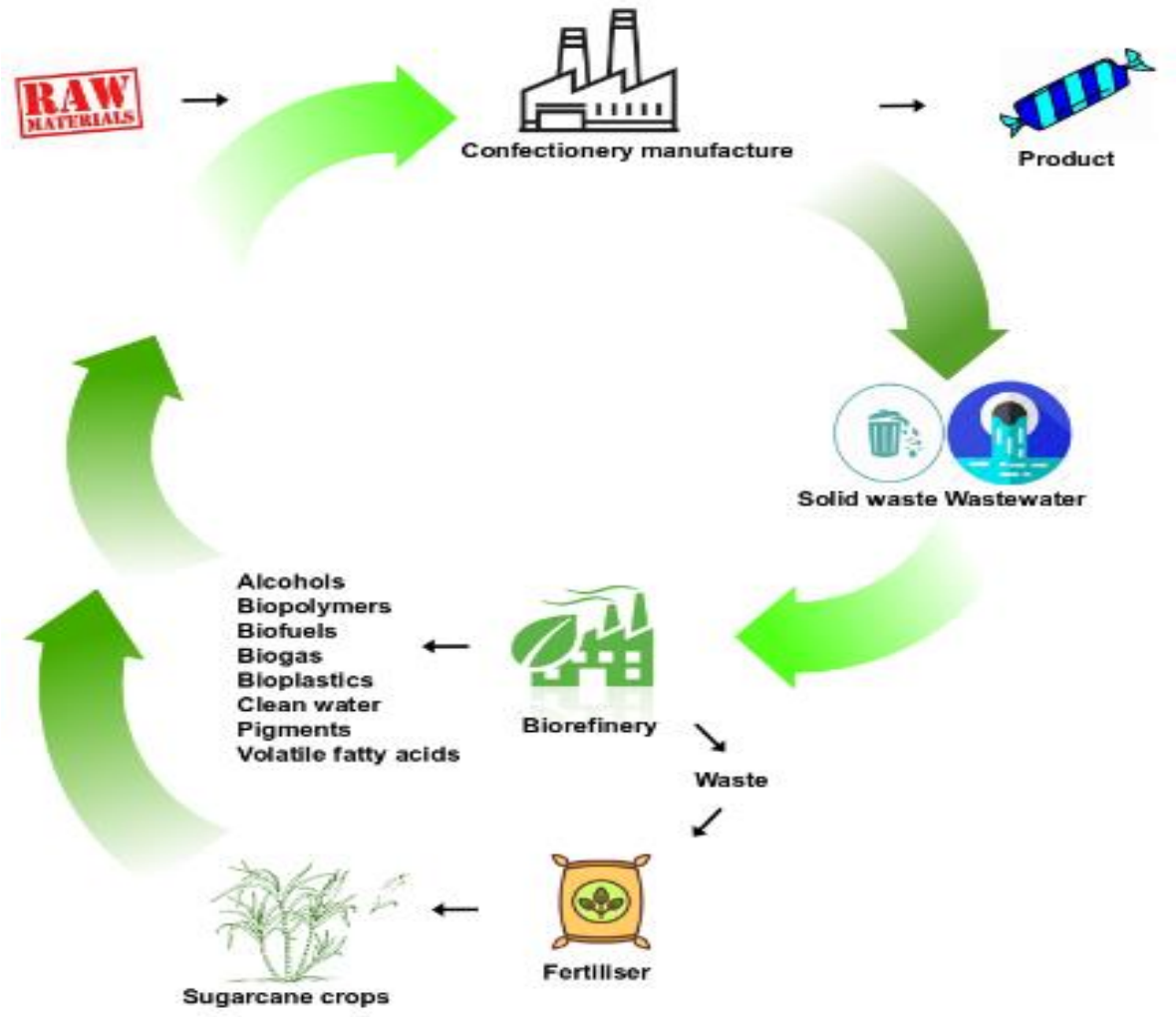

Figure 3-5 The conceptual confectionery waste biorefinery

3.5.2 Characterisation of sample South African confectionery waste streams

To determine key properties of confectionery waste, several confectionery waste streams were obtained from an anonymous confectionery factory site; these contained defective chocolate, marshmallow, and hard candy. The macronutrients identified for cell growth as well as bioenergy, biopolymer, organic acid and fine chemical production (see Section 4) included a carbon, nitrogen and phosphorus source, as well as key trace elements, namely, Ca, Fe, K, Mg, and Mn (Madonsela, 2013). To determine the supplementation of candy waste required to support microbial growth, it required characterisation. Table 3-1 is a summary of the analytical techniques used for the analyses and characterisation of the confectionery waste.

The confectionery waste was characterised in terms of trace elements, sugars and free amino nitrogen. Confectionery waste is known to contain a large proportion carbohydrate (Bussiere and Serpelloni, 1985), and acidulants (Sortwell, 2004) which can form the carbon source for the cultivation of various microorganisms. Confectionery waste was characterised to determine whether supplementation with nutrients was required. The characterisation of confectionery waste was conducted using the analyses found in Table 3-2. The aim was to quantify the nutrients in the

waste and compare these with the nutrients needed for production of potential bioproducts (see Section 4.2); these included nutrients such as free amino nitrogen, fermentable sugars, and trace elements.

Table 3-1 Analytical techniques used for the analysis and characterisation of confectionery waste

Nutritional Component	Method/Instrument	Reference
Total Kjeldahl Nitrogen (TKN)	Kjeldahl method	Spedding et al. (2012)
Elemental analysis: C, H, O, N, P, K, Ca, Na, Mg.	ICPOES	Boss and Fredeen (2004)
Total organic carbon (TOC)	TOC analyser	HACH 10128 (n.d.)

The N, TOC and trace element results obtained for confectionery waste are tabulated in Table 3-2. The results show that chocolate, marshmallow and hard candy waste consisted largely of total organic carbon (TOC). The chocolate waste contained the most TKN, the most N at 0.5% on a mass basis. However, the concentrations were too low to be used as a nitrogen source as the requirement of at least 1 g of NH_3 per litre has been reported in many microbial systems. The C:N ratios of chocolate, marshmallow and hard candy were calculated and are presented in Table 3-2. Hard candy had the highest C:N ratio of 400:1. This high value indicated that the waste consisted mostly of carbon with little to no nitrogen. From the ICP-OES data, the concentration of trace elements was so low that they were considered to be negligible. Since the waste consisted mostly of organic carbon, it has to be supplemented with additional nutrients for the cultivation of microbial systems.

Table 3-2 Characterisation of chocolate, marshmallow and hard candy waste

Parameter	Chocolate	Marshmallow	Hard Candy
Total Kjedahl nitrogen (g/g as N)	0.005	0.003	0.001
Total organic carbon (g/g as C)	0.551	0.410	0.430
C:N ratio	110:1	124:1	400:1
Trace elements			
Calcium (g/g)	0.036	0.057	0.069
Copper (g/g)	0	0	0
Iron (g/g)	0.004	0	0.005
Manganese (g/g)	0	0	0
Magnesium (g/g)	0.013	0.011	0.001
Potassium (g/g)	0.136	0.002	0.001
Zinc (g/g)	0	0	0

4 Product Selection for the Confectionery Waste Biorefinery

4.1 Introduction to Product Selection

Product selection for a waste biorefinery must meet the processing requirements highlighted by Harrison et al. (2017) in addition to standard bioprocess requirements. These include the appropriateness of the pre-defined concentration ratios of C:N:P in the feedstock, its variability, its availability, and restrictions on process conditions. Primarily these restrictions focus on limited potential for sterilisation, limited potential for high energy mass transfer processes and the applicability of downstream processing options that permit early stage, low energy product recovery to high concentration prior to its purification in a smaller volume process. Minimal need for supplementary raw materials is also desirable. Figure 4-1 presents a framework for choice of process in a wastewater biorefinery (WWBR) (Harrison et al., 2017) from which components can used in decision making for the waste biorefinery. Additionally, bioprocesses used must not hindered by the presence of contaminants present e.g. stabilisers, acidulants and preservatives. In a waste biorefinery, optimising operations in terms of energy and materials inputs is critical.

In Figure 4-2, potential product classes for the WBR are provided and classified into six levels. These levels are organised on product value and product volume. It is important to note that in a WBR, selection of high value products with small markets only will not allow the processing of the full waste burden. Hence inclusion of large volume products, potentially of lower value, is important.

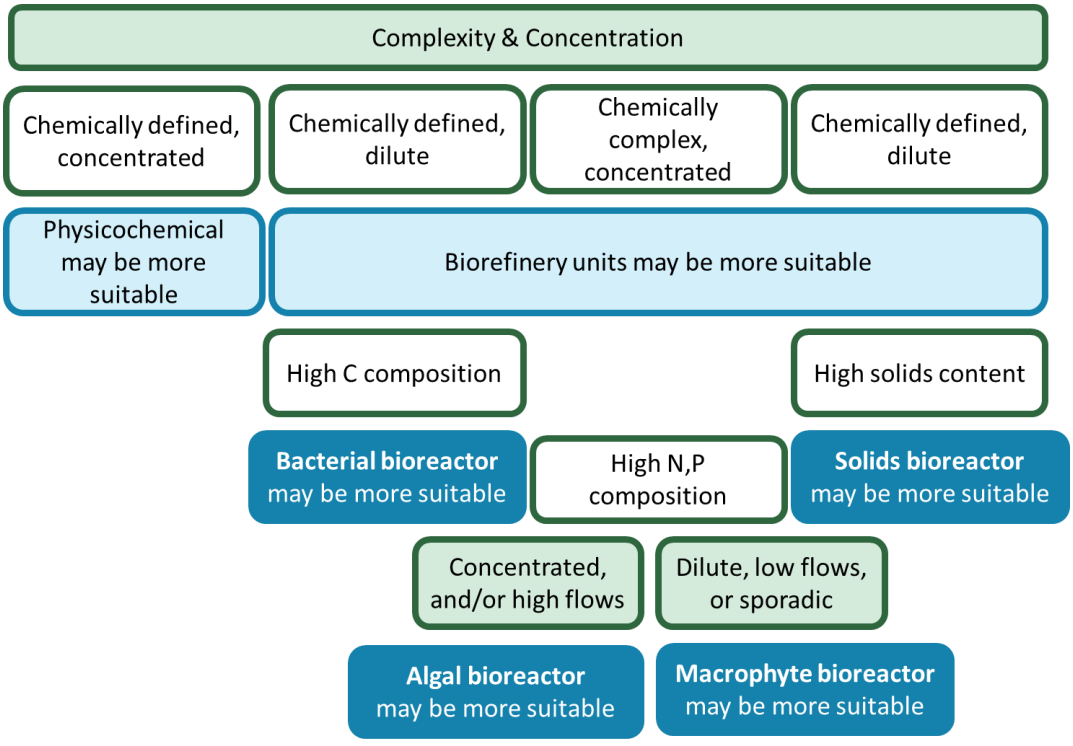

Figure 4-1 Decision making matrix to guide selection of priority bioreactor in the WWBR (Harrison et al. 2017)

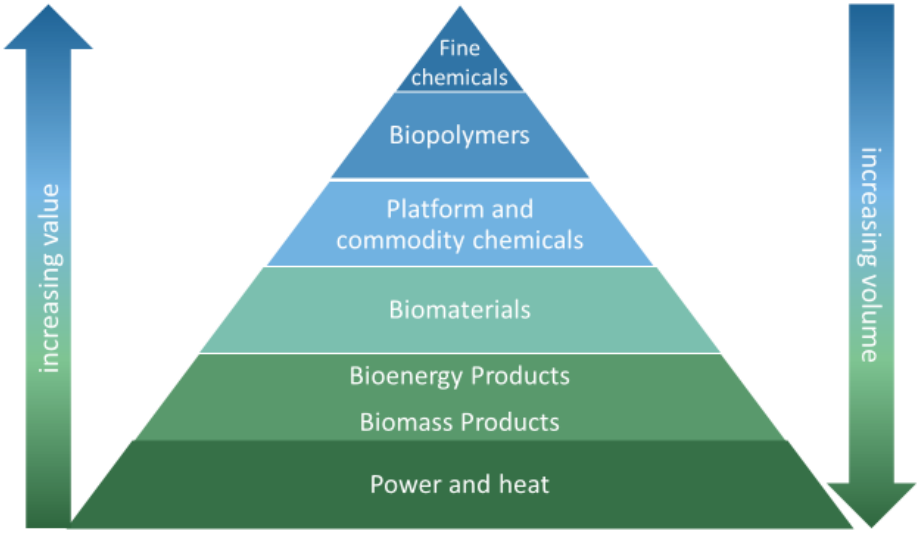

Figure 4-2 Potential products for a waste biorefinery (WBR), categorised on a volume vs value hierarchy

In addition to meeting the techno-economic requirements to which all processes must comply, the product selection for a waste biorefinery requires that a series of requirements are met. These include market compatibility, waste biorefinery requirements, environmental sustainability and process complexity and performance (Landucci et al., 1994), as defined in Figure 4-3.

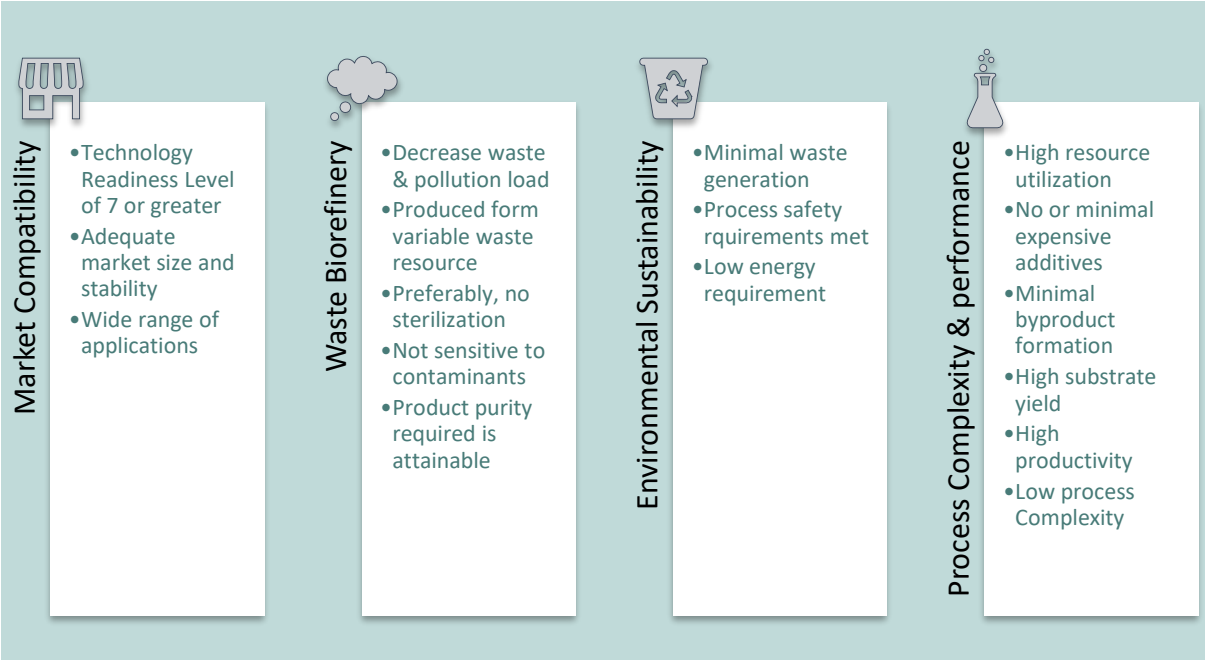

Figure 4-3 Product selection guide for a WBR: adapted from Harrison et al. (2019)

Technology Readiness Levels (TRL) are used to classify the maturity of a technology across nine levels. **Error! Reference source not found.** shows the definitions of these levels as defined by the Research Contracts and Innovation department at the University of Cape Town. A high TRL,

preferably of 7 or above (Harrison et al., 2016) but certainly of TRL 3 and above, is desirable for consideration for implementation into the WBR.

Table 4-1 Technology readiness levels (TRL) definitions (http://www.rci.uct.ac.za/technology-readiness-levels).

TRL 1	Basic idea
TRL 2	Concept developed
TRL 3	Experimental proof of concept
TRL 4	Lab demonstration
TRL 5	Lab scale validation
TRL 6	Prototype demonstration
TRL 7	Pilot scale
TRL 8	Commercial design
TRL 9	Ready for full deployment

In South African context, the recently compiled report on the feasibility of bio-based bioproducts for the bio-economy sector (Harrison et al., 2016), commissioned by the Department of Science and Technology (DST) can be used as a tool to select products with high market potential. Bioproducts considered include platform chemicals, biopolymers, nutraceuticals including bioactives, pigments, niche enzymes, biosurfactants and biolubricants, animal and aquafeeds and organic fertiliser. The product potential in South Africa was ranked based on its market demand in South African and global markets, current use and future applications, complexity of production routes, technology readiness and barriers to market, using a combination of technology review, market literature, import–export data, application analysis, and expert opinion.

In the waste biorefinery based on confectionery waste, several specific factors must inform product selection. These include:

- As solid waste, the organic loading of the bioprocess can be manipulated to be high and high concentration processes can be operated, unlike the wastewater biorefinery.
- There is feedstock variability.
- The feedstock is rich in carbon, with potential for protein and fat components, depending on the product range of the plant.
- The use of the product within the production process or related activities can ensure market uptake.
- The industry is not highly seasonal.
- A single, or at most dual, product system is sought to ensure a focussed business opportunity while reducing the waste load to landfill. This differs from the WWBR in which multiple units are required to ensure delivery of compliant water.

In accessing the potential of bio-based products that can be produced and used in the food and related industries, the following product categories were considered: biomaterials, biocatalysts, bioenergy and biochemicals. These are expanded in Table 4-2. Factors were considered as the basis

for the evaluation framework for the selection of the potential products include: i) the technology readiness level of South Africa to implement the technologies required; ii) the demand for the product within South Africa's confectionery production; iii) the market or potential market for products; iv) the potential of drop-in bio-based chemicals. Pharmaceuticals and products going directly into the food chain may be included provided particular attention is given to the source of the carbon source and nutrients, the required standards and policies, the cost of sterilizing the waste stream or product or both. Each of the above-mentioned factors were assigned a weighting factor to assist in the decision-making process.

To inform product selection for the waste biorefinery, a decision matrix is needed to determine the product of most value that is possible on each site. Figure 4-1 provides an early approach to considering the feasible products. Factors of technology readiness, market demand, competition, environmental burden or benefit, process complexity, process performance and economic feasibility must be super-imposed on these, including the hurdles highlighted in Figure 4-3. According to the Green European Foundation, products can be categorised according to a range in value and market size (Figure 4-2). The lower value bioenergy tier has the lowest value per weight compared to other value-added products but can, however, often be produced in larger volumes to meet the larger market demand. Similarly, chemicals and materials have a lower market value than fine chemicals (Davis et al., 2017), but higher market demand. The waste biorefinery is best designed to incorporate products from both zones to facilitate conversion of the full waste stream owing to sufficiently high market demand while ensuring sufficient value creation to ensure economic feasibility.

Table 4-2 Potential products from confectionery waste

Category		Type of product	Production process	Application
Biomaterials	Biopolymer	Bio-PGA Levan	Microbial bioprocess	Packaging Presentation Preservation Transportation
	Bioplastics	Bio-PHB Bio-PET		
Biocatalysts	Enzymes	Invertase Glucose Oxidase Lipases Amylase Protease L-Asparaginases		Confectionery production
Bioenergy	Biofuels	Bioethanol Biobutanol Biodiesel		Energy recovery Manufacturing Transportation Storage
	Biogas	Biohydrogen Biomethane	Anaerobic Digestion	
Biochemicals	Organic acids	Volatile fatty acids		Commodity products
		Acetic acid Malic acid/Citric acid Succinic Acid	Microbial bioprocess	Confectionery additives Packaging
	Sugar Alcohols	Sorbitol Xylitol		
	Biopigments	Astaxanthin Beta-Carotene *Monascus* pigments Riboflavin		

4.2 Product Selection for the confectionery waste biorefinery

In exploring the potential for the confectionery waste biorefinery, we have selected products for investigation across different ranges of the volume – value spectrum (Figure 4-2) and across the functional categories considered in Table 4-2. The products selected for consideration are detailed in Table 4-3.

Table 4-3 Product for investigation for selection in the confectionery waste biorefinery

Product	Product category	TRL	Use	Primary feedstock
Poly-glutamic acid (PGA)	Biopolymer	TRL 6	Soil conditioner Water retention	C6 sugars NH_4^+
Poly-β-hydroxyalkanoates (PHA)	Biopolymer	TRL 9	Biodegradable plastic for healthcare products, packaging and one-use plastic products	C6 sugars Organic acids CO_2 & H_2
Citric acid	Organic acid	TRL 9	Acidulant	C6 sugars
Purple Monascus pigment	Biopigment	TRL 4	Colorant	C6 sugars
Bio-ethanol	Alcohol	TRL 9	Biofuel Platform chemical Precursor to polymer	C6 sugars
Biogas	Biomethane	TRL 9	Bioenergy	C6 sugars, C5 sugars, organic acids etc

5 Bioenergy from Confectionery Waste

5.1 Introduction to bioethanol and biogas production from sugar-rich feedstocks

In this chapter, the feasibility of using confectionery waste streams as feedstocks for bioenergy production is reported based on experimental data. Confectionery waste has a carbon content between 60 and 90 % (Pleissner et al., 2013) with variation in the proportions of simple and complex carbohydrates, depending on the origin of the wastes. Candy and chocolate confectionery waste streams contain large quantities of simple sugars such as sucrose, glucose and fructose. These may be suitable for use in 'clean' single strain bioprocesses. Flour confectionery products such as cakes and biscuits contain lesser proportions of simple sugars and a greater proportion of more complex carbohydrates, mostly in the form of starch. The objective of this research study was to use confectionery waste in different process technologies for energy recovery. Here we considered bioethanol production through the microbial fermentation of sugars (sucrose, glucose and fructose) by *Zymomonas mobilis* and biogas recovery from a mixed confectionery waste stream and flour confectionery product through anaerobic digestion (AD).

5.1.1 Biofuel production

Biofuels can be classified as 1st, 2nd and 3rd generation biofuels, differentiated on a basis of the type of feedstock used. The 1st generation biofuels are produced from organic matter with high sugar or starch content and other simple compounds such as grains and seeds. Typically, these organic compounds can also serve as food. Grains containing high starch content are firstly hydrolysed to glucose to make the carbohydrates accessible to fermentative organisms. The 2nd generation biofuels are produced from biomass waste streams or non-food biomass crops, often considering the more complex carbohydrates such as agricultural lignocellulosic material (Nigam and Singh, 2011). When utilising lignocellulosic feedstocks, the production of 2nd generation biofuel requires extensive pre-treatment of the lignocellulosic material prior to bioconversion. These pre-treatment methods use harsh conditions and are energy extensive (Huang et al., 2015). Third generation biofuels use purpose-grown energy crops, especially algal biomass, with desired properties for energy product recovery as well as non-agricultural waste streams. Conversion of waste organics such as confectionery waste to biofuels form part of 3rd generation biofuels; no extensive pre-treatment is required.

5.1.1.1 Fermentation of feedstocks

Biofuels such as bioethanol and biobutanol are produced by microbial fermentation of biomass containing significant amount of accessible sugars using fermentative facultative anaerobes (Nigam and Singh, 2011). Bioethanol is most commonly produced by yeast fermentation of carbohydrates under anaerobic conditions using S*accharomyces cerevisiae* which ferments hexose sugars anaerobically to bioethanol using the Embden-Meyerhof (EM) or glycolytic pathway (Lin and Tanaka, 2006). Alcohol fermentation by *S. cerevisiae* is a well-developed process applied at industrial level. Recently, studies have focused on developing and/or identifying potential microorganisms for bioethanol production that can utilise a wider range of substrates. *Pichia stipitis* was identified as the most promising yeast alternative to *S. cerevisiae* for ethanol production due

to its ability to utilise both hexoses and pentoses . Du Preez et al. (1986) demonstrated *P. stipitis* fermentation of various sugars liberated from the pre-treatment of lignocellulosic biomass. Findings showed that the yeast was able to produce ethanol from glucose, mannose, galactose, xylose and cellobiose. However, *P. stipitis* produces ethanol aerobically and therefore requires continuous aeration during large-scale fermentation resulting in increased costs (Agbogbo and Coward-Kelly, 2008). Interestingly, bacteria have also shown desirable properties in sugar to bioethanol fermentations.

Zymomonas mobilis has attracted attention as suitable industrial bioethanol production strain. *Z. mobilis* is a naturally occurring gram negative fermentative ethanogenic bacterium (Yang et al., 2016), capable of ethanol production from glucose, fructose and sucrose substrates (Borsari et al., 2006; Ruanglek et al., 2006). Benefits of *Z. mobilis* bioethanol production systems include high ethanol producing capabilities and tolerance to high ethanol concentration (Ruanglek et al., 2006). Sugar fermentation is achieved via the Entner-Dondoroff (ED) pathway to produce ethanol and release CO_2 (Zm et al., 2005). The ED pathway, pyruvate decarboxylase and two alcohol dehydrogenase isoenyzmes form the central metabolism of *Z. mobilis* (Pentjuss et al., 2013). Compared to *S. cerevisiae*, *Z. mobilis* has a high specific sugar uptake rate and has the ability to co-ferment mixed sugar substrates (Kim et al, 2010) i.e. it does not demonstrate the glucose effect, also known as catabolite repression, as seen in *S. cerevisiae*. Additionally, during the ED pathway, fermentation is carried out with 50% less ATP relative to the EM pathway active in *S. cerevisiae*, resulting in better ethanol yields (De Kok et al., 2012).

Various comparative studies between *Z. mobilis* and *S. cerevisiae* for bioethanol production have been reported. He et al. (2014) highlighted that one of the advantages of *Z. mobilis* as an ethanologen includes the organism's high specific rate of sugar intake. This was supported and further elucidated by Yang et al. (2016)'s review that *Z. mobilis* has a high-specific cell surface area and therefore high substrate consumption rate compared to *S. cerevisiae*; this leads to a higher productivity. As ethanol production by *Z. mobilis* is achieved via the ED pathway which requires less ATP than the EMP pathway, as mentioned above, higher ethanol yields are achieved at lower biomass concentrations using *Z. mobilis* as fermentation strain. In a study conducted by Ofosu-Appiah (2016), *Z. mobilis* had a higher ethanol yield compared to that of *S. cerevisiae* using pito mash (sorghum spent grain generated from brewing) as substrate. This was similar to results reported by Rogers et al. (1979) where *Z. mobilis* had higher ethanol yields compared to a flocculent yeast *S. carlsbergensis* from high glucose concentrations (10-25%).

5.1.2 Anaerobic digestion of carbohydrate-rich wastes

Anaerobic Digestion (AD) of food wastes has proven to be an effective method for bioenergy recovery, as biogas or biomethane, from these mixed solid wastes due to their high moisture content and biodegradability. AD is considered as an alternative energy process as it results in the production of methane-rich biogas suitable for energy production as heat or power or both, supplementing or partially replacing the use of fossil fuels. As a result, the AD of food waste has become a research area of interest for the treatment of solid waste, preventing its disposal to landfill, with co-production of alternative renewable energy in the form of biogas (Kader et al., 2015; Zhang et al., 2007).

5.1.2.1 Anaerobic digestion process

Anaerobic digestion is a promising bioconversion technology for the production of renewable energy (Scarlat et al., 2015). Organic solid or liquid waste is degraded into biogas, containing predominately methane (60%), carbon dioxide (40%) and traces of hydrogen sulphide and water vapour (Surendra et al., 2015 Chynoweth et al. 2000). This process is carried out by a microbial consortium at mostly moderate temperatures (Chynoweth et al., 2000), although thermophilic processes are reported. AD for biogas production is a four stage process (Figure 5-1), including hydrolysis, acidogenesis, acetogenesis and methanogenesis (Khalid et al., 2011). During hydrolysis, facultative anaerobes hydrolyse high molecular weight compounds such as complex carbohydrates, lipids and protein, using extracellular hydrolytic enzymes, into small soluble compounds such as sugars, amino acids and free fatty acids. In the acidogenesis step, the hydrolysed products are fermented into alcohols and organic acids such as volatile fatty acids (acetate, propionate, butyrate and lactate). The acetogenesis step is relatively rapid; VFAs are further digested into acetate, H_2 and CO_2 which are used as substrates for methanogenesis (Zhang et al., 2014).

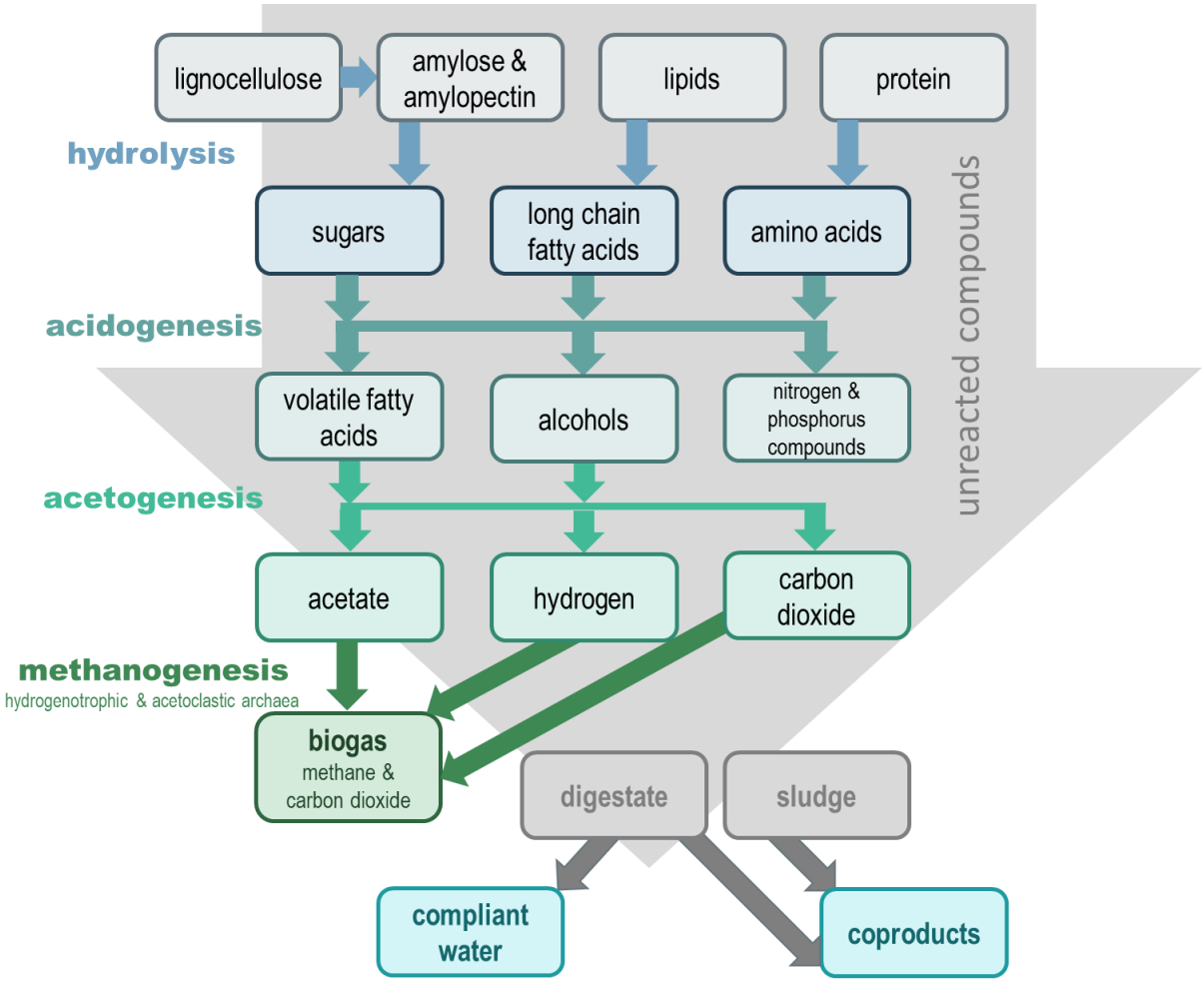

Figure 5-1 The four stages of the process of anaerobic digestion: adapted from Harrison et al. (2019)

5.1.2.2 Anaerobic digestion of confectionery waste

Globally, confectionery industries have implemented a range of strategies for waste treatment. Nestle-UK developed an anaerobic system for wastewater treatment with production of renewable energy to meet with 10% of site energy demand (See Section 1.2). Solid waste such as chocolates and sugar confectionery milled into small particles and mixed with cleaning water to create a 'chocolate soup' for anaerobic digestion ("Breathing new life into leftovers: how one factory achieved zero waste," 2018). A limiting factor to using confectionery waste in anaerobic systems is the high carbohydrate content of the waste stream. Break down of this readily degradable substrate can generate products that can acidify the digester, inhibiting methanogenesis (Lafitte-Trouqué and Forster, 2000). A solution to this challenge is to use a continuous two stage process, as illustrated by Magnusson (2010) in a study using confectionery waste to produce biogas.

Mars-UK has also found means to reuse, recycle and generate fuel from waste products, reducing the amount of waste going to landfills (Section 1.2). In 2014, Mars announced the development of an anaerobic wastewater treatment plant for the purification of both solid waste and waste water in biological processes. This initiative reduced waste to landfills by 250 tons and the biogas recovered is used to power the onsite chocolate factory ("Mars reaches zero-waste-to landfill milestone, helped by converting waste to energy," 2016).

Bioenergy recovery from confectionery waste may be a sustainable approach to the valorisation of these waste streams. Both liquid fuel, such as bioethanol or biobutanol, and gaseous energy, such as biogas, can be derived from confectionery waste using specific technologies. Energy can be derived from biofuels and biogas through direct heating in boilers to generate steam, while combined heat and power (CHP) can be co-generated from low carbon energy sources in electrically powered steam generators located alongside processing equipment in a confectionery plant (Adendorf, 2009).

5.2 Experimental approach

5.2.1 Bioethanol production from confectionery products by *Zymomonas mobilis*

5.2.1.1 Culture and cultivation methods

Zymomonas mobilis ATCC 31821 was used as the bioethanol production strain. The strain was maintained by cultivating in RM (Rich Media) containing 20 g/L glucose as a carbon source, 1 g/L yeast extract, KH_2PO_4 and MgCl (Goodman et al., 1982). *Z. mobilis* was cultivated overnight at 30°C under anaerobic conditions with agitation at 180 rpm. It was then stored in 20% glycerol stocks at -80°C and subcultured periodically. All media used in this study were sterilised by autoclaving at 121°C for 20 mins. For anaerobic fermentation studies, 1 mg/L of Resazurin was included as oxygen indicator. Resazurin turns pink in the presence of oxygen under the medium conditions used here.

For fermentation inoculum preparation, the glycerol stocks were streaked onto RM agar plates containing glucose as substrate and incubated at 30°C. To prepare the pre-inoculum, a single colony was aseptically transferred from the agar plate to 10 ml RM with glucose. Cultures were incubated at 30°C with shaking at 120 rpm for 12 - 16 hrs. The pre-inoculum was used to inoculate 100 ml

inoculum media and incubated at 30°C with shaking at 120 rpm for 12 - 16 hrs. Initial experiments were performed with a glucose-supported inoculum; however, in later studies the inoculum preparation was modified. For sucrose and mixed sugar studies, the bacterial cells were pre-exposed to sucrose by serial sub-culturing in medium containing varying proportions of glucose to sucrose, with increasing sucrose levels over time. The aim was to promote the production of the extracellular sucrose hydrolysing enzyme for improved sucrose utilisation by the bacterium. The pre-inoculum was used to inoculate medium containing a ratio of 4:1 glucose to sucrose, cultivated overnight at 30°C with shaking (120 rpm) for 12 - 16 hrs. This culture was used to inoculate medium containing a 1:1 ratio of glucose to sucrose, cultured as described, and subsequently used as inoculum for medium containing a 1:4 ratio of glucose to sucrose. Overnight cultures to be used as inocula for the fermentation studies were prepared from the culture pre-exposed to sucrose and in media containing synthetic carbon compositions closely resembling the carbon source to be tested in the fermentation study i.e. sucrose or mixed sugars for confectionery waste studies.

5.2.1.2 Analytical methods

Sugar and ethanol quantification

Detection and quantification of residual sugars and ethanol produced was carried out using HPLC equipped with a BioRad HPX-87H ion exclusion column and RI detector. The mobile phase was 5 mM H_2SO_4, used at a flowrate of 0.50 ml/min with a detector temperature of 40 °C. A calibration curve was generated by preparing a stock solution of 1 g/L each of sucrose, glucose, fructose and ethanol. The stock solution was diluted using mobile phase to achieve a concentration range of 1, 0.8, 0.6, 0.4, 0.2 and 0 g/L. Samples were prepared by dilution in mobile phase, filtered through 0.22 µm syringe filters and analysed in triplicate. For chocolate samples, the lipid component of the chocolate was extracted prior to HPLC analysis. This was achieved using the lipid extraction method modified from Bligh and Dyer (1959).

Extraction of lipids for sugar and ethanol quantification from chocolate tests

Samples obtained from *Z. mobilis* fermentations with chocolate as substrate were extracted with a mixture of chloroform and methanol to achieve a miscible liquid. This was done by adding 300 µL sample to 300 µL chloroform and 600 µL methanol. Samples were mixed by vortexing at high speed for 3 min. A further 300 µL chloroform and 300 µL dH_2O were added and samples again vortexed at highspeed for 1 min followed by centrifugation at 14 400 *x*g for 10 mins. This approach results in the formation of two layers, the chloroform layer containing all the lipids and the methanolic layer containing sugars, ethanol and other aqueous soluble compounds. This was followed by a washing step by addition of an equal volume of chloroform into the methanol extract, vortexing and centrifugation (as described above) to remove remaining lipids. This step was repeated until all lipids were removed. For ethanol analysis, the chloroform was omitted as it interferes with the ethanol retention time in the HPX-87H column.

The recovery of sugars and ethanol from the extraction method was validated by preparing the calibration stock solutions in the background of chocolate media. The sugar and ethanol standards in chocolate media were extracted and diluted in mobile phase to generate a calibration curve. Further validation included spiking samples with known ethanol and sugar concentration prior extraction to test for complete recovery of known spiked concentrations.

Biomass quantification

Cell biomass was quantified using various methods depending on the substrate tested. For synthetic sugars, candy and marshmallow based media, cell biomass was measured using Optical Density (OD) at 600 nm. A standard curve of OD_{600} as a function of cell dry weight was prepared to determine the cell concentration in the sample. Cell dry weights (CDW) were determined by centrifugation of a known volume of culture, up to 10 mL for low cell density cultures and 4 mL for high cell densities, at 14 400 g for 10 min. Cell pellets were washed twice with 1x Phosphate Buffered Saline (PBS, containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, pH 7.4) and dried at 80°C in pre-weighed microcentrifuge tubes for at least 48 hrs before weighing for CDW determination. For experiments conducted using chocolate as the basis of the media, particulates remained in suspension hence biomass was quantified via direct cell count. Cell counts were conducted using a Thoma counting chamber under oil emersion at 1000x magnification on an Olympus BX-40 phase contrast microscope.

5.2.1.3 Small-scale serum bottle fermentations

Z. mobilis growth and ethanol production was tested in selected confectionery products expected to represent key components of confectionery waste: hard candy, chocolate and marshmallows. A simulated sugar synthetic mix comprising of the sugars found in hard candy: 50% sucrose, 25% glucose, 25% fructose was used as a control to rule out the effect of unknown additives present in confectionery waste on the fermentation process. Confectionery products were added to achieve a sugar concentration of 20 g/L sugar. Every batch of confectionery materials used for fermentation studies were characterised by HPLC analysis as described in Section 5.2.1.2 in terms of sugar content and composition. Sugar or confectionery-based solutions were supplemented with essential nutrients including 10 g/L yeast extract, 1 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$ and 1 g/L MgCl. The carbon sources and confectionery products were sterilised separately from the growth medium by pasteurisation at 105°C for 30 mins. This was done to prevent Maillard reaction of the sugars and nitrogen compound in the growth medium, and possible sugar hydrolysis at higher temperatures.

Small scale preliminary studies were conducted in serum bottles containing 100 mL fermentation medium. The serum bottles were sealed with a butyl rubber septum and crimp sealed with an aluminium cap following the Hunsgate technique. The medium was sterilised by autoclaving at 121°C for 20 min. Sugar solutions were autoclaved separately as described above preceding inoculation of the medium with an *Z. mobilis* overnight culture to achieve a starting OD 600 nm of 0.1 (0.06 g/L CDW) prepared as described in Section 5.2.1.1. Fermentation was performed at 30°C under anaerobic conditions with agitation at 120 rpm. Samples were taken at hourly intervals with a sterile syringe and needle to monitor cell biomass concentration, sugar utilisation and ethanol production following the techniques outlined in Section 5.2.1.2.

5.2.1.4 5 L bioreactor fermentations

Large scale fermentations studies were conducted following a similar set-up as for the small-scale fermentations (Section 5.2.1.3) in 7 L New Brunswick Bioreactors with 5 L working volumes. The bioreactor experiments were conducted at 30°C under anaerobic conditions with no sparging and an agitation speed of 200 rpm to keep the culture homogeneous. pH, dissolved oxygen and

temperature were monitored throughout the fermentation period. During later experiments the fermentation process was enhanced by increasing the fermentation temperature to 37°C.

5.2.2 Anaerobic digestion of confectionery waste streams

5.2.2.1 Analytical methods

Chemical oxygen demand (COD)

COD analyses were performed at the beginning and end of the experiment by using COD reagents A (Merck, 114679) and B (Merck, 114680). The reaction volume was halved compared to the manufacturer's instruction to reduce the amount of toxic waste generated. Validation of the results achieved with half reaction volumes has previously been confirmed by performing standard curves with full and half reactions. Similar curves were obtained. Briefly, reagents A and B were mixed in COD vials at a volume of 1.10 mL and 0.90 mL. Thereafter, 0.50 mL of sample was added to the vial. Deionised water was used as a blank. Vials were incubated in a COD block (HI 839800 COD reactor 2008 series) at 150°C for 2 hours. Samples were removed from the block and once room temperature had been reached, the absorbances were measured spectrophotometrically at 605 nm. For total COD measurements, representative samples were taken using P1000 tips with cut ends to allow for homogeneous sampling of larger particles. Soluble COD was measured from the supernatant collected following the centrifugation of samples at 14 400 *x*g for 15 min. Potassium hydrogen phthalate (PHP) (1.175 g COD/g PHP) prepared in de-ionised water was used as a standard.

pH measurements

The pH in the BMP test bottles was measured at the beginning and end of the test using a Jenway 3510 pH meter (Lasec). The pH was corrected to approx. 7.8 at the beginning of the test by the addition of NaOH.

Gas chromatography

Daily gas volumes were measured using a 50 mL syringe and the collected gas was used for methane determination. A Perkin-Elmer Auto system gas chromatography instrument equipped with a Supelco wax column (1.2 mm x 37 m) and flame ionisation detector (FID) was used for the determination of the methane fraction in the biogas. The FID and oven temperatures were set at 280°C and 50°C respectively. Nitrogen was used as a carrier gas at a flow rate of 1.5 mL/min. Standards containing 25% and 50% methane were injected with each sampling point to allow for the quantification of the methane contained within the biogas from the BMP tests. Measurements were performed by injecting 50 µL of gas from each sample in triplicate.

5.2.2.2 Substrate and inoculum preparation

BMP tests were initiated with a granular sludge previously obtained from an anaerobic digester operated at Prospecton Brewery (SA Breweries, KZN) from Tabolt and Tabolt (Durban). The sludge had been maintained on a synthetic wastewater containing sucrose, glucose, yeast extract, propionic acid, acetic acid and soluble starch as carbon sources. The inoculum was maintained at 37°C preceding use as inoculum for the BMP tests. Two substrates were used for BMP tests: 1) a mixed waste stream obtained from a South African confectionery company and 2) flour

confectionery. For the mixed waste, a solution was prepared by melting the confectionery waste (approx. 500 g/L) in tap water until a homogenous solution was achieved. The mixture was thereafter pasteurised as described in Section 5.2.1.3. For the flour confectionery, biscuits were blended to achieve a homogenous powder. The COD of the feedstocks were determined as described in Section 5.2.2.1, except for the biscuits for which small quantities of powder were weighed into COD vails and 0.5 mL water added instead of a sample volume.

5.2.2.3 Biomethane potential reactor set-up

Biomethane potential (BMP) test reactors consisted of 250 mL Schott bottles, with a working volume of 200 mL, with airtight caps fitted with metal inserts containing two ports. The first port was connected to an 8 mm internal diameter tube submerged two thirds of the way into the reactor fluid to allow for the sampling of the digester slurry. The outside of the port was connected to a 10 mL syringe allowing for the reactor fluid to be withdrawn. This tube was clamped shut except during sampling periods. The second port was connected to a 50 mL syringe to allow for the collection and volume measurement of any gas produced. BMP tests were initiated by the introduction of 10 g/L COD from the mixed confectionery waste or flour confectionery to a 30% (v/v) inoculum slurry and the remaining volume adjusted with tap water. BMP tests were performed in triplicate. The reactors were maintained in a 37°C constant temperature room. Preceding gas volume measurements and sampling of the BMP reactors, the reactors were mixing thoroughly to allow a homogenous representation of the solid and liquid phases of the reactors and release any biogas trapped within the sludge. Gas volumes and methane content (gas chromatography) were recorded daily. The reactors were sampled and analysed for soluble and total COD at the beginning and the end of the experiment. The end of the experiment was marked by no more gas production for two consecutive days. Sampling at the end of the experiment involved the removal of 4 mL of the liquid from the reactor for the measurement of pH and COD.

5.3 Results and Discussion

5.3.1 Bioethanol production by *Zymomonas mobilis*

5.3.1.1 Small-scale ethanol fermentation

Sugar analysis of the confectionery products showed that these waste streams contained a mixture of sugars comprising of glucose, fructose and predominately high in sucrose. Hard candy consisted of 0.8 g sugar per g confectionery product, of which 0.4 g was sucrose and the other 0.4 g was equally attributed to glucose and fructose. Marshmallow contained 0.6 g sugar per g confectionery product consisting of 0.45 g sucrose, 0.12 g glucose and 0.03 g fructose. Chocolate contained 0.5 g sugar in the form of sucrose only.

Small-scale fermentations with a *Z. mobilis* inoculum culture prepared using glucose as a carbon source, were performed in medium containing 20 g/L sugar from hard candy as a carbon source. Figure 5-2 shows that the bacterium was able to utilise the sugars from hard candy, although at different efficiencies, for growth and ethanol production.

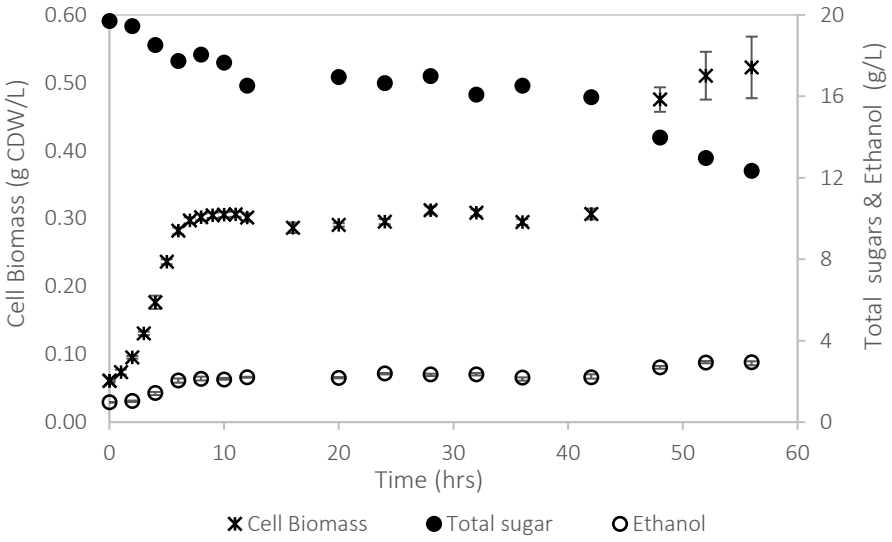

Figure 5-2 *Growth, sugar utilisation and ethanol production of Z. mobilis cultivated using hard candy as carbon substrate. Data points represent the average of results obtained from three independent experiments with error bars indicating standard deviation.*

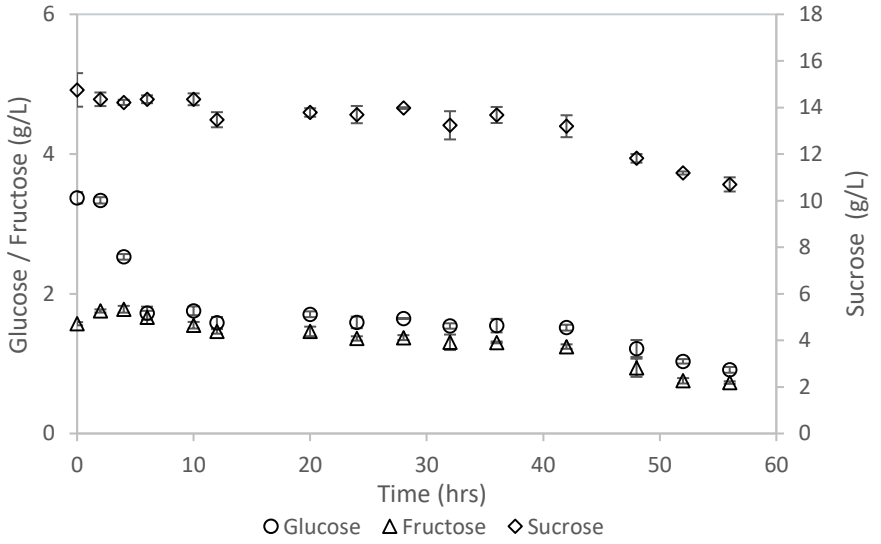

Figure 5-3 *Utilisation of individual sugars contained within hard candy confectionery during Z. mobilis fermentation. Data points represent the average of results obtained from three independent experiments with error bars indicating standard deviation.*

Growth was sustained for the first 10 hours of incubation after which, it entered stationary phase. No increase in growth or ethanol production was observed between 10 and 42 hrs of the fermentation. Between 42 and 48 hrs a second increase in bacterial biomass was observed, indicative of diauxic growth. The individual sugar utilisation profiles shown in Figure 5-3 indicated minimal hydrolysis of sucrose during the first 42 hrs of the fermentation. The growth observed between 0 and 10 hrs was possibly supported by the glucose present within the hard candy. The absence of fructose from the hard candy batch used here was possibly the result of the age of the

candy confectionery used. Between 42 and 48 hrs, where diauxic growth was observed, the utilisation of sucrose was observed (Figure 5-3).

Results from the small-scale fermentation study using hard candy as substrate for bioethanol production suggested that under the conditions tested here, sucrose hydrolysis by *Z. mobilis* was identified as the rate limiting step of fermentation. Between 42 and 48 hrs was required for the bacterial cells to adapt to the sucrose rich medium by expressing and producing the extracellular enzymes required for sucrose hydrolysis. After 56 hours of incubation fermentation was incomplete with a residual sugar concentration of 12.3 g/L remaining in the medium. From the initial total sugar concentration of 19.6 g/L, only 7.3 g/L was utilised during this period. The fermentation was stopped at 56 hrs. Should fermentation have been continued, sucrose utilisation may have continued until other essential growth components may have become limited in the growth media due to the extended fermentation period.

To verify if sucrose hydrolysis was the rate limiting step during the hard candy study, the bacterium was grown in medium containing 20 g/L sucrose for 24 hours. Poor growth and low ethanol yields were obtained. This is in accordance with Sprenger (1996) who reported the lack of any evident active sucrose transportation mechanism within *Z. mobilis*. *Z. mobilis* sucrose utilisation requires the hydrolysis of the disaccharide into glucose and fructose monomers which can diffuse through the cell membrane. Sootsuwan *et al.* (2013) found that during fermentation with sucrose as carbon substrate, *Z. mobilis* does not only produce ethanol and carbon dioxide but also levan. Levan is an extracellular fructose-polymer whose formation is catalysed by the levansucrase enzyme. This enzyme consists of two subunits each with its own catalytic site. The first site catalyses the hydrolysis of sucrose and the second the polymerisation of fructose into levan. The production of levan compromises the stoichiometric ethanol yields achievable from *Z. mobilis* fermentations with sucrose as substrate. This has been the major limitation in the use of *Z. mobilis* for ethanol production from low-cost industrially available sucrose as substrate. As the application of *Z. mobilis* for bioethanol production from sucrose rich feedstocks would be beneficial for the bioconversion of confectionery waste to bioenergy, the current research aimed to develop strategies to improve the bioethanol production efficiencies of *Z. mobilis* from sucrose. Firstly, the rate of sucrose utilisation was improved by acclimatising *Z. mobilis* to sucrose rich media prior fermentation as described in Section 5.2.1.1.

Following the acclimatisation of *Z. mobilis* cells to sucrose during the inoculum preparation phase the growth, small-scale fermentation studies were again performed with sucrose as substrate. Sucrose utilisation and bioethanol yields were improved to achieve 95% of the theoretical bioethanol yield expected for the sucrose utilised (Figure 5-4).

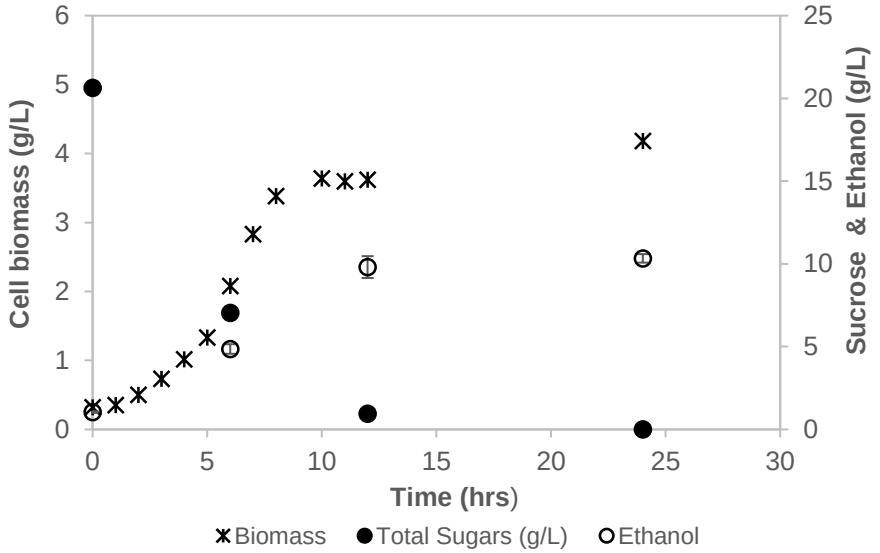

Figure 5-4 Growth, sugar utilisation and ethanol production from small-scale fermentation studies of Z. mobilis cells on sucrose as carbon source following pre-exposed of the inoculum to sucrose. Data points represent the average of results obtained from three independent experiments with error bars indicating standard deviation.

Improved growth and sugar utilisation were also observed in hard candy with the absence of a diauxic growth pattern and higher final ethanol concentrations compared to the unacclimated culture (Figure 5-5).

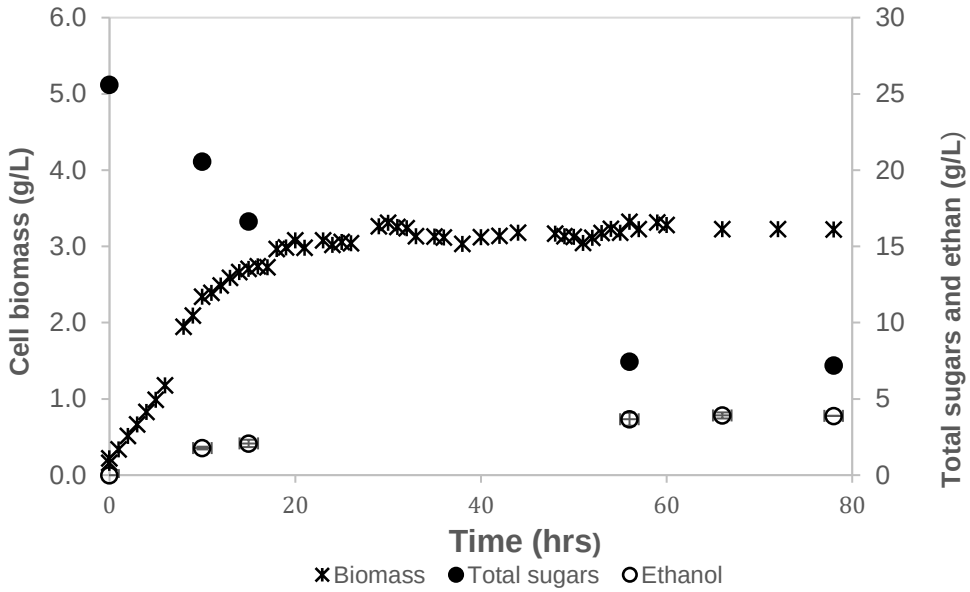

Figure 5-5 Growth, sugar utilisation and ethanol production from small-scale fermentation studies of Z. mobilis cells hard candy as carbon source following pre-exposed of the inoculum to sucrose. Data points represent the average of results obtained from three independent experiments with error bars indicating standard deviation

However, after 78 hours of fermentation a significant amount of residual sugars remained, and a relatively low ethanol yield was achieved compared to the results achieved using sucrose or a synthetic sugar mixture as substrate (Figure 5-4 and Figure 5-6). It is speculated that the presence of possible inhibitors in the hard candy, such as acidulants and additives, may be contributing to the poor sugar utilisation and bioethanol yields in these studies.

To test for the presence of inhibitors in hard candy, fermentations were performed in a synthetic sugar mix simulating the sugars present in hard candy. Figure 5-6 shows efficient sugar utilization, with all sugars utilised simultaneously, and bioethanol yields slightly less than that of sucrose only fermentation.

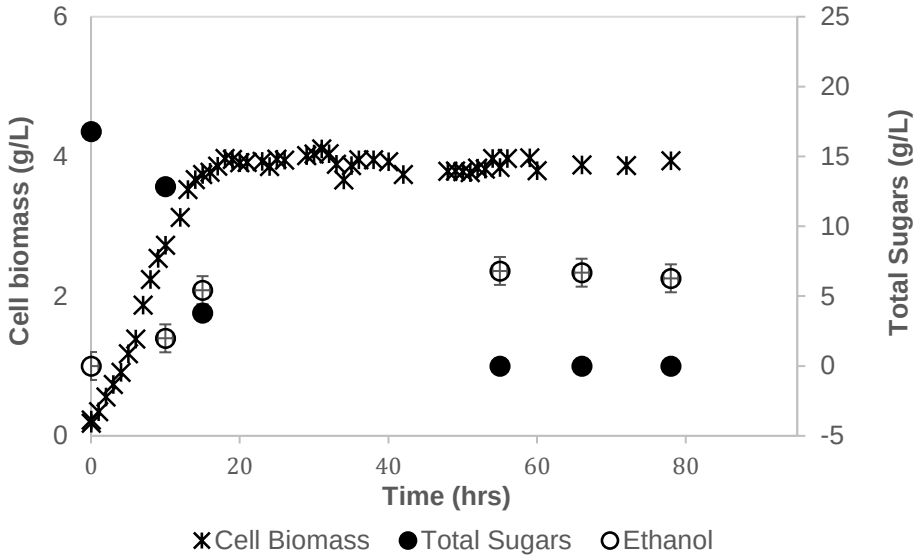

Figure 5-6 *Growth, sugar utilisation and ethanol production from small-scale fermentation studies of Z. mobilis cells on synthetic sugar mix as carbon source following pre-exposed of the inoculum to sucrose. Data points represent the average of results obtained from three independent experiments with error bars indicating standard deviation*

Comparative fermentations were performed using other confectionery products, marshmallows and chocolates. As observed with hard candy, incomplete sugar utilization and lower bioethanol yields were observed with the other confectionery-based media. Growth and ethanol production in the marshmallow-based medium were similar to that obtained in marshmallows. The maximum biomass and ethanol concentration obtained in marshmallow-based media were 3.18 g/L and 3.59 g/L respectively (Figure 5-7)

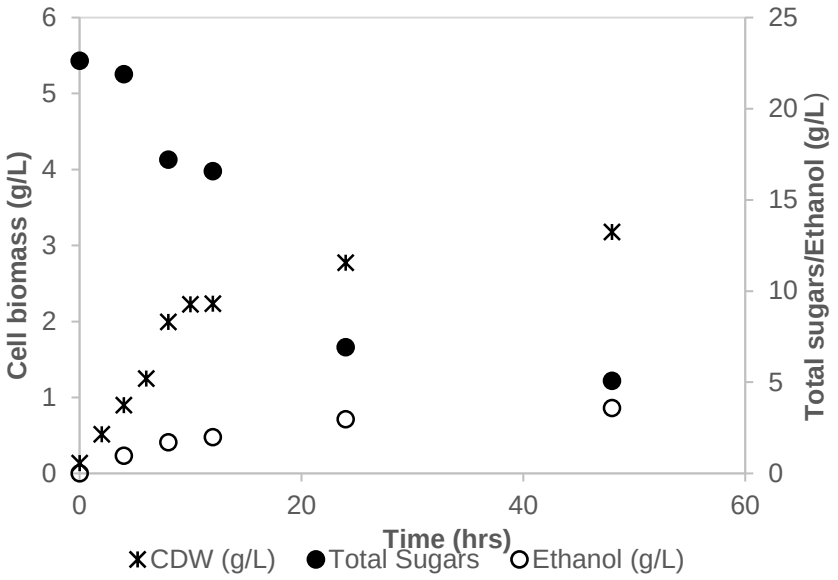

Figure 5-7 Growth, sugar utilisation and ethanol production from small-scale fermentation studies of Z. mobilis cells on marshmallows as carbon source following pre-exposed of the inoculum to sucrose. Marshmallows study were conducted in duplicate.

Efficient sugar utilisation was observed in media containing chocolate as a carbon source (Figure 5-8). As a result, the biomass concentration obtained in chocolate was higher than that obtained in both marshmallows and hard candy. However, a maximum ethanol concentration of 3.12 g/L was obtained comparable to the ethanol concentration obtained in using hard candy- and marshmallow-based media (Figure 5-5 and Figure 5-7).

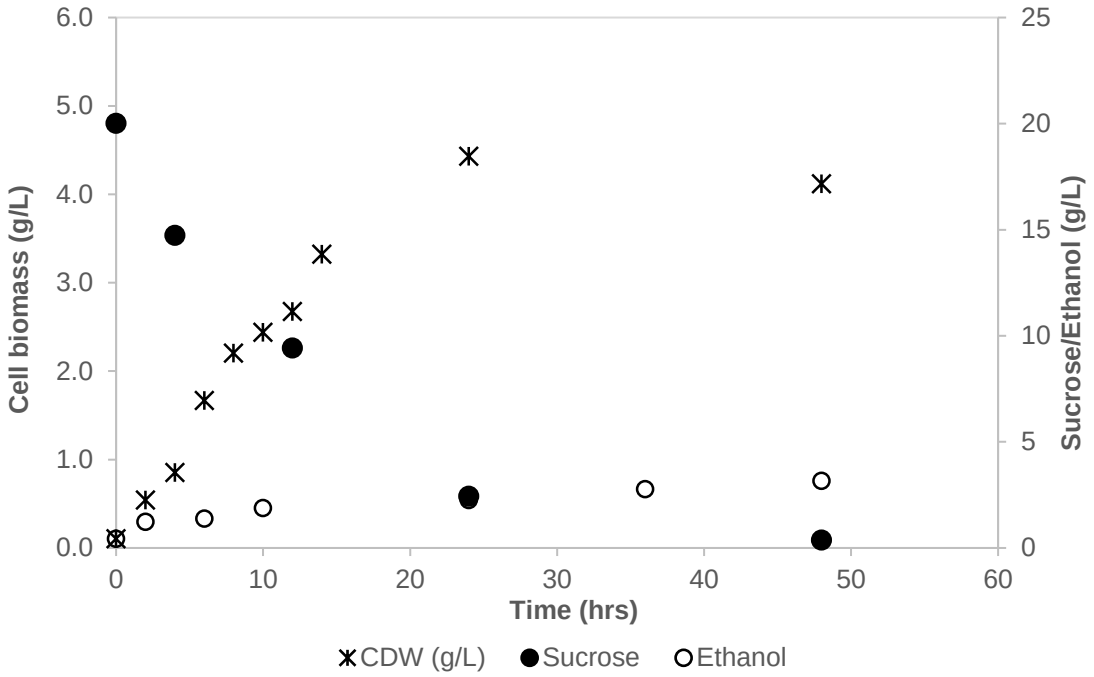

Figure 5-8 *Growth, sugar utilisation and ethanol production from small-scale fermentation studies of Z. mobilis cells on chocolate as carbon source following pre-exposed of the inoculum to sucrose. Chocolate studies were conducted in duplicate.*

Z. mobilis could co-ferment the mixed sugars in confectionery products for growth and subsequent ethanol production. A preacclimation to sucrose was required to ensure that the required enzymes were present to hydrolyse the disaccharide to glucose and fructose which could be utilised for growth and bioethanol production. Higher substrate conversion yields for biomass production ($Y_{B/S}$) and product formation ($Y_{P/S}$) as well as ethanol concentrations were obtained in the simulated mix compared to the confectionery waste products. This suggests that there may be inhibitors present within the confectionery products negatively affecting *Z. mobilis* growth and bioethanol production during fermentation. This inhibition may be overcome during larger scale fermentation.

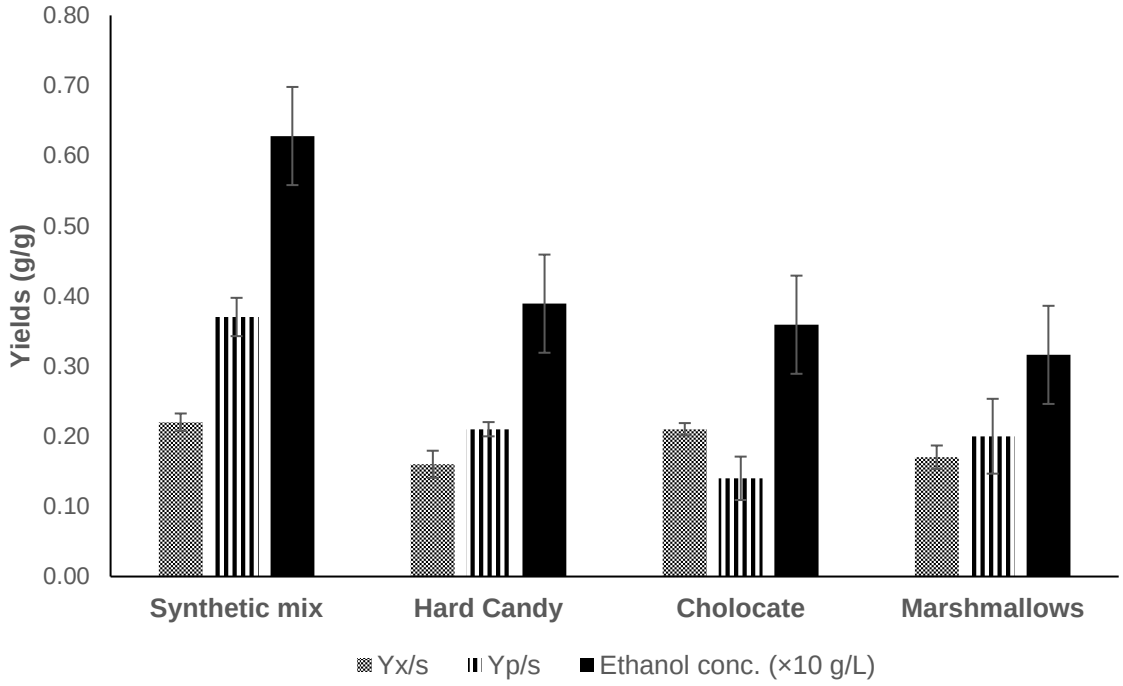

Figure 5-9 Biomass ($Y_{X/S}$) and bioethanol product ($Y_{P/S}$) yield coefficients and bioethanol concentrations achieved by Z. mobilis fermentations on various confectionery products and a synthetic sugar mixture simulating hard candy (10 g/L sucrose, 2.5 g/L glucose and 2.5 g/L fructose).

5.3.1.2 Large scale fermentations

Laboratory-scale bioreactor fermentations of 5 L working volume were performed to test the feasibility of bioethanol production from confectionery using *Z. mobilis* as production strain. Fermentations using glucose as sole carbon source resulted in the theoretical biomass and bioethanol yields, as shown in Figure 5-10.

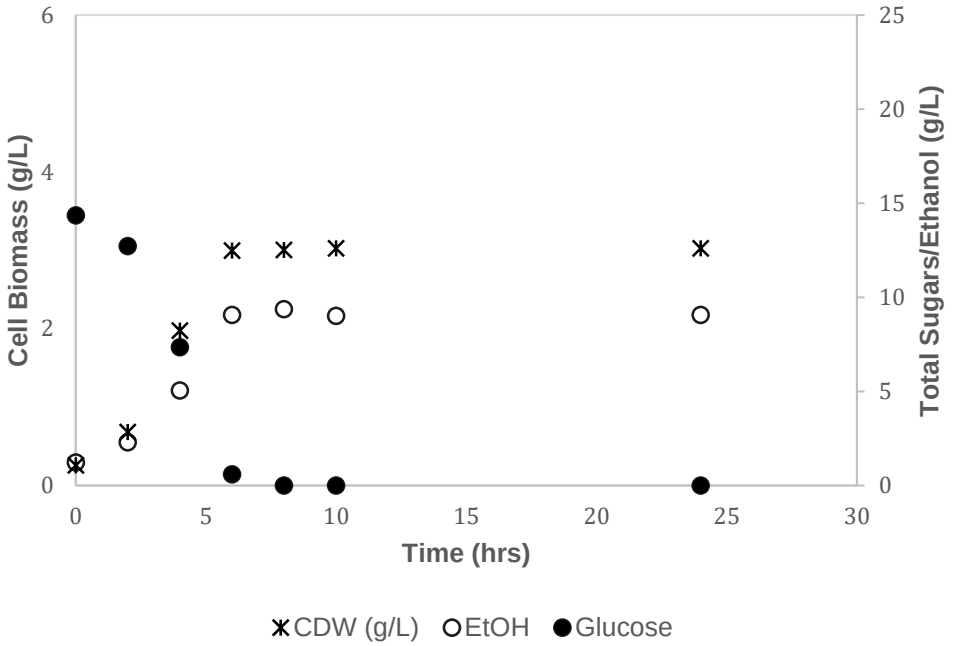

Figure 5-10: Growth and ethanol production by Z. mobilis on glucose as carbon source in New Brunswick bioreactors at 30°C. Data represents the averages of two replicate bioreactor fermentations

However, when sucrose and the synthetic sugar mixture was used as carbon source, low ethanol yields were achieved, reduced from ~ 9 g/L to 4 – 5 g/L, as shown in Figure 5-11 and Figure 5-12.

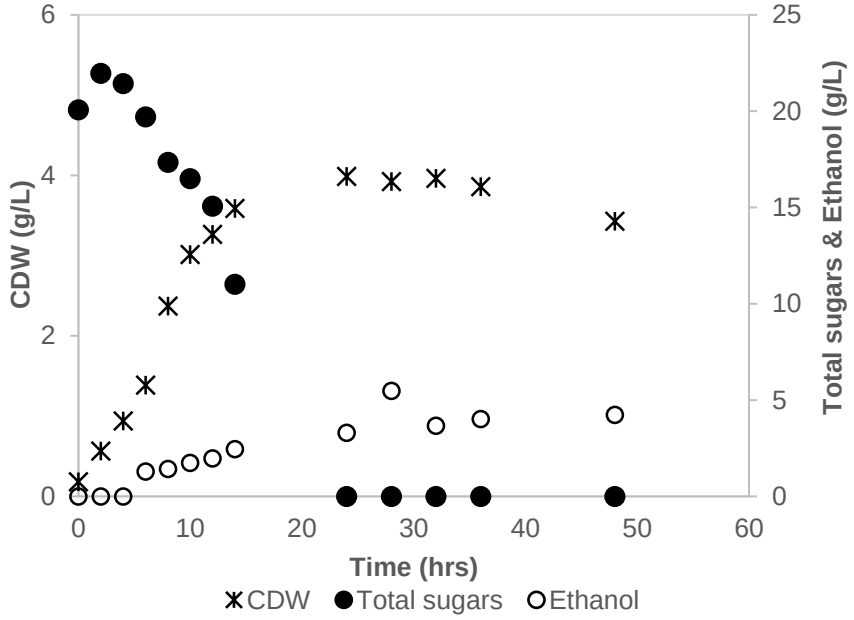

Figure 5-11 Growth and ethanol production by Z. mobilis on sucrose as carbon source in New Brunswick bioreactors at 30°C. Data represents the averages of two replicate bioreactor fermentations

The growth and ethanol production profile in sucrose and synthetic mix (Figure 5-12) followed a similar trend. In both carbon sources, sugars were completely utilised within 24 hours of incubation, despite the relatively low ethanol concentration after extended fermentation periods. This suggested deviation of sugar to a by-product.

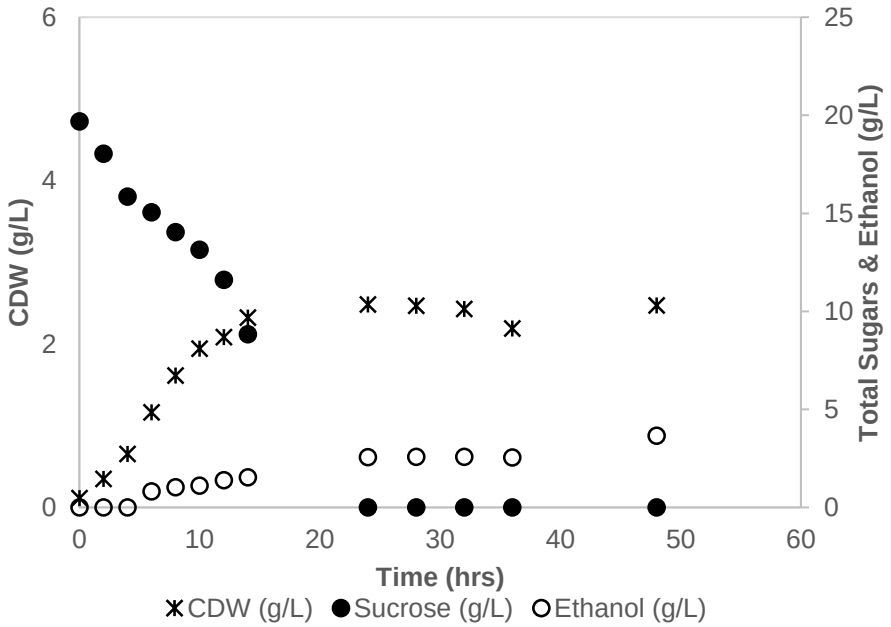

Figure 5-12 Growth and ethanol production by Z. mobilis on synthetic sugar mix as carbon source in New Brunswick bioreactors at 30°C. Data represents the averages of two replicate bioreactor fermentations

The poor growth and bioethanol production of *Z. mobilis* when large quantities of sucrose were available as carbon source for growth was possibly the result of levan formation, compromising ethanol yields under the fermentation conditions used. As mentioned before, levan is an extracellular polysaccharide produced by the polymerisation of fructose catalysed by the second active site present within the levansucrase enzyme (Lee et al, 1981). During this study we considered two approaches towards reducing levan production to optimise bioethanol production in large-scale bioreactors from sucrose-rich carbon sources. Increasing the fermentation temperature (Sangiliyandi et al, 1999) and pH (Goldman et al., 2008) have been shown to inhibit the polymerisation activity of the enzyme. Increasing the pH of the *Z. mobilis* fermentation resulted in excessive foaming of the culture and was therefore disregarded as an optimisation method. However, increasing the fermentation temperature from 30°C to 37°C resulted in efficient fermentation with 95% theoretical yield conversion efficiency (Figure 5-13).

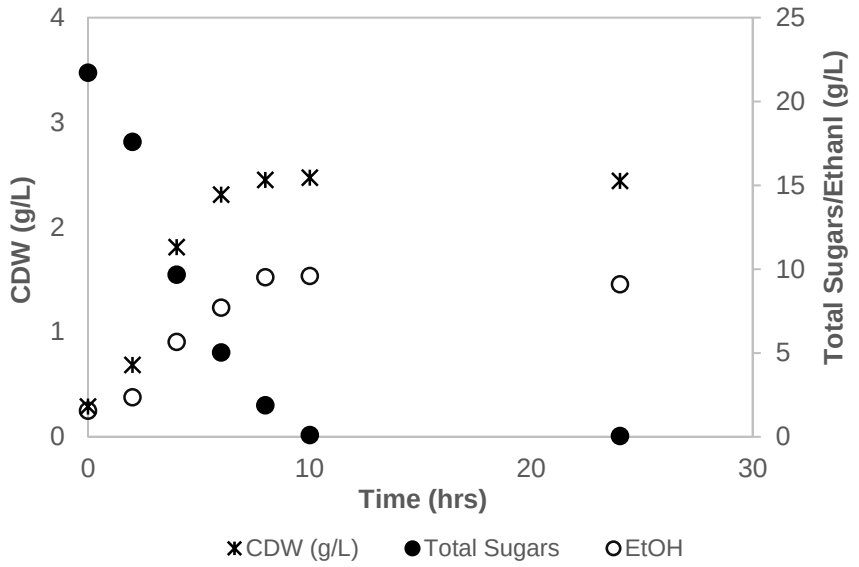

Figure 5-13 Growth and ethanol production by Z. mobilis on sucrose as carbon source in New Brunswick bioreactors at 37ºC. Data represents the averages of two replicate bioreactor fermentations.

For fermentation of the synthetic mix of sugars at 37°C, the highest ethanol yield obtained was 8 g/L which was comparable to the ethanol concentration in small scale fermentation (Figure 5-6), also slightly less than that obtained in sucrose fermentation only at 37°C.

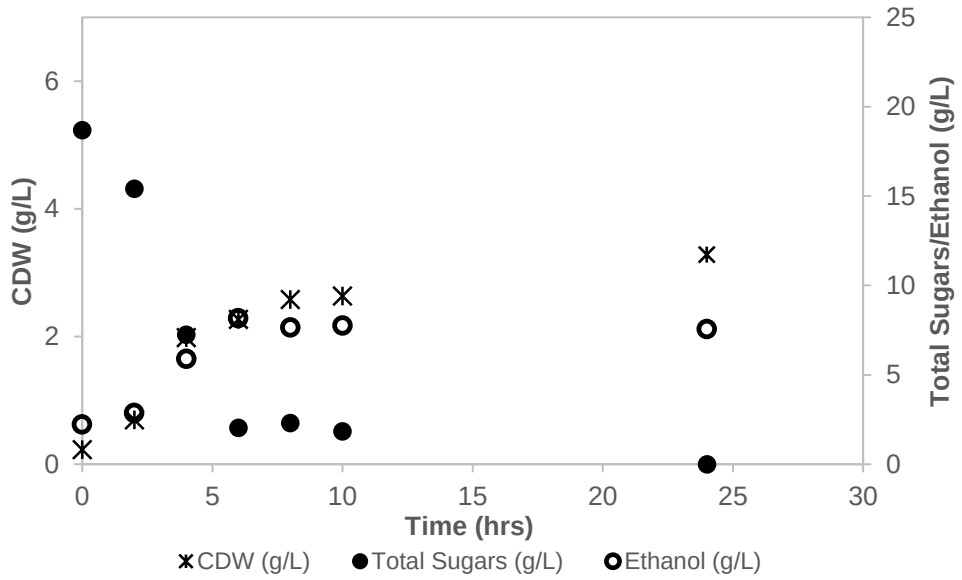

Figure 5-14 Growth and ethanol production by Z. mobilis on synthetic sugar mix as carbon source in New Brunswick bioreactors at 37ºC. Data represents the averages of two replicate bioreactor fermentations.

Growth and ethanol production of *Z. mobilis* using confectionery waste products were further investigated using the optimised bioreactor conditions at 37ºC. Figure 5-15 shows the growth and ethanol production profile in hard candy at 37ºC. A biomass yield coefficient of 0.2 g/g was achieved,

comparable to that achieved in the small-scale studies (Figure 5-9). A diauxic growth pattern was again observed; however, the duration of the stationary phase was reduced compared to the small-scale hard candy studies performed using sucrose un-adapted cultures (Figure 5-2). Between 10 and 24 hrs the biomass concentration increased by 0.5 g/L and continued to increase until 30 hrs when approx. 3 g/L CDW was reached. The diauxic growth observed here may be as a result of the inhibition of the levansucrase enzyme by glucose as demonstrated by Lyness and Doelle, (1983). An ethanol concentration of 6 g/L was obtained with a product yield coefficient of 0.37 g/g. This was higher than the maximum ethanol concentration and product yield coefficient obtain in small-scale studies performed with the sucrose adapted inoculum at 30°C (Figure 5-5).

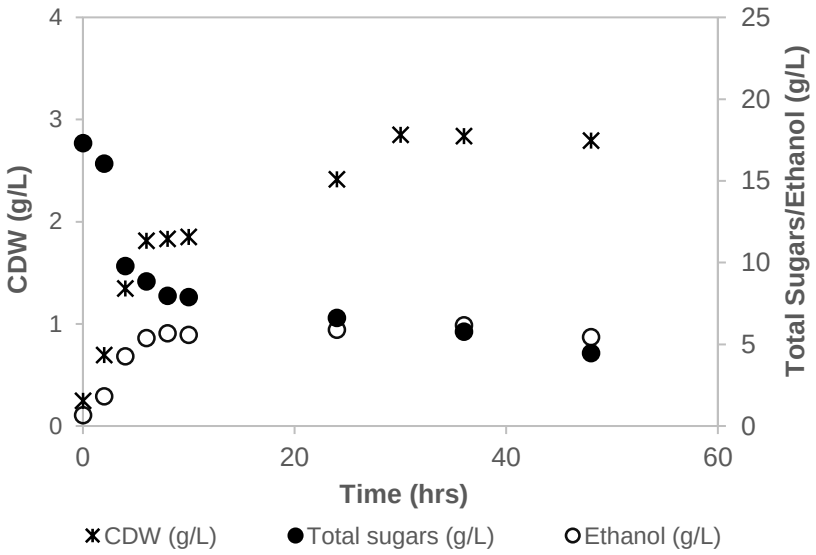

Figure 5-15 Growth and ethanol production by Z. mobilis with hard candy as carbon source in 5 L bioreactors at 37°C. Data represent the averages of two replicate fermentations.

Similar results as those obtained for hard candy fermentation were achieved using marshmallows as carbon substrate (Figure 5-16). A maximum ethanol concentration of 7 g/L was obtained in the lab scale bioreactor, whereas only 4 g/L was produced in the small scale study performed with marshmallow substrate (Figure 5-7). No diauxic growth profile was observed in the medium containing marshmallow-based medium as substrate, possibly due to the specific ratio of sugars present within the substrate. Marshmallows have a higher proportion of sucrose present within the total sugars, 75%, compared to hard candy, 50%.

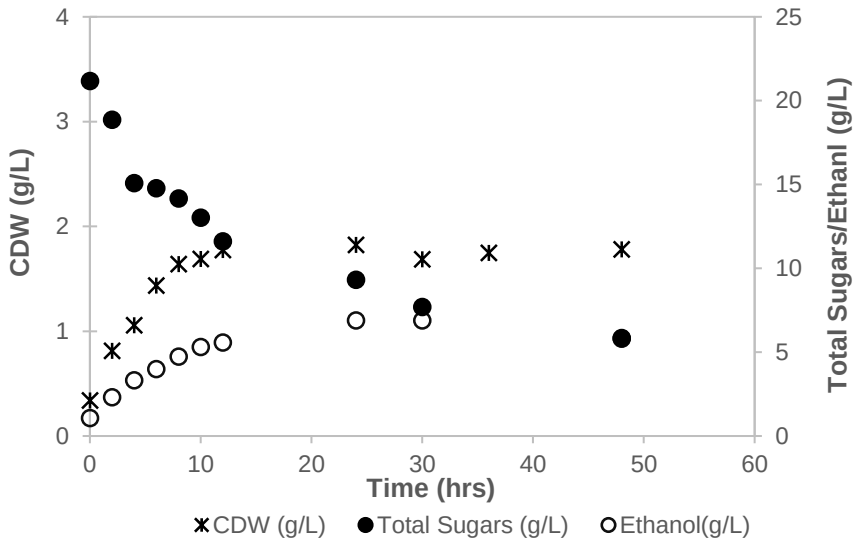

Figure 5-16 Growth and ethanol production of Z. mobilis fermentations performed with marshmallows as carbon substrate in bioreactors at 37ºC. Data represent the averages of two replicate fermentations.

A higher maximum ethanol concentration of close to 9 g/L was achieved with chocolate-based media as carbon substrate (Figure 5-17), compared to marshmallows (Figure 5-16) and candy fermentations (Figure 5-15). Sugar analysis of chocolate showed that the sugar in chocolate is comprised only of sucrose. As no sucrose utilisation inhibition would have occurred because of the presence of hexose sugars, *Z. mobilis* was able to utilise the sucrose more efficiently achieving a bioethanol yield coefficient of 0.45 g/g and a theoretical product yield conversion efficiency of 83%.

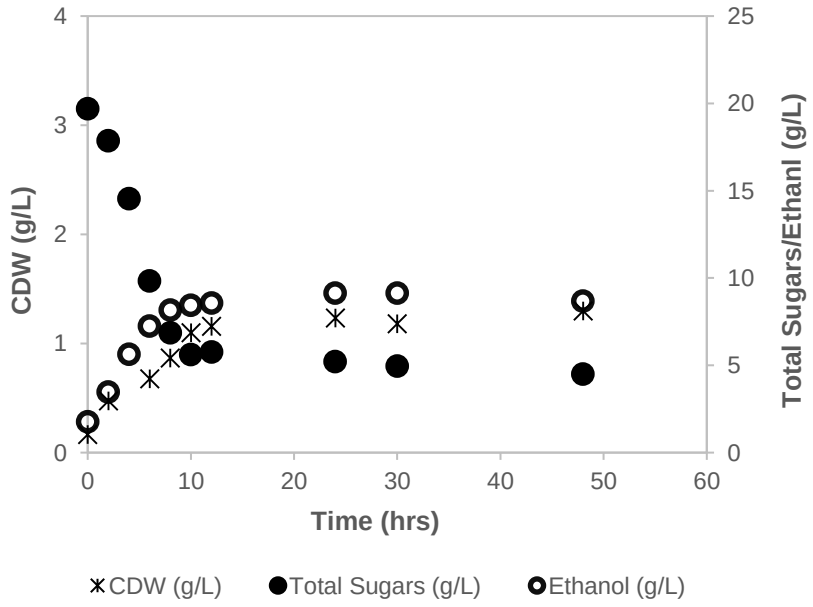

Figure 5-17 Growth and ethanol production by Z. mobilis fermentations performed with chocolate as substrate in large scale bioreactors. Data represent the averages of two replicate fermentations.

Z. mobilis showed the ability to utilise the sugars in confectionery waste to produce ethanol achieving higher yields and ethanol concentration in the lab scale New Brunswick bioreactors at 37°C optimised for bioethanol production compared to small-scale tests. However, for all fermentations performed with confectionery products to date, *Z. mobilis* was only able to utilise approx. 75% of the sugars present within the fermentation, while 25% remained as residual sugars at the end of the fermentation period. This is thought to be as an effect of inhibition from a confectionery additive. A similar observation was reported in a study conducted by You et al. (2017) where slow conversion of glucose from dairy manure to ethanol by *Z. mobilis* ZMT2 was attributed to presence of inhibitors. Different fermentation strategies i.e. fed-batch can be used to overcome inhibition by unknown additives in confectionery products while allowing complete utilisation of the sugars.

5.3.2 Anaerobic digestion of flour and mixed confectionery streams

Preliminary studies suggested that feedstocks rich in simple sugars such as sucrose, glucose and fructose resulted in the acidification of the anaerobic system owing to the acidogenesis and acetogenesis occurring more rapidly than methanogenesis, leading to the accumulation of weak acids. Methanogens are pH sensitive and operate strictly between pH 6.5 and 8.0 (Cheng, 2010; Chynoweth & Isaacson, 1987). Outside this pH range, the products of hydrolysis and acidogenesis will less easily be converted to methane, aggravating acidification. This suggested that confectionery waste may be unsuitable for single stage anaerobic digestion. The applicability of AD has been demonstrated on a commercial scale; hence reactor operation approaches are necessary to control and prevent acidification.

Here we considered two substrate types with a combination of simple and more complex carbon compounds present: a flour confectionery stream, simulated by using biscuits as feedstock, and a mixed confectionery waste stream obtained from a South African confectionery producer. These feedstocks were applied to the BMP tests at a 10 g/L COD loading to test the feasibility of biogas production from similar wastes.

The total biogas volume produced from the flour confectionery was in excess of 1 L over an 18 day period for the 200 mL reactor volume, while the mixed confectionery waste feedstock only produced 700 ml of biogas, as shown in Figure 5-18.

Assuming the only COD containing compound in the biogas is CH_4, we would expect 1.5 L of CH_4 from 2 g of COD included as feedstock in these BMP tests. Total biogas produced from the flour confectionery feedstock was less than the expected CH_4 yield. On average, 60% CH_4 was present within the biogas on analysis by GC. This equates to approx. 629 ml CH_4 from flour confectionery and 452 ml CH_4 from the mixed confectionery waste. COD analyses of the BMP tests following termination of the experiment on day 33 showed approx. 0.24 and 0.62 g COD remaining in the soluble fractions of the flour confectionery and mixed confectionery waste BMPs respectively. Taking the remaining COD into account, the expected CH_4 yields were re-calculated. Even considering the soluble COD remaining, the COD conversion efficiencies for flour and mixed confectionery streams were only 47 and 43 % respectively. This may, however, be an under-representation of the COD conversion efficiencies, as the total COD measurements did not produce definitive results, and COD may have remained in the insoluble fraction. For accurate measurement

of the total COD in a system it is necessary to blend the sludge and soluble fraction to allow representative sampling. This was not done at the end of this experiment as the sludge is to be used as inoculum for the next set of experiments.

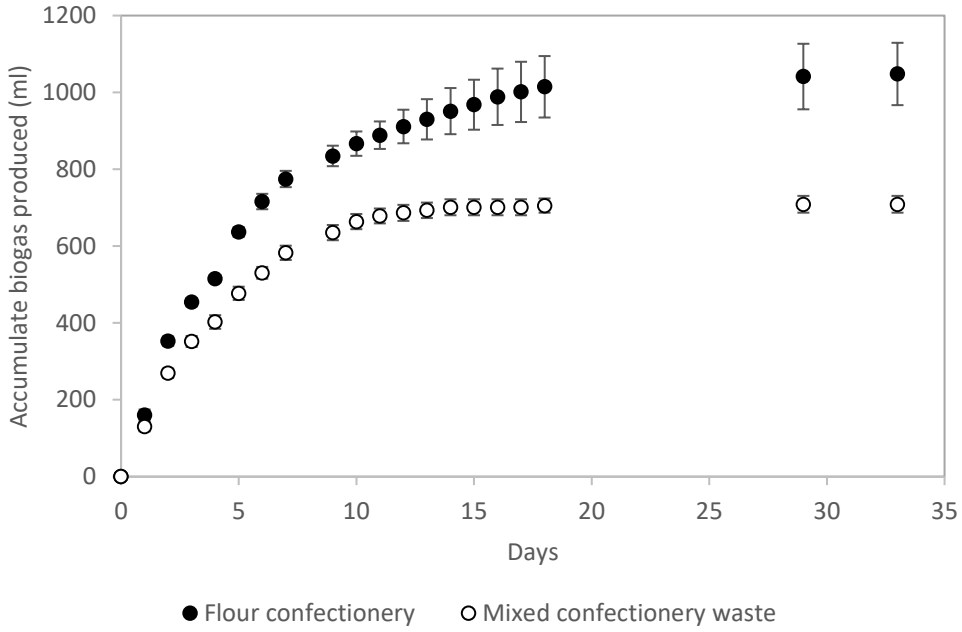

Figure 5-18 Total biogas production from the anaerobic digestion of flour confectionery and mixed confectionery feedstocks. Data points indicate the average of three independently performed BMP tests and the error bars indicate standard error.

At the end of the BMP test, marked by sluggish or negligible biogas production, the pH of all ADs was higher than 7 but lower than 8, which falls within the pH range suitable to methanogens. It is therefore possible that the COD remaining from the initial 10 g/L used as feedstock for these BMP tests may be inaccessible to the microbial consortium present within the system, or that the microbes are still adapting the composition of the 'new' feedstocks. Further validation of the suitability of mixed confectionery waste and flour confectionery waste as feedstocks for biogas production will be obtained from repeated feeding experiments.

5.4 Concluding remarks

When deciding the most feasible route for bioenergy production from confectionery waste, consideration needs to be given to the feedstock composition. Here we presented results achieved using feedstocks such as waste rich in hard candies, marshmallows or chocolate-based materials for bioethanol production using *Z. mobilis* as fermentation strain. All these feedstocks contain high proportions of sucrose (40 to 50 %). The bioethanol yield coefficient achieved under the optimal conditions for *Z. mobilis* growth was low but was improved 2 fold by increasing the fermentation temperature to 37°C to inhibit the polymerisation of fructose into levan. Results suggest that bioethanol production in *Z. mobilis* is growth-associated and further work will concentrate on the optimisation of the fermentation parameters to obtain improved sugar utilisation and bioethanol

production. Although flour confectionery was not tested as a substrate for bioethanol production during this study, it would be possible to utilise the starch from the flour confectionery as substrate for *Z. mobilis* fermentation following the hydrolysis of the starch molecular into monomeric glucose units. Here a two-phase system can be considered. Firstly, the hydrolysis of the starch in the flour confectionery can be achieved by addition of amylases under the optimal conditions for their activity. Thereafter, the hydrolysed product can be used as substrate for *Z. mobilis* fermentation. Should it be possible to identify amylases with sufficient activity under the fermentation conditions applied for bioethanol production, simultaneous enzymatic hydrolysis and fermentation may be performed within the same reactor. Here the enzyme activity will need to be carefully matched to the sugar utilisation rate of *Z. mobilis* to prevent limitation and resultant lower bioethanol yield.

As an alternative bioenergy production strategy, we investigated the utilisation of a less well-defined 'mixed' confectionery waste obtained from a confectionery manufacturer and flour confectionery as substrates for biogas production through anaerobic digestion. The results reported here were the preliminary results from BMP tests. Biogas containing approx. 60% methane was produced from both the feedstocks tested, however the % conversion efficiency of COD to methane was <50% for the initial BMP test. The inoculum may perform better in subsequent BMP tests following acclimatisation i.e. with a re-feed of the BMP tests with the same feedstocks, as a period of adaption is often required to allow the evolution of the microbial community to best access the COD in the feedstock.

6 Polyhydroxyalkanoates (PHA) from Confectionery Waste

6.1 Introduction to PHAs

Poly-β-hydroxyalkanoates represent a family of bio-based biodegradable plastics synthesised microbially. For the microorganism, they represent a carbon and energy storage product, produced in response to nutrient limitation. PHAs were first recognised by the French Scientist LeMoigne (Chee et al., 2010). These storage compounds can accumulate up to more than 70% of the bacterial cell mass (Harrison, 1990). As polyesters, the PHA chemical backbone is made up of the ester linkage between alcohol and acid functional groups. When the polymer is made of a single monomer type, it is known as a homopolymer (e.g. poly-β-hydroxyalkanoate PHB); where two types of monomers are present, it is known as a copolymer (e.g. PHB/HV made up of hydroxybutyrate (HB) and hydroxyvalerate (HV)monomers). Figure 6-1 shows the general structure of a polyhydroxyalkanoate where m represents the number of monomeric units and R the side chain e.g. $-CH_3$ in the HB monomer and $-CH_2CH_3$ in the HV monomer (Ebnesajjad, 2013). The side chain R impacts the polymer's properties.

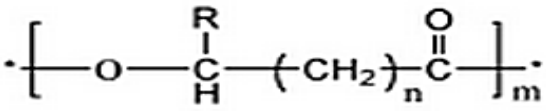

Figure 6-1 General structure of PHA (Ebnesajjad, 2013)

PHAs are subdivided into two broad categories, namely short chain length (scl) monomers which consist of between 3 to 6 carbon atom monomers (e.g. hydroxybutyrate, hydroxyvalerate) and medium chain length (mcl) monomers which consist of monomers with 6 to 14 carbon atoms e.g. hydroxy-octanoate, and hydroxy-hexanoate. There is variation in polymer properties with the nature of the monomer. For example, polymers of mcl monomers are elastomers with low crystallinity and tensile strength, lower melting point and glass transition temperature while polymers of scl monomers are brittle with high melting point and low glass transition temperature (Akaraonye et al., 2010). The most common homopolymer PHB represents the standard bacterial storage compound and is a brittle, non-ductile polymer with properties similar to polypropylene. By forming the copolymer PHB/HV, the ductility improves and brittleness decreases (Harrison, 1990).

PHA has potential to mitigate the current crisis with respect to disposal of plastics materials, particularly single use plastics, due to properties mimicking conventional plastics, biodegradability and production from renewable material allowing use in various applications, including applications previously reserved for only petroleum derived plastics. PHA can be applied in the following categories

- Medical: used for implants, temporary 'pins' and sutures due to being biocompatible and immunologically inert as well as slowly biodegradable in human tissues

- Industrial biotechnology: used as metabolic regulators, slow release and as resistance enhancer
- Chemical industry: intermediate products forming starting material for the production of a range of bioproducts, platform chemicals and fine chemicals
- Packaging: packaging materials used for daily consumables and electronic appliances like mobile phones as well as thin films
- Barrier material: used as impermeable barrier in personal hygiene products, diapers etc.
- Single use plastics: used in disposal eating utensils and containers etc.

6.2 PHA production

PHAs are synthesized intracellularly as carbon and energy storage components under nutrient limiting conditions. Both wild type bacteria and recombinant bacteria engineered to host the PHA synthesis pathway can accumulate PHAs. Some wild type bacteria accumulate PHA during their growth phase while, more commonly, PHA is accumulated as a storage product under nutrient limiting conditions. The biosynthesis of PHA can use three pathways: that initiated through the tricarboxylic acid cycle, fatty acid biosynthesis and the β oxidation pathway, shown in Figure 6-2.

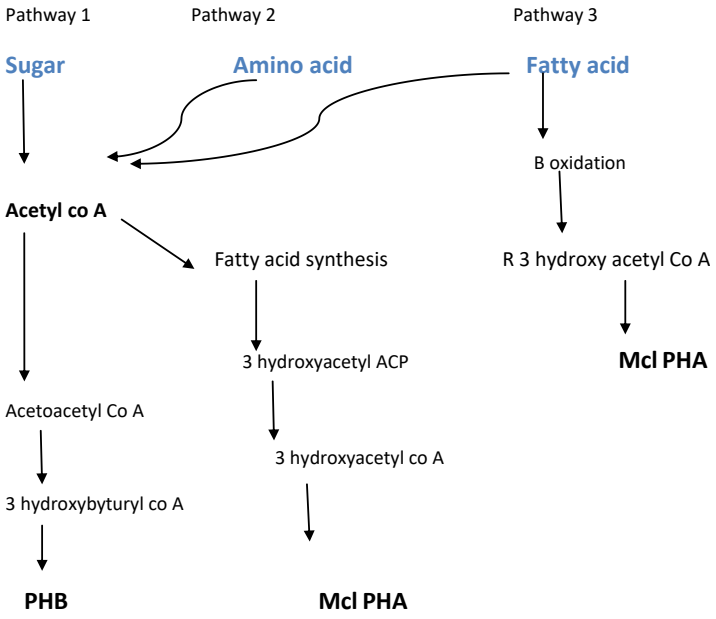

Figure 6-2 Different biosynthesis pathways of PHA

The diversion of carbon into PHA synthesis in the wild type bacteria occurs in response to nutrient limitation, most commonly nitrogen, but also oxygen and phosphorus. Owing to its insolubility in water and hence no impact on the osmolarity of the cytoplasm, very high intracellular

concentrations are reached. The key enzymes involved in PHA synthesis via the acetoacetyl CoA intermediate are 3-ketothiolase to divert acetyl CoA from the TCA cycle into acetoacetyl CoA, acetoacetyl CoA reductase resulting in 3-hydroxybutyryyl CoA and PHB synthase forming the polymer. Typically, the production is initiated by bacterial cell growth to achieve a high cell concentration. Thereafter, nutrient limitation is incurred, and the polymer accumulates, causing the swelling of the rod-shaped cells into nearly spherical cells with the maximum volume to surface area ratio. The range of bacteria able to produce PHA is wide, with a selection listed in

Table 6-1.

6.3 Commercial production of PHAs

Typically, PHAs have been produced from the monosaccharide glucose, although some bacterial strains have allowed the use of sucrose. Production on fatty acids is also reported. However, despite the numerous potential applications of polyhydroxyalkanoates, their commercial production is still limited to niche applications as the cost ($4-9/kg) (Bolck, 2014) remains substantially higher than conventional plastics such as polypropylene ($1-2/kg).

The economics of PHA production is affected by PHA productivity and yield, final biomass concentration and PHA content of the biomass, cost of raw materials and downstream processing costs (Choi and Lee 1999).

Despite the cost of production, polyhydroxyalkanoates have been produced on large scale by companies such as ICI / Zeneca, U.K. (in the early 1990s) as Biopol; Metabolix, USA (1990s to ~2016); Telles,USA; P&G, USA; Meridian, USA; Liangi Biotech, China; Tianjin North Food, China; Nigho, China; Jiang Su Nan Tian, China; Chemie Linz, Austria; Biomers, Germany, just to name a few with production capacities between 200-50 000 tons (Chanprateep, 2010; Chen, 2009). These facilities used one of glucose (most common), sucrose, lauric acid or other fatty acids as the major carbon source with addition of propionate or 1,4-butanediol for copolymer production (Chen, 2009). Final biomass yield exceeds 60 g/L in all cases and typically exceeds 100 g/L with the highest concentration reported of 200 g/L while PHA content in these industrial facilities typically exceeded 75% of biomass (Chen, 2009; Harrison, 1990).

6.4 PHA production from waste materials

The high price of PHA is attributed to both production costs and raw material costs with the latter accounting for 20-50% of the total production cost (Choi and Lee, 1999; Strong et al., 2016). It has been proposed that the costs may be reduced on using cheap carbon-rich raw materials.

Renewable raw materials and waste streams rich in carbohydrates or fats or both have attracted attention to mitigate the raw material cost. Lignocellulosic materials, molasses, bagasse as well as wastewater are some of the waste stream demonstrated for PHA synthesis.

Table 6-1 lists the various micro-organisms that can produce PHA as well as their respective PHA productivities from various waste resources. Further examples are provided by Nielsen et al. (2017), focussing on food waste as raw material.

Table 6-1 Various micro-organisms that produces PHB from renewable resources

Substrate	Microorganism	PHB (%) and Cell concentration (g/L)	Productivity (g/(Lh) and Yield (gPHA/gCDW)	Limiting nutrient	Reference
Sugar beet juice	*Alcaligenes latus* ATTC 29714	38.7% CDW 4.0 g /L	0.39 g PHB/g CDW 0.22 g/L/h	Nitrogen	Wang et al. (2013)
Potato starch, saccharified waste	*Ralstonia eutropha* NCIMB 11599	230 g/l	0.22 g PHB/g starch 1.47 g/L/h	Phosphate	Haas et al. (2008)
Sugar cane molasses	Mixed microbial cultures (70% of *Candidatus competibacter* and 30% *Defluviicocus vanus*)	37% (g/g TSS)	0.47-0.66 C mol PHA per C mol total substrate	Nitrogen (ammonia)	Bengtsson et al. (2010)
Molasses	*Cupriavidus necator*	20			Beaulieu et al. (1995)
Bagasse hydrolysate	*Burklolderia cepacian* IPT 048	53% CDW 0.299g /g	0.09 g/l/h	Nitrogen and phosphorous	Silva (2004)
Bagasse hydrolysate	*B. sacchari* IPT 101	62% CDW 0.39g/g	0.11 g/l/h	Nitrogen and phosphorous	Silva (2004)
Molasses	Mixed microbial culture	6.4% 23%	0.18 C mol PHA/C mol VFA 0.59 C mol PHA/ C mol VFA	Nitrogen (ammonia)	Alburquerque et al. (2010)
Margarine waste	*Cupriavidus necator* DSM 428	55.8% 6.4 g/l	0.50g /g 0.061 g/g/h	Phosphorous	Morais, (2013)
Paper mill waste water	Mixed microbial cultures	48%	0.27 cmol/cmol vfa	Nitrogen and phosphorous	Bengtsson et al. (2008)
Date syrup	Bacillus megatorium	25% 0.85g/l	Not reported	Nitrogen (ammonia)	Omar (2001)
Glycerol - waste	Cupriavidus necator DSM 545	62% 82.5 g/L			Cavalheiro et al. (2009)
Soya waste, malt waste	Alcaligenes latus DSM 114	32.6-70% 18 – 33 g/L			Yu et al. (1999)
Soybean oil	Cupriavidus necator WT	74% 122 g/L			Kahar et al. (2004)

6.5 Potential for PHA production from confectionery waste

Several researchers have reported on the production of PHAs from food waste. In the work of Cai et al. (2008), PHA synthesis on confectionery waste, ice-cream waste, milk waste, malt waste and sesame oil are considered. Koutinas et al. (2014) report extensively on the potential of food waste, generated at 1.3 x 10^9 t p.a. globally, as a resource for valorisation to chemicals and biopolymers,

including PHAs and bacterial cellulose. While exact waste numbers are not available for food waste generated in particular categories, 8 x 10^6 t of rusks, biscuits and preserved pastries and cakes, 26.4 x 10^6 t of fresh baked goods including pastries and cakes and 8.8 x 10^6 t of chocolate, cocoa and sugar confectionery are sold each year in the EU (Koutinas et al., 2014).

Interest in the processing of these waste materials to products beyond bioenergy is increasing. Starbucks Hongkong is working with their local university to evaluate the production of succinic acid and PHAs from their bakery waste (Zhang et al., 2013). Flour-rich waste streams from the confectionery industry have also been used for PHB production, with promising results reported, inspiring future research toward biodegradable plastics (Lopez *et al*., nd). One of the leading chocolate companies in the Netherlands (Mars factory) in collaboration with Delft University has conducted research on using their chocolate-based wastewater as the carbon substrate for PHB production. The polymer can be used as packaging material for the confectionery industry with simultaneous treatment of the wastewater (Delta, 2013). Alternatively, where the properties of the polymer are less defined owing to the variable feedstock, other uses such as cement coatings have been proposed or its use as an intermediate for further processing. This work is now in the commercialisation phase with Paques, Netherlands (personal communication, 2019).

The chocolate waste stream from the confectionery industry is rich in both carbohydrates and fat, hence may be an ideal substrate for the microorganisms that are used in biopolymer synthesis, particularly PHAs.

6.6 Experimental assessment of PHA production on confectionery waste

6.6.1 Medium development for PHA production

Bacteria have a requirement for both macro- and micro-nutrients. The nutrients potassium, sodium, and magnesium are typically added in the form of potassium dihydrogen phosphate, sodium hydrogen phosphate and magnesium sulphate. The concentrations used across several researchers are included in Table 6-2.

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Table 6-2 Mineral salt media in g/L (unless otherwise specified) for Cupriavidus necator (adapted from Aramvash et al. (2015)).

	Aragao et al. (1996)	Khanna et al. (2005)	Baei et al. (2011)	Aramvash et al. (2015)	Aramvash et al. (2015)	Anusha et al., (2016)
Glucose	40		40	20		25
Fructose		20			20	
$(NH_4)_2SO_4$	5	2	1.35	2	2	3
Citric acid				1.7		2
KH_2PO_4	37	2	1.5	1.54	2	12
$MgSO_4.7H_2O$	0.5	0.2	2.2	1.2	0.2	1.5
Na_2HPO_4	2.24	0.6	3.75		0.6	
$CaCl_2$	0.01	20 mg/l			20 mg/l	
Trace elements	1m/l	10 ml/l	10 ml/l	10 ml/l	10 ml/l	
$FeSO_4.7H_2O$		0.2mg/L	0.1 g/l	10 mg/l	10 mg/l	10
$CuSO_4.5H_2O$		1.3 mg/L	0.08	1 mg/l	1 mg/l	1
$ZnSO_4.6H_2O$		1.3 mg /l		2.25 mg/l	2.25 mg/l	2.25
$MnSO_4.4H_2O$			0.1	0.6 mg/L	0.6 mg/l	0.5
$CoCl_2$	0.2 g/l					
ZnCl	0.1					
$CaCl_2.2H_2O$	0.001					2
$NiCl_4$	0.02 g/l					
$(NH_4)_6Mo_7O_{27}$		0.6 mg/l		0.6 mg/l	0.6 mg/l	
$MnCl_2.4\ H2O$	0.03 g/l					
H_3BO_3	0.3	0.6 mg/L	0.02			0.23
$Na_2B_4O_7$		0.23 mg/l				0.1
Max biomass (g/l)	4.5	3.75	Not rep	5.14	4.76	5.01
PHB conc (g/l)	0.742	1.4	3.3	1.38	2.16	2.34
Yield gPHB/g sugar	0.018	0.07	0.08	0.069	0.108	0.093

6.6.2 Evaluating PHB production on confectionery waste

Initial shake flask experiments for the improvements of biomass as well as sugar utilisation of *Alcaligenes latus* DSM 1123 were carried out with sugar-based media. *A. latus* was then grown in mineral medium containing chocolate or candy as the carbon source, supplemented with a trace elements solution. Initially these shake flask experiments resulted in low biomass concentration and high residual sugar with starting sugar concentrations of 20 and 10 g/L for both candy and chocolate. However, proof of concept was demonstrated in that the bacterium was able to use the sugar contained in both candy and chocolate. The starting concentration was further decreased to 5 g/l with regular carbohydrate feeding at 4 h intervals. Similar experiments were performed on candy and chocolate-based media to test whether the low sugar utilisation results from substrate inhibition.

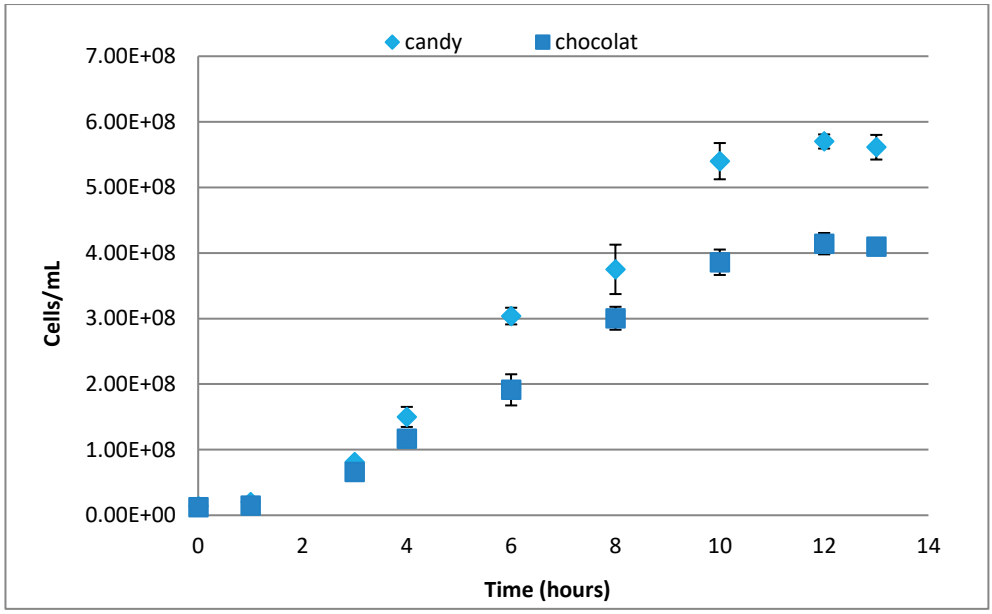


Figure 6-3 Cell growth of Alcaligenes latus *on candy and chocolate*

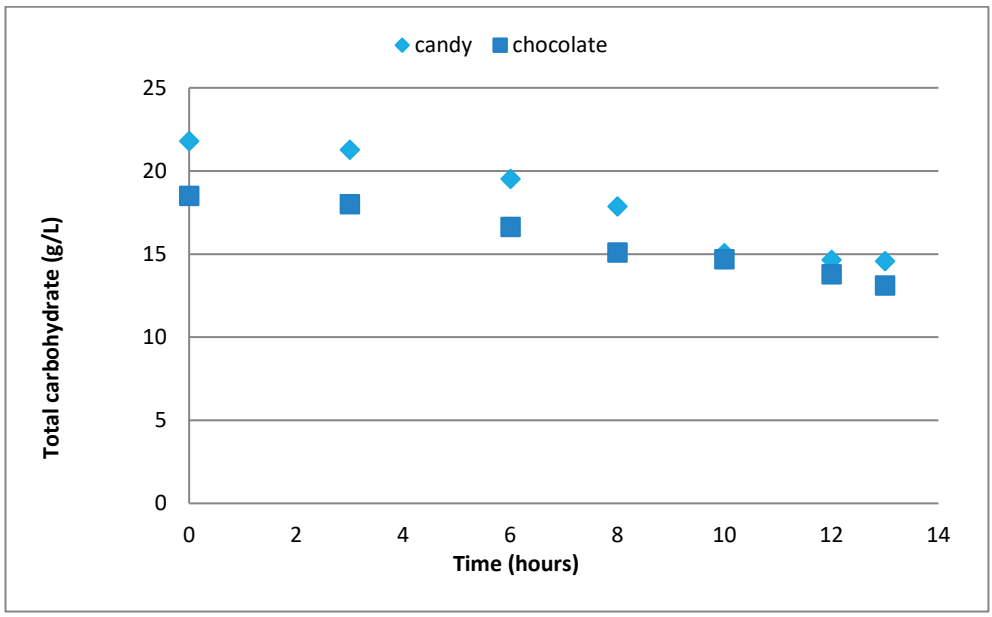


Figure 6-4 Sugar utilisation of Alcaligenes latus *from candy and chocolate*

A second approach to improve the biomass concentration was investigated, focussing on the addition of the carbohydrate feed at a specific time point. It has been reported that the time at which the excess carbon or limited nutrient is introduced affects the biomass formation of *Alcaligenes latus* ATCC 29714 (Wang et al., 2013). These experiments were conducted using candy as the carbon source with all other parameters similar to previous experiments. Two time points were selected, 10 h and 20 h corresponding to mid to late exponential phase and stationary phase of *Alcaligenes latus* DSM1123. It was observed that the time of addition of further sugar did not impact biomass accumulation. Oxygen limitation was removed by lowering growth medium volume in the shake flask to increase the oxygen transfer.

Further work was directed towards the growth of *Cupriavidus necator* DSM 428, an alternative PHB producer with preferred growth on sugars. Growth was tested on sucrose, glucose, fructose, a sugar mixture comprising of sucrose (50%) glucose and fructose (25 % each) to mimic candy, as well as on candy. *Cupriavidus necator* is one of the most common PHB producers and has been reported to produce PHB from pentose and hexoses as well as other substrates. The experiments were performed in shake flasks with a starting sugar concentration of 20 g/L and 2 g/L ammonium sulphate as the nitrogen source in a minimal medium solution. It was observed that *Cupriavidus necator* is able to grow on all three constituent sugars as well as on candy. The growth rate observed on the three media did not differ significantly with a highest growth rate of 0.302 h^{-1} observed for sucrose compared to growth on candy and the sugar mixture at 0.281 h^{-1} and 0.291 h^{-1} respectively. The highest biomass obtained was 2.5 g/L for the sugar mixture after 28 h of cultivation. It was, however, observed that the buffering capacity of the selected minimum medium was not as effective as anticipated. From a starting pH of 6.8 -7.0, the highest final pH recorded was pH 8.5 in glucose followed by pH 7.8 on candy. This might have hindered the growth of the microorganism and resulted in the termination of growth. *Cupriavidus necator* has been reported to grow for up 48 h or even more.

6.7 Investigating the potential of low cost, waste carbon sources on the PHA technoeconomic potential

While use of waste carbon sources represent improved resource efficiency and provide a savings on the raw material cost of the process, these may also affect the process performance. A lower productivity or lower PHA content or both may negatively impact the process economics, negating the benefit of reduced raw material cost in economic terms. This possibility has been particularly highlighted by Kim and Chang (1995) as well as Ramsay et al. (1995). This scenario is considered for PHA production in this section.

Rumjeet and Harrison (n.d.) overview the techno-economic simulation studies reported to date on use of waste materials such as dairy whey (García et al., 2011; Van Wegen et al., 1998), rice bran (García et al., 2011) and wastewater (Fernández-Dacosta et al., 2015). In all these cases, the simulations assumed process performance levels equivalent to the pure carbon sources used and resulted in costs in the range $3-7 per kg, except in the case of the wastewater in which the otherwise required wastewater treatment costs were offset against process costs in an appropriate manner, resulting in a net final cost of $1.60 – 2.20 per kg PHA.

We developed a generic production model for the PHA homopolymer, PHB, by *Cupriavidus necator*, using CeBER Bioprocess Modeller (Harding and Harrison, 2016a, 2016b) which is constructed for development of mass and energy inventories at the early design stage analysis and requires minimal inputs. This allows initial techno-economic and environmental burden assessment at a much earlier stage of process development than possible with ASPEN or SuperPro Designer. In this, PHB is produced in a fed-batch configuration at the 20 ton per batch scale and product recovery and purification occurs through a surfactant – sodium hypochlorite process, according to process data presented in Harrison (1990) and Harding (2008). The details of the model development are described by Rumjeet and Harrison (n.d.) and the process flowsheet used is given in Figure 6-5.

Further, equipment costing and the basis of the techno-economic study is also given in Rumjeet and Harrison (n.d.).

In this study, we first explore the impact of biomass concentration pre-PHB accumulation, PHB concentration, PHB content of the biomass and rate of production on the cost of PHB production. This is done using a two level factorial approach, detailed in Table 6-3. We then consider the impact of the cost of the major carbon-rich raw material on production cost, allowing the potential compromises to be explored. The latter is done by scaling the raw material cost from $0.33 per kg sugar to a "no-cost" resource.

Table 6-3 Conditions for the factorial design of influence of process performance on production cost of PHB

Variable	Low input spec.	High input spec.	Base case
Final non-PHB biomass concentration (g/L)	40	50	44
PHB content %	67	75	70
Production time (bioreactor stage only) (h)	40	50	44

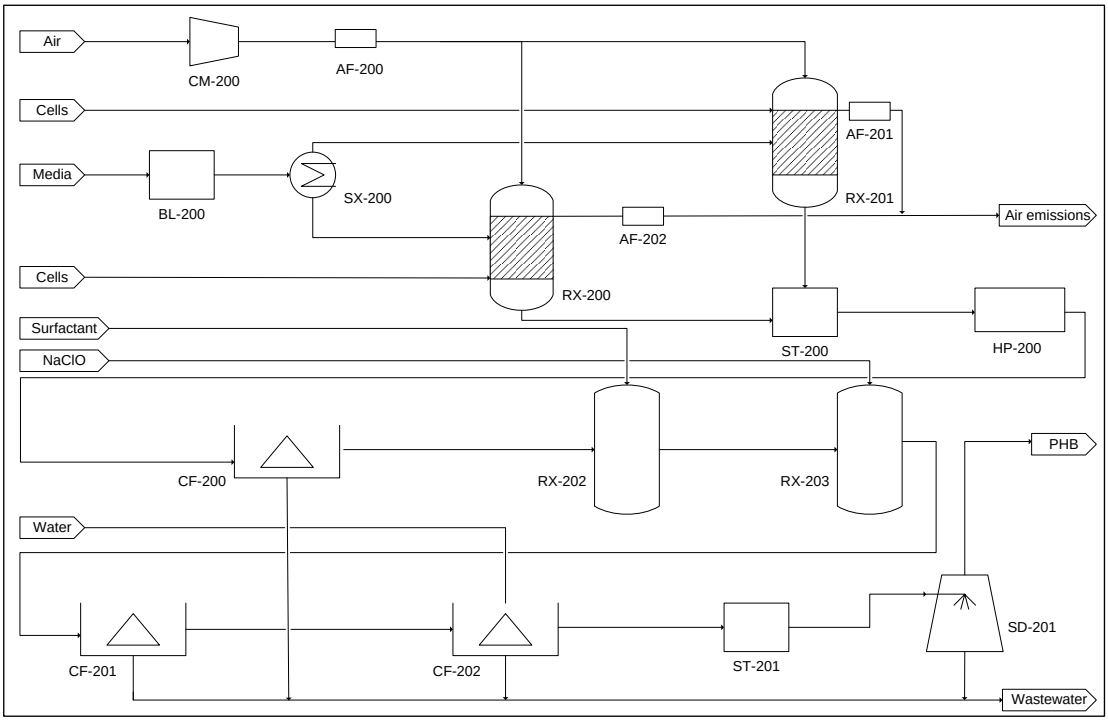


Figure 6-5 Process flow diagram for the production of PHBusing in the CeBER Bioprocess Modeller simulation (adapted from Rumjeet and Harrison, in prep b). Key: SX-200 – steam steriliser, RX-200 and RX-201 – bioreactor, CM-200 – compressor, HP-200 – high pressure homogeniser, CF-200 and CF-201 – centrifuge, RX-202 – DSP reactor 1, RX-203 – DSP reactor 2.

Based on the data presented in Harrison (1990) and Harding (2008), using the flowsheet developed by Harding (2008) and Harding et al. (2007) and refined in Rumjeet and Harrison (n.d.), a production cost of $6.5 per kg was calculated for the base case performance. The breakdown of these costs in

presented in Table 6-4, showing the raw material to contribute 25% of overall production costs, mostly due to the cost of glucose.

Table 6-4 Contribution of production costs for the base case conditions

Production costs (%)			Raw material costs (%)	
Raw materials	25		Glucose	77
Utilities	8		Ammonium sulphate	5
Waste disposal	6		Antifoam	1
Labour	12		DSP: Detergent	5
DFC dependent costs & plant overheads	49		DSP: Hypochlorite	12

On varying the process performance in terms of final PHB concentration, PHB content of the biomass and rate of cultivation, substantial changes in production cost are shown in Table 6-5. Based on a 20% variation in non-PHB cell concentration and cultivation time and a 12% change in PHB content achieved, a variation in production cost of 64% is found. The maximum and minimum costs found under these conditions represent 127% and 78% of the base case production cost of $6.54 / kg. The latter agrees well with estimates recorded for industrial processes in the range $4-9 per kg (Liew et al., 2014; Telis, 2012).

Table 6-5 Impact of process performance on the production cost of PHB

	Scenario conditions	PHB productivity (g/(Lh))	Production cost ($/kg PHB)	% contribution of raw materials to production cost
1	Non-PHB biomass = 40 g/L PHB content = 67% Culture time = 40 h	2.00	6.93	23.8
2	Non-PHB biomass = 50 g/L PHB content = 75% Culture time = 40 h	3.75	5.09	25.7
3	Non-PHB biomass = 40 g/L PHB content = 67% Culture time = 50 h	1.60	8.35	23.6
4	Non-PHB biomass = 50 g/L PHB content = 75% Culture time = 50 h	3.00	6.12	25.8
Base case	Non-PHB biomass = 44 g/L PHB content = 70% Culture time = 44 h	2.33	6.54	24.6

Table 6-6 Impact of cost of carbon source and process performance on raw materials. The shades of green indicate a reduction in production cost in steps of 10% relative to the base case while the shades of red indicate an increase in production cost in steps of 10%

Price of C ($/kg)	Cost of PHB production ($/kg)				
	Scenario 1	Scenario 2	Scenario 3	Scenario 4	Base case
0.33	6.93	5.09	8.35	6.12	6.54
0.20	6.44	4.66	7.77	5.61	6.04
0.10	6.08	4.34	7.33	5.23	5.67
0.00	5.71	4.03	6.89	4.85	5.29

The impact of the cost of the carbon source was super-imposed onto this performance analysis with results presented in Table 6-6. From the base case data, it is seen that a savings of 19% (the contribution of glucose to the process costs = 77% of 25%) is achieved on use of 'free' carbon source where the same performance is attained. However, should performance decrease, as shown between the base case with productivity of 2.33 g/(Lh) and Scenario 3 with productivity of 1.60 g/(Lh), then the reduction in carbon source cost is offset by the decrease in performance. This analysis shows clearly the need for an excellent understanding of the impact of waste carbon source, or nitrogen source not considered here, on process performance.

A key aspect of this cost of production not superimposed on this study yet is the cost of disposal of the waste carbon rich material. In the confectionery industry in South Africa at present, much of this is disposed of to landfill at a cost. This cost must be offset against the production cost of PHB.

Landfill costs reported in South Africa varied from R421 to R117 per ton (2017). In Cape Town, the increase in landfill costs has increased from R292 and R387 per tonne in 2007 to R421 and R558 in 2017/18, depending on landfill site. Ongoing increases are estimated at 7 to 12%, suggesting costs ranging from R492 to R593 per ton for general waste and R652 toR786 per ton for special waste in 2020/21 (Basson et al., 2018). Despite costs, legislation is also expected to drive alternatives.

6.8 Conclusion

The polyalkanoates are well-recognised as an interesting family of bio-based biodegradable plastics with widely ranging applications. In order to improve their process economics, cheap carbon sources are sought. Both through this work and through emerging studies globally, confectionery waste materials are being shown to be potential feedstocks for this important set of polymers with wide-ranging properties and the potential to process as conventional polymers. The reduction in cost of carbon source has potential to contribute positively to the process economics. Further the offsetting of waste handling charges is expected to bring the process economics of these plastics into a range competitive with conventional plastics and of particular interest where the niche properties of PHAs are explored. It is cautioned, however, that on substitution of carbon source the maintenance of acceptable process performance is key to ensure process competitiveness. This factor, relevant for all processes considered I this report, is explored in more depth in this section.

7 Polyglutamic acid (PGA) from Confectionery Waste

7.1 Introduction to PGA

Poly-γ-glutamic acid (γ-PGA) is an anionic polypeptide biopolymer (Figure 7-1) that has many attractive properties and industrial applications (Ogunleye et al., 2015). It is non-toxic, hygroscopic and can be used as a flocculent in the wastewater treatment industry. Since this biodegradable polymer poses no threat to the environment or to humans, it can replace chemically synthesised flocculants. At the optimum pH, and with the availability of multivalent cations such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Al^{3+} and Fe^{3+}, the flocculation activity can increase (Shih and Wu, 2009). It also aids dispersion of pigment in the paper making industry (Shih and Wu, 2009). Owing to its hygroscopic nature, it can be used as a soil conditioner as well as a fertiliser.

Owing to the nature of the feedstock (waste), applications are limited to the agricultural and wastewater industries. Production of PGA from solid confectionery waste would divert waste from going to landfill, thus preventing methanogenesis in the landfill, acidogenic conditions with associated toxic leachates developing and potential for pathogenesis, each causing harm to the environment.

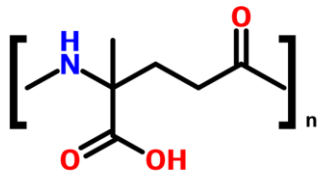


Figure 7-1 Structure of PGA

7.1.1 Carbon sources for production of PGA

PGA is commercially produced by *Bacillus subtilis* with L-glutamic acid as a carbon source (Kreyenschulte et al., 2014). However, this process is costly owing to the costly starting material, glutamic acid. Emerging studies focus on producing γ-PGA directly from renewable sugar sources. To the authors' knowledge, no literature has described the use of confectionery waste for the production of PGA; however, researchers have investigated the use of other waste-based renewable resources as feedstock. Zhu et al. (2014) studied corncob hydrolysate containing glucose, xylose and arabinose as a carbon source for the production of PGA using *Bacillus subtillis* HB-1. Production of PGA from xylose exceeded that from glucose. They concluded that corncob fibre hydrolysate can be used as a suitable carbon source in place of glucose. The use of a waste stream as a nutrient source has also been explored by Zhang et al. (2012), using cane molasses. It was found that this was a suitable carbon source for production of PGA using *Bacillus subtilis* NX-2, and that the molasses did not require hydrolysis before addition to the fermentation. *Bacillus subtilis* has a well-characterised sucrose utilisation system responsible for the hydrolysis of the sucrose molecule by sucrose hydrolases. It was speculated that the untreated cane molasses contained unidentified nutrients which aided in the production of PGA (Zhang et al., 2012). *Bacillus subtilis* GXC-36 was cultivated on cassava starch and corn steep liquor in a non-sterile batch reactor and produced high yields of PGA using these low-cost feedstocks (Zeng et al., 2018).

7.1.2 Biosynthesis of PGA

Bacillus is a renowned robust workhorse used in many industrial applications such as production of heterologous proteins, antibiotics, nucleotides, biosurfactants, biofuels and biopolymers (Meissner et al., 2015). It produces PGA in the presence of excess nitrogen and carbon sources (Ogunleye et al., 2015). The industrial production of PGA is typical run as a continuous process in a stirred tank reactor (CSTR) with a steady supply of nitrogen (Bending et al., 2015). Table 7-1 provides a summary of PGA-producing *Bacillus* strains reported.

Table 7-1 Bacillus *species producing PGA*

Strain	Reference
Bacillus licheniformis	Du et al. (2005)
Bacillus licheniformis A13	Mabrouk et al., (2012)
Bacillus licheniformis NCIM 2324	Kumar and Pal (2015)
Bacillus licheniformis SAB-26	Soliman et al. (2005)
Bacillus methylotrophicus	Peng et al. (2015)
Bacillus subtilis C1	Shih and Wu (2009)
Bacillus subtilis CGMCC1251	Huang et al. (2011)
Bacillus subtilis GX-28	Zeng et al. (2014)
Bacillus subtilis GXC-36	Zeng et al. (2018)
Bacillus subtilis HB-1	Zhu et al. (2014)
Bacillus subtilis NX-2	Zhang et al. (2012)
Bacillus subtilis TAM-4	Ito et al. (1996)
Bacillus velezensis NRRL-23189	Moraes et al. (2012)
Bacillus TISTR 1010	Kongklom et al. (2017)

PGA-producing bacteria can be grouped into two categories: (i) L-glutamic acid-dependent microorganisms, where PGA cannot be synthesised without the presence of this amino acid in the cultivation medium and (ii) L-glutamic independent bacteria, where they are able to synthesise the polymer in the absence of L-glutamic acid in the medium because of the *de novo* pathway of L-glutamic acid synthesis (Xu et al., 2005). PGA biosynthesis takes place in two steps. The first step involves the synthesis of L- and D- glutamic acid monomers via the tricarboxylic acid (TCA) cycle. The second step joins these monomers into a polymer. The size of the polymers differs from organism to organism and is also dependent on the nutrients in the cultivation medium (Huang et al., 2011). The presence of precursors such as L-glutamic acid and α-ketoglutaric acid increases PGA productivity (Sirisansaneeyakul et al., 2017).

The glutamate-dependent strains need an organic nitrogen source in the form of glutamate for PGA synthesis, making the production medium costly. Glutamate-independent strains, such as *Bacillus velezensis* NRRL B-23189, are able to utilise an inorganic nitrogen source for PGA synthesis. Moraes et al. (2012) supplemented molasses with citric acid and ammonium sulphate was used as a nitrogen source in place of glutamate. Madonsela (2013) demonstrated that *Bacillus licheniformis* JCM2505

was able to use an inorganic nitrogen source, NH_4Cl, to produce PGA. In addition to the carbon source, the key nutrients identified were citric acid, a phosphate and nitrogen source, as well as trace elements which are all present in minimal medium E (MME), a medium optimised from medium E (ME) (Birrer et al., 1994), by Madonsela (2013) for the optimum carbon, nitrogen, and phosphorus ratio for the PGA production from *B. licheniformis. Other r*esearchers (Feng et al., 2016; Tang et al., 2015; Zhang et al., 2012) used a "basal medium", which contained several micronutrients, as well as glutamate as a nitrogen source.

7.1.3 *Bacillus* on starch

Starch is the staple food of most cultures and comes in many different forms; it is the main source of stored energy for plants. Starch sources for human consumption include corn, cassava, potato and wheat. Crops for starch are commercially grown and are used for food as well as in many industrial processes as feedstock to produce bioproducts such as bioethanol and biobutanol and other biobased chemicals (Zhang et al., 2016). Starch itself has industrial applications such as gelling, thickening, adhesion, moisture retention and texturizing.

Starch is a large molecule that consists mainly of the polysaccharides amylose (AM) and amylopectin (AP). AM consists mainly of linear chains of α-1,4 glucose, AP is branched with linear α-1,4 and α-1,6 glucose (Horstmann et al., 2017). The starch molecule comprises mostly AM and AP with small amounts of protein, lipid, phosphorus and trace elements. The ratio between AM and AP varies between the different sources but generally consist of 70-80% AP and 20-30% AM (Schirmer et al., 2013). Table 7-1 is a summary of the characterisation of common starches. The ash component consists of trace elements and phosphorus.

Table 7-2 Physiochemical properties of starches from different sources (Adapted from Schirmer et al., (2013))

	Protein (%w/w on dry basis)	Lipid (%w/w on dry basis)	Ash (%w/w on dry basis)	Starch (%w/w)	AM (%w/w)
Maize	0,37	0,21	0,07	96,3	22,7
Potato	0,08	0,19	0,33	93,4	20,9
Wheat	0,19	0,14	0,16	92,8	27,8
Barley	0,32	0,18	0,18	94,8	24,7

Cassava is a crop that is grown commercially in China for starch production and was characterized as 20% AM and 80% AP. Cassava starch (CS) consists long-chained structures which can be hydrolysed into glucose and maltose and used as substrate to commercially produce many products such as bioethanol, polyhydroxyalkanoates and succinic acid. This process needs an intermediate treatment step to liberate sugars before use, making this process costly and not economically viable (Zeng et al., 2018; Zhang et al., 2016). *Bacillus subtilis* GXC-36 was able to utilize CS as substrate for PGA production at a yield of 33.51 g/L (Zeng et al., 2018). *Bacillus* sp naturally produce amylases which are enzymes that can break down the starch into smaller chains such as dextrin, maltose, and in some cases glucose (Asgher et al., 2007; Baysal et al., 2003; Haq et al., 2003). *Bacillus* is grown for amylase production through submerged fermentation (SmF) and solid-state fermentation (SSF).

Table 7-3 provides a list of *Bacillus* species used for production of this enzyme. Other species used for amylase production are fungal species such as *Aspergillus niger; Aspergillus oryzae; Penicillium fellutanum* and *Penicillium roquefortii*.

Table 7-3 Bacillus *species used for amylase production (adapted from Sundarram et al. (2014)).*

Strain	Reference
Bacillus licheniformis	Babu and Satyanarayana (1995)
Bacillus licheniformis GCB-U8	Sodhi et al. (2005)
Bacillus licheniformis M27	Amoozegar et al. (2003)
Bacillus amyloliquefaciens	Oboh et al. (2005)
Bacillus coagulans	Prakash et al. (2009)

7.2 PGA from Confectionery Waste: Materials and Methods

7.2.1 Experimental approach

The *Bacillus* species investigated were selected according to specific criteria, tailored to meet the requirements for processing the confectionery waste characterised. Both species and waste stream informed the medium requirements. *B. licheniformis* was cultivated in shake flasks using a nutrient medium and then adapted to the chemically defined medium. Following the establishment of appropriate production conditions under defined conditions using a nutrient and a defined medium, growth and product kinetics and yields were determined. Thereafter the system was tested on and adapted to confectionery streams.

Figure 7-2 Experimental approach to investigating production of PGA from confectionery waste

7.2.2 Experimental groundwork

7.2.2.1 Strain selection

In the selection process for a *Bacillus* species, the following selection criteria were used:

- A well-characterised microorganism was preferred.

- The species needed to not be fastidious about media requirements and to be robust with respect to process perturbation
- The microorganism needed to be capable of the biosynthesis of PGA.
- L-glutamic acid independent biosynthesis was important for cost-effectiveness.

B. licheniformis JCM 2505 was selected based on the criteria above. Madonsela (2013) demonstrated that this strain was able to produce PGA without glutamate as a nitrogen source, using a two-level Plackett–Burman factorial design to assess the impact of C:N:P ratios. The medium was optimised from ME to MME (Subsection 7.2.2.3, Table 7-4) by determining the ideal proportions needed to facilitate growth and PGA production. The strain was originally obtained from Riken, the Japanese research institute.

Bacillus species ubiquitous in nature are also able to produce γ-PGA. These organisms can use a variety of substrates, including dilute nutrients in wastewater to produce γ-PGA, thereby improving the quality of the water from the waste treatment plants with the simultaneous production of an additional product of value. In a previous project (WRC K/5 2000) in the CeBER research centre (Verster et al., 2014), thirteen *Bacillus* species were isolated from Mitchell's Plain's wastewater treatment plant. Two of the most likely PGA-producing isolates were selected (Madonsela, 2013). Since these isolates are facultative, *Isolate 1* was selected as the most promising *Bacillus* species for PGA production on starch, forming the second selection.

7.2.2.2 Matching the waste resource to media requirements

The confectionery waste obtained from an anonymous confectionery factory site, containing defective chocolate, marshmallow, and hard candy, was characterised and is presented in Table 3-2. The macronutrients identified for cell growth as well as PGA production include a carbon, nitrogen and phosphorus source, as well as key trace elements, namely, Ca, Fe, K, Mg, and Mn (Madonsela, 2013). The characterisation allowed us to determine the supplementation of candy waste required to support microbial growth. The aim was to quantify the nutrients in the waste and compare these with the nutrients needed for PGA production, such as free amino nitrogen, fermentable sugars, and trace elements, identified in Section 7.2.2.3.

The confectionery waste consisted largely of total organic carbon (TOC). According to Madonsela (2013), the required C:N ratio (based on the composition of MME) was calculated to be 14:1. The chocolate waste contained the most TKN, the most N at 0.5% on a mass basis. The C:N ratios of chocolate, marshmallow and hard candy are presented in Table 3-2. Hard candy had the highest C:N ratio of 400:1. Owing to the high C:N ratios, it had to be supplemented with additional nutrients for the cultivation of *B. licheniformis.* A minimum of 1.1 g of NH_3 per litre is needed for PGA production.

7.2.2.3 Medium selection

To grow *B. licheniformis* JCM 2505 on confectionery waste, the different approaches taken using renewable resources as feedstock for the production of PGA were compared (Birrer et al., 1994; Feng et al., 2016; Kumar and Pal, 2015; Madonsela, 2013; Moraes et al., 2012; Tang et al., 2015; Zhang et al., 2012). The confectionery waste required supplementation with a "basal medium". A screening approach to determine whether *B. licheniformis* was able to grow on the different types of waste (candy, marshmallow, chocolate and starch) was used. The control was MME, the medium

composition optimised by Madonsela (2013) and detailed in Table 7-4, which contained 20 g/L sucrose.

Table 7-4 Composition of the basal medium

Component	Concentration (g/L)
$CaCl_2.2H_2O$	0.15
Citric acid	12
$FeCl_3.6H_2O$	0.04
Glycerol	1
K_2HPO_4	2.99
$MgSO_4.7H_2O$	0.5
$MnSO_4.H_2O$	0.104
NH_4Cl	3.48

7.2.2.4 Growth profiling

B. licheniformis was cultivated on 20 g/L solid hard candy waste supplemented with basal medium MME. A medium containing 20 g/L sucrose supplemented with basal medium MME was used as a control. The basal medium composition is summarised in Table 7-4. The cultivations undertaken are detailed in Table 7-5 and extended for 24 h.

Table 7-5 Cultivation of Bacillus *on hard candy waste*

Parameter	Run 1	Run 2	Run 3	Run 4
Organism	B.licheniformis	B.licheniformis	B.licheniformis	B.licheniformis
Substrate	Sucrose	Sucrose	Candy	Candy
Sugar concentration (g/L)	20	20	20	20
Temperature (° C)	37	37	37	37

B. licheniformis and *Isolate 1* were cultivated on 20 g/L dextrin suspended in deionised water, supplemented with basal medium. Table 7-6 provides a summary of the conditions for the cultivation of *Bacillu*s on starch. Experiments took place in three 1 L Erlenmeyer shake flasks containing 250 mL medium and at an orbital shaking frequency at 150 rpm for the duration of 24 h. A 2 mL sample was taken every two hours for the first 14 h to determine the growth profile.

Table 7-6 Cultivation of Bacillus on starch waste

Parameter	Run 1	Run 2	Run 3	Run 4
Organism	B.licheniformis	B.licheniformis	Isolate 1	Isolate 1
Substrate	Starch	Starch	Starch	Starch
Sugar concentration (g/L)	20	20	20	20
Temperature (° C)	37	37	37	37

7.2.3 Bioreactor studies for growth kinetics

Microorganisms, including *B. licheniformis,* need a sufficient nutrient supply to ensure proper cell function (Birrer et al., 1994; Feng et al., 2016; Kumar and Pal, 2015; Madonsela, 2013; Moraes et al., 2012; Tang et al., 2015; Zhang et al., 2012). PGA production is growth associated. The amino acid monomer is produced from intermediates of the TCA cycle; hence a supply of C_6 sugars and NH_4^+ ions is needed (Kongklom et al., 2017, 2015). The shake flask data was used to determine the growth behaviour of *B. licheniformis* to inform the operation of the bioreactor system. Based on these findings, the pH was maintained at a pH of 6.5 as it fluctuated during the different stages of growth. The stirred tank bioreactor system was selected as it enabled control of parameters pH, temperature and dissolved oxygen at the optimum determined for growth and provided enhanced oxygen transfer to ensure that the cells within the system were not oxygen-limited.

The batch reactor studies provided a baseline for understanding how *B. licheniformis* responds to a more controlled environment. The relationship between the microorganism and substrate, as well as product formation, was investigated. The subsequent fed-batch experiments investigated enhancing PGA production by preventing substrate limitation. Table 7-7 is a summary of the substrates and conditions used to produce PGA from confectionery waste.

Table 7-7 Parameters used for bioreactor studies

Parameter	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6
Organism	B.licheniformis	B.licheniformis	B.licheniformis	B.licheniformis	Isolate 1	Isolate 1
Substrate	Sucrose	Sucrose	Candy	Candy	Starch	Starch
Sugar conc. (g/L)	20	20	20	20	20	20
DO (% sat)	≥20	≥20	≥20	≥20	≥20	≥20
pH	6.5	6.5	6.5	6.5	6.5	6.5
Temperature (° C)	37	37	37	37	37	37
Working volume (L)	5	5	5	5	5	5

7.3 PGA from Confectionery Waste: Experimental Results

7.3.1 Growth of *B. licheniformis* on confectionery waste

In order to compare the results obtained from growth of *B. licheniformis* on the modified medium E (MME) (Section 7.2.2.3), the stoichiometric amount of carbon was calculated. The total carbon contribution from the glucose, glycerol, and citric acid sources from the MME was 12.83 g C/L (Table 7-8). From Table 3.2, the TOC values were multiplied by 12.83 g C/L to calculate the respective amount of waste required (Table 7-9) to ensure that the amount of carbon added from the confectionery waste was equal to the amount of carbon added from the MME.

Table 7-8 The individual carbon requirements of glucose, citric acid and glycerol

Carbon source	Stoichiometric ratio of C	C required (g/L)
Glucose (20 g/L)	0.40	8.00
Citric acid (12 g/L)	0.37	4.44
Glycerol (1 g/L)	0.39	0.39
Total C required		**12,83**

Table 7-9 Respective carbon amounts added from various confectionery waste

Waste source	TOC (g Carbon /g waste)	Waste required (g)
Marshmallow	0.41	0.41 x 12.83 = 31.29
Chocolate	0.55	0.55 x 12.83 = 23.32
Hard candy	0.43	0.43 x 12.83 = 29.83
Mixture	*0.46*	*0.46 x 12.83 = 28.00*

Multiwell plate experiments were conducted using confectionery waste added as carbon source in basal medium inoculated with *B. licheniformis.* Figure 7-3 shows the plates prior to inoculation, while Figure 7-4 depicts the growth after 30 h. The 1st, 2nd, 3rd and 4th columns represent samples of glucose, chocolate, hard candy, and marshmallow, respectively, in triplicate (A, B, C). The 5th column consists of equal proportions of mixed candy, chocolate, and marshmallow. From Figure 7-3, it is seen that the chocolate imparts a brown colour and some turbidity to the medium. Figure 7-4 shows that glucose, hard candy as well as marshmallow displayed growth, monitored by OD at 600 nm blanked against media (Table 7-10), as well as colour change.

Figure 7-3 Multiwell plates containing 1) glucose, 2) chocolate, 3) hard candy, 4) marshmallow, and 5) mixture prior to inoculation with B. licheniformis

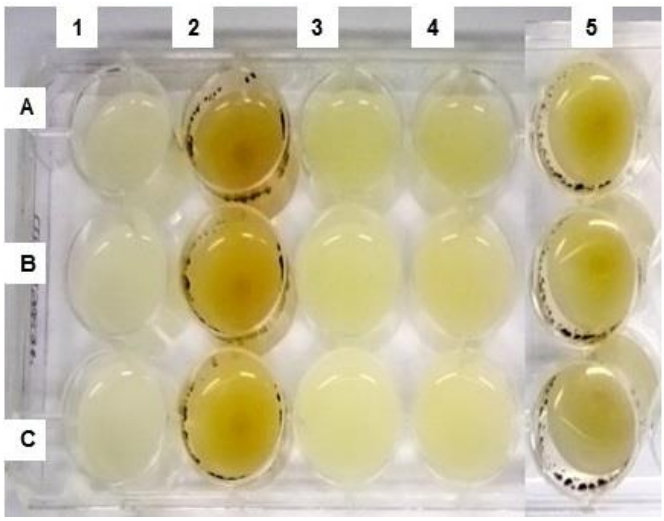


Figure 7-4 Multiwell plates containing 1) glucose, 2) chocolate, 3) hard candy, 4) marshmallow, and 5) the mixture after 30 h cultivation

Optical densities of the multiwell plate experiments at 30 h, blanked against their respective medium, as well as the starting pH and the pH after 30 h of cultivation are shown in Table 7-10. Marshmallow waste showed the best growth over all substrates. Chocolate, which was the most complex substrate, produced the second highest cell concentration. The glucose and hard candy produced similar, but lower, cell concentrations. The mixture which contained equal proportions of the three had a final OD between those of chocolate and hard candy. The starting pH ranged between 6.77 and 6.89. After 30 h of cultivation, the medium containing marshmallow was the most acidic, with a final pH of 4.2 ± 0.02. Glucose and hard candy had similar pH values of 4.46 ± 0.03 and 4.47 ± 0.06 respectively. The chocolate and the mixture had similar final pH values of 4.93 ± 0.08 and 4.86 ± 0.09 respectively. Hence, the screening of confectionery waste for growth showed promising results.

OD at the end of the cultivation was measured with a deionised medium blank. Where necessary, samples were diluted in deionised water. The chocolate and marshmallow contained undissolved solids which caused interference in obtaining an accurate reading. Furthermore, the chocolate and marshmallow could not easily be filtered. This challenge required investigation into how to extract the sugars for analysis easily and was beyond the scope of this project. The fatty deposit seen above the chocolate-containing medium demonstrated that fat was not broken down by *Bacillus* which preferred to use the sucrose over the complex fats as a carbon source.

Table 7-10 Growth of B. licheniformis *in multiwell plates after 30 h cultivation*

	OD			pH		
Carbon source	**Starting**	**Final**	**STDEV**	**Starting**	**Final**	**STDEV**
Chocolate	0.10	5.20	0.31	6.85	4.93	0.08
Glucose	0.10	4.29	0.34	6.89	4.46	0.03
Hard candy	0.10	4.23	0.24	6.77	4.47	0.06
Marshmallow	0.10	5.62	0.36	6.82	4.20	0.02
Mixture	0.10	5.07	0.12	6.80	4.86	0.09

For the purpose of this study, hard candy was used as a model substrate as it was the simplest carbon source. Figure 7-5 represents the sugar profile of hard candy used in this study. The largest proportion of the sugars was sucrose, a disaccharide consisting of glucose and fructose molecules; the remainder of the sugars were the simple monosaccharides, glucose and fructose. *Bacillus* species has natural ability to break down sucrose (Zhang et al., 2012).

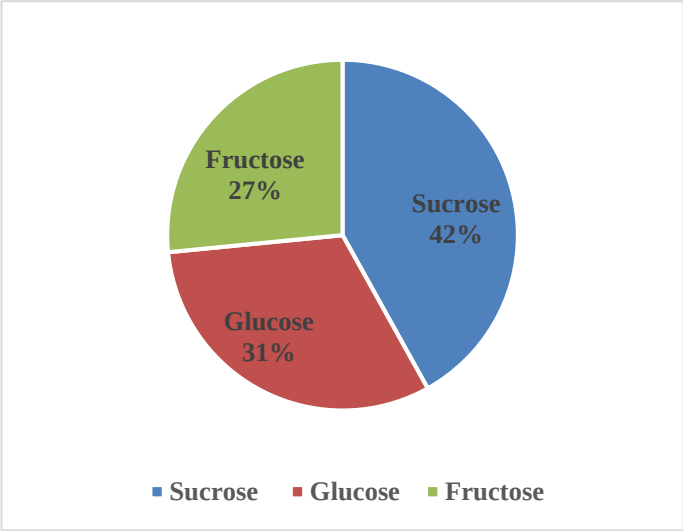


Figure 7-5 Sugar profile of hard candy waste

7.3.2 Growth of *B. licheniformis* on a nutrient-rich medium

A nutrient-rich medium consists of all nutrients essential for microorganisms to grow and to function optimally. To provide a baseline, *B. licheniformis* was cultivated on a tryptone-soy medium in shake flasks at a 120 rpm orbital shaking frequency and 37 °C to determine the growth profile, observe strain adaptation to the nutrients and monitor the fluctuation in pH. The results are shown in Figure 7-6.

The starting cell concentration of 0.056 g/L CDW was used. The initial exponential growth phase lasted for 6 h, after which they transitioned through a late exponential phase to reach the stationary phase at 10 h. The final cell concentration of 1.40 g/L ± 0.70 was reached at 10. The pH decreased from a starting pH of 6.9 to a minimum pH of 6.2 in late exponential phase, whereafter the pH increased to pH 6.6 in the stationary phase.

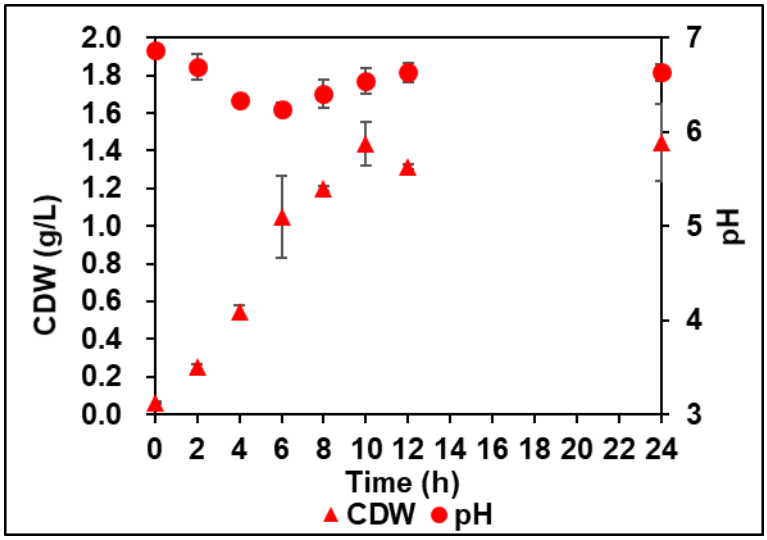


Figure 7-6 Growth of B. licheniformis on nutrient-rich medium

7.3.3 Growth of *B. licheniformis* on sucrose

Since candy consisted mostly of sucrose, *B. licheniformis* growth was profiled in shake flasks containing sucrose-supplemented basal medium MME, pH 6.5 (Section 7.2.2.3). Sampling was done at 2 h intervals for the first 14 h to monitor growth and physicochemical conditions.

During sampling, the pH was measured using a Eutech CyberScan pH 2100 pH probe. The pH remained constant over the first 2 h, as shown in Figure 7-7. A steady drop in pH was seen for the next 10 h, whereafter it remained constant at pH 5.6 ± 0.2 between 12 and 14 h and increased to pH 5.82 at 24 h. The starting cell dry weight (CDW) was 0.056 g/L. During the first two hours, the cell concentration increased by 0.026 g/L, which is not as fast as growth on tryptone soy. This may be due to the adaptation phase needed for the cells in a minimal medium. The exponential phase continued until 10 h. Thereafter a diauxic growth pattern was observed between 10 and 16 h, which may be due to the depletion of monosaccharides, namely, fructose and glucose released by hydrolysis on autoclaving (10 h) and the activation of the internal sucrose utilisation system (Zhang et al., 2012). This was validated by sugar analysis on a larger scale. The maximum cell concentration at 24 h was 1.85 g/L.

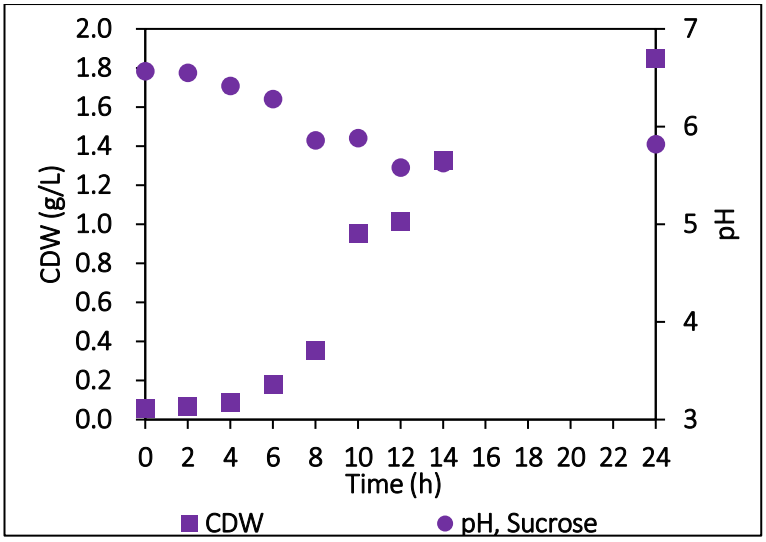


Figure 7-7 *Growth of B. licheniformis on sucrose in shake flasks (pH is given as (●) while biomass concentration is given as (■))*

7.3.4 Growth of *B. licheniformis* on hard candy waste

Hard candy waste contains acidulants added as flavourants; hence is slightly acidic. Prior to autoclaving, its pH was adjusted to pH 6.5, using 5 M NaOH. Similarly, the pH of the basal medium, autoclaved separately to prevent the Maillard reaction, was adjusted to pH 6.5 (Martins et al., 2000). In Figure 7-8, the pH profile followed a similar trend to that of the sucrose shake flask experiments presented in Figure 7-7. For the first 4 h, the pH remained constant at pH 6.3, whereafter it decreased gradually to pH 5.6 at the end of the exponential phase, increasing slowly on entry into the stationary phase, and reaching a pH of 6.04 at 24 h. Similarly, the biomass growth on hardy candy material resembled that on sucrose, increasing exponentially to 1.05 g/L at 10 h, prior to exhibiting diauxic growth and a second exponential increase to 1.63 g/L at 14 h.

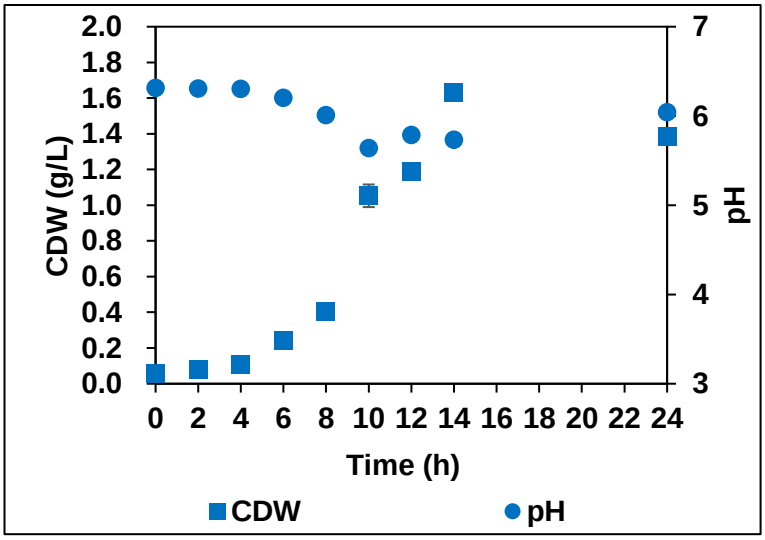


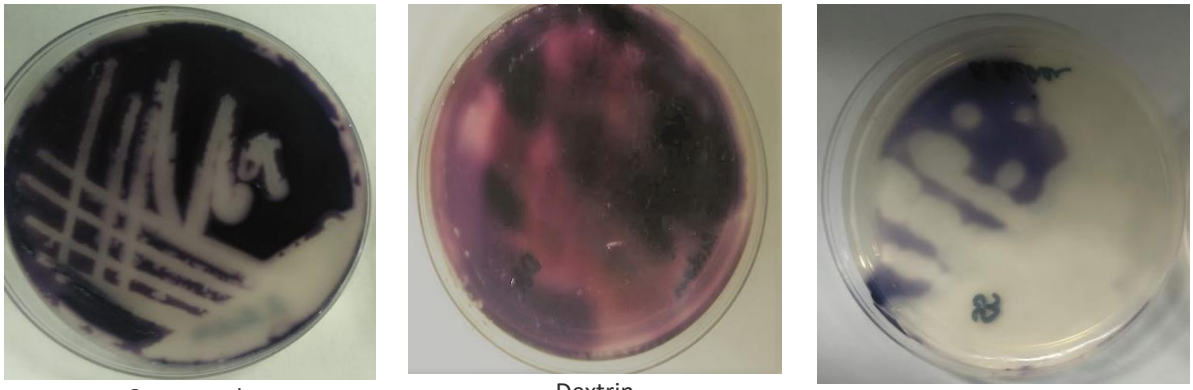
Figure 7-8 *Growth of B. licheniformis on candy waste in shake flasks(pH is given as (●) while biomass concentration is given as (■))*

7.3.5 Growth of *B licheniformis* on starch

The cultivation of *Bacillus* on starch waste as a potential feedstock was investigated. A new *Bacillus* strain named Isolate 1 (Section 7.2.2.1) was selected for the production of PGA on confectionery waste as Madonsela (2013) demonstrated that it was able to produce high yields of biopolymer using an inorganic nitrogen source and dilute nutrients found in wastewater. Isolate 1 was cultivated on potato dextrin in shake flasks to determine if it was able to use starch as substrate for cultivation and PGA production.

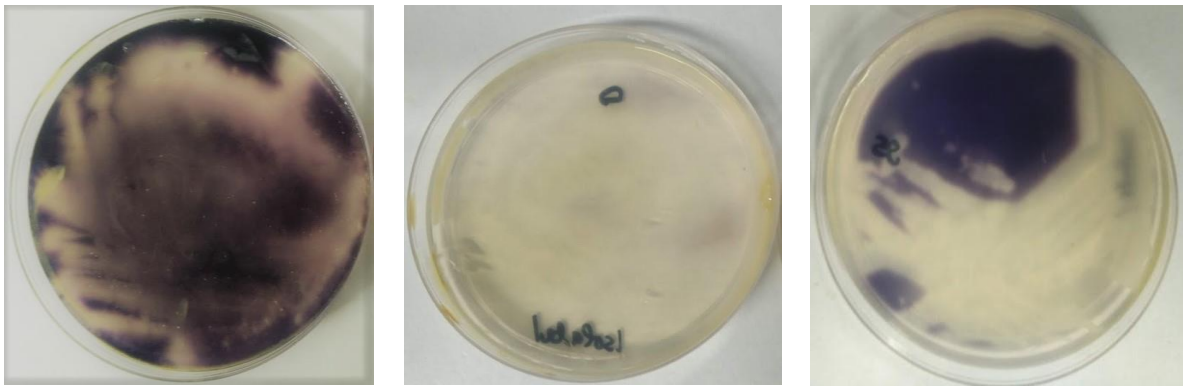
7.3.5.1 Amylase plate assays

Isolate 1 and *B. licheniformis* were plated onto agar plates containing 20 g/L of different starches, namely corn starch, potato dextrin and soluble starch supplemented with 0.15 % (w/v) yeast extract. The plates were incubated for 48 h, the cells were then scraped off the plates, and iodine was used to flood the plates. Iodine stains the AM molecule by interacting with the helical shape, producing a blue-black colour. When the polysaccharide is hydrolysed into its glucose monomers, the colour changes from blue-black to colourless. The "zones of clearance" indicate amylase activity. Figure 7-9 shows that *B licheniformis* was able to produce amylase. The amylase activity for Isolate 1 can be seen Figure 7-10. The dextrin plate was completely clear, indicating the starch degrading activity of Isolate 1.



Corn starch Dextrin Soluble starch

Figure 7-9 B. licheniformis *amylase activity on different starch sources*



Corn starch Dextrin Soluble starch

Figure 7-10 Isolate 1 amylase activity on different starch sources

7.3.5.2 Growth profiling

The cultivation of *B. licheniformis* and Isolate 1 on 20 g/L potato dextrin supplemented with basal medium took place in shake flasks for 24 h. Figure 7-11 is shows the results for the cultivation of *B licheniformis* and Isolate 1 on dextrin. A shorter lag phase was seen compared to the candy. Isolate 1 produced more biomass than *B. licheniformis* at the end of the cultivation. Both strains exited exponential phase between 9 and 10 h but continued growth until between 16 and 23 h, with Isolate 1 reaching 2.4 g/L and *B.licheniformis* 2.0 g/L. The pH profile decreased to a minimum of pH 5.2 in late exponential phase, thereafter increasing to pH 6.3.

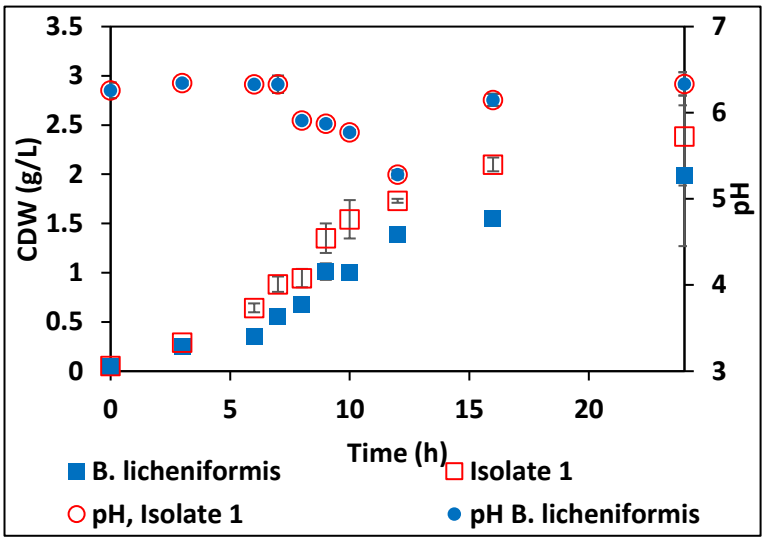


Figure 7-11 Cultivation of Isolate 1 and B. licheniformis *on dextrin in shake flasks*

The comparison of growth kinetics of *B. licheniformis* in shake flasks using different substrates is summarised in Table 7-11. Dextrin yielded the highest biomass with a maximum CDW of 1.99 ± 0.72 g/L for *B. licheniformis* and 2.39 ± 0.50 for Isolate 1 after 24 h of cultivation, followed by sucrose with a CDW of 1.85 g/L. Growth on candy and sucrose had identical μ_{MAX} values and both had exponential phases that lasted 10 h, while tryptone soy had a short exponential phase of 6 h at a higher maximum growth rate of 0.48 h^{-1}. The growth rates for both Isolate 1 and *B. licheniformis* were low compared to candy and sucrose at 0.17 and 0.24 h^{-1} respectively. Overall, Isolate 1 was the better performer on dextrin and was selected as the strain for cultivation on starch waste.

Table 7-11 Kinetic parameters for shake flasks

	B. licheniformis				**Isolate 1**
Substrate	Tryptone soy	Sucrose	Hard candy	Dextrin	Dextrin
CDW (g/L)	1.44 ± 0.20	1.85 ± 0.00	1.38 ± 0.02	1.99 ± 0.72	2.39 ± 0.50
µmax (h^{-1})	0.48 ± 0.01	0.32 ± 0.01	0.32 ± 0.02	0.17 ± 0.00	0.24 ± 0.01

7.4 Bioreactor studies

The bioreactor system provides better control of pH, temperature and dissolved oxygen at a larger scale. The batch reactor configuration provides a baseline for understanding how *B. licheniformis* responds to a more controlled environment. The relationship between the microorganism and substrate, as well as product formation, was investigated. The subsequent fed-batch experiments investigated the prospect of enhancing PGA production by preventing substrate limitation. The results and discussion on cultivating *B. licheniformis* in a batch bioreactor setup are presented here.

7.4.1 Batch reactor cultivation of *B. licheniformis* on sucrose

The results of the cultivation of *B. licheniformis* on basal medium with sucrose as carbon source are given in Figure 7-12. Exponential growth was observed from the start of the cultivation till 12 h. During the exponential phase, total sugar utilisation took place in proportion to biomass formation, with depletion at 14 h. This growth follows the Monod model, where exponential growth is seen under balanced nutrient conditions, after which a decrease in growth rate occurs owing to exhausted nutrients (Shuler and Kargi, 2002). The onset of the stationary phase was between 12 and 14 h, after which the biomass concentration remained constant at 5.3 g/L. The error bar on the sugar concentration at 12 h show that the reduction in substrate concentration happened rapidly, with a high concentration of biomass within the system that had adapted to the cultivation conditions. Between 10 h and 14 h, the sugars within the system were completely exhausted. Following this phase, *B. licheniformis* may start to break down extracellular polymeric substances (EPS) such as PGA for growth and maintenance.

In Figure 7-13, the pH and PGA concentration are shown. The pH was maintained at pH 6.5 with 5 M NaOH during the exponential growth phase. According to Kongklom et al. (2015), an increase in pH is attributed to the depletion of sugars within the system, while pH decrease is due to the formation of byproducts such as organic acids. After 12 h, the pH began to increase as the cells entered the stationary phase and organic acid production was complete. As there was no pH control, the final pH ranged between 8.42 and 8.53 ± 0.08.

The PGA concentration started at 0.49±0.16 g/L, owing to carry over with the inoculum. During the exponential phase, a steady increase in PGA concentration was seen. At 12 h, the PGA concentration was 3.1 g/L and increased to 3.42 ± 2.22 at 14 h. The final PGA concentration was 3.68 ± 2.38 g/L.

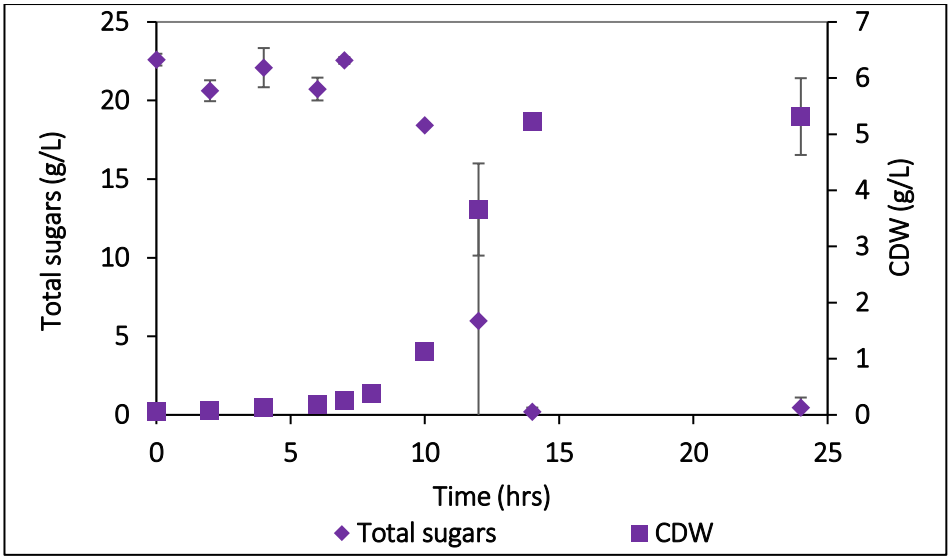


Figure 7-12 Sugar utilisation of B. licheniformis on sucrose at pH 6.5, aeration at 1 vvm and 37 °C with agitation starting at 500 rpm. (Biomass concentration is given as (■) and total sugars given as (◆))

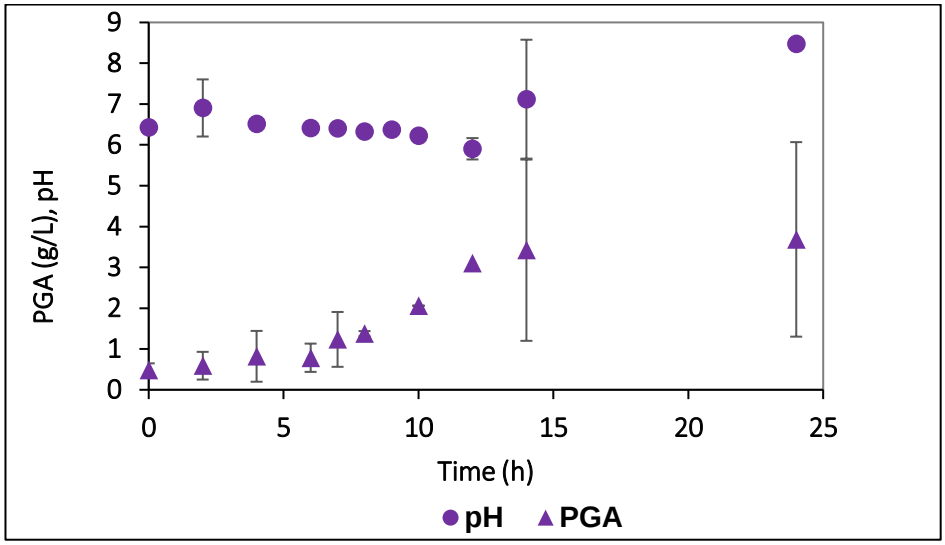


Figure 7-13 PGA formation and pH of a B. licheniformis culture grown in sucrose at pH 6.5, aeration at 1 vvm and 37 °C with agitation starting at 500 rpm. (PGA concentration is given as (▲), while pH is given as (●))

The relationship between biomass formation (CDW) and PGA formation during the first 12 h of the cultivation is shown in Figure 7-14. A linear relationship was seen which suggested that the PGA product is growth associated *i.e.* as the cell concentration increased in the system, so did the PGA.

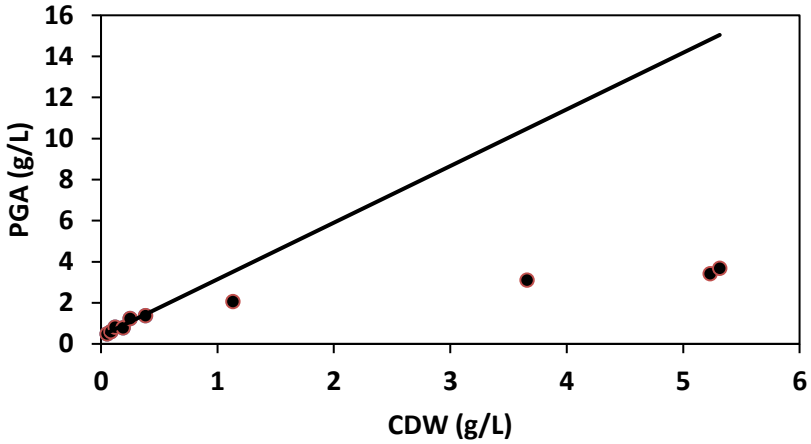


Figure 7-14 Correlation between CDW versus PGA

7.4.2 Batch reactor cultivation of *B. licheniformis* on candy waste

The 24 h cultivation of *B. licheniformis* on a basal medium supplemented with hard candy waste to an equivalent sugar concentration in the 7 L New Brunswick BioFlo 110 stirred tank reactor had the same sampling procedure as stated above; data are presented in Figure 7-15. A slightly longer lag phase was seen. When prepared, the hard candy waste-based medium was acidic (pH 3 to 4); hence a significant amount of 5 M NaOH was used to neutralise the waste. The Na^+ concentration may have inhibited the growth and required a longer adaptation time. Sibanda (2009) demonstrated such an effect when the yeast *S. cerevisiae* was subjected to molasses with an elevated monovalent cation concentration, especially Na^+. At 14 h, the cells were still in exponential phase. Alternatively, acidulants, preservatives or stabilisers may have slowed growth and required adaptation. At 24 h, the maximum cell concentration was 5.86 ± 0.41 g/L.

The relationship between pH and PGA formed is shown in Figure 7-16. The peak PGA concentration measured was 3.04 g/L at 14 h and a pH of 6.2 ± 0.18. During the stationary phase, the pH increased to 8.67 ± 0.09. The PGA concentration decreased to 2.49 g/L ± 0.73 following the depletion of the sugar, suggesting that it is metabolised in the presence of carbon limitation to support cell maintenance. The growth of *B. licheniformis* on candy followed similar trends to that on sucrose, thus supporting the use of hard candy waste as a potential feedstock.

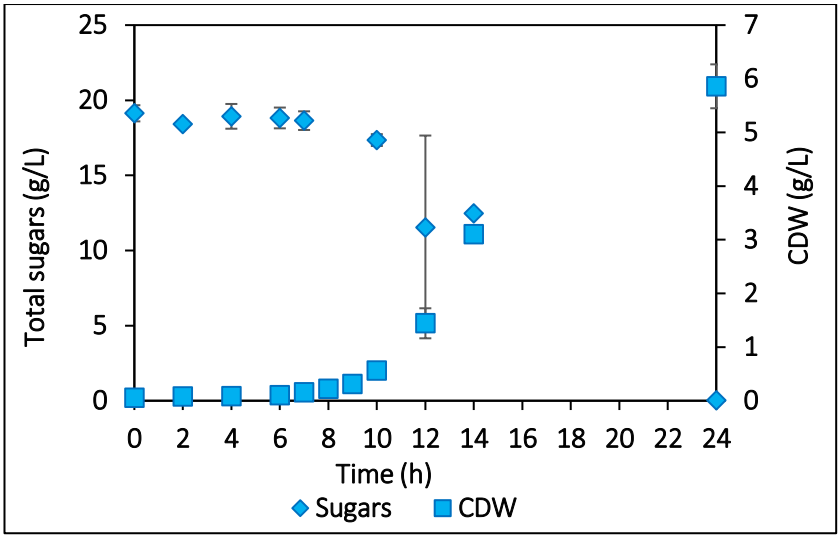


Figure 7-15 *Growth of B. licheniformis and associated sucrose utilisation when grown on hard candy waste at pH 6.5, aeration 1 vvm and 37 °C with agitation starting at 500 rpm. Total sugar concentration is given as (◆), while biomass concentration is given as (■).*

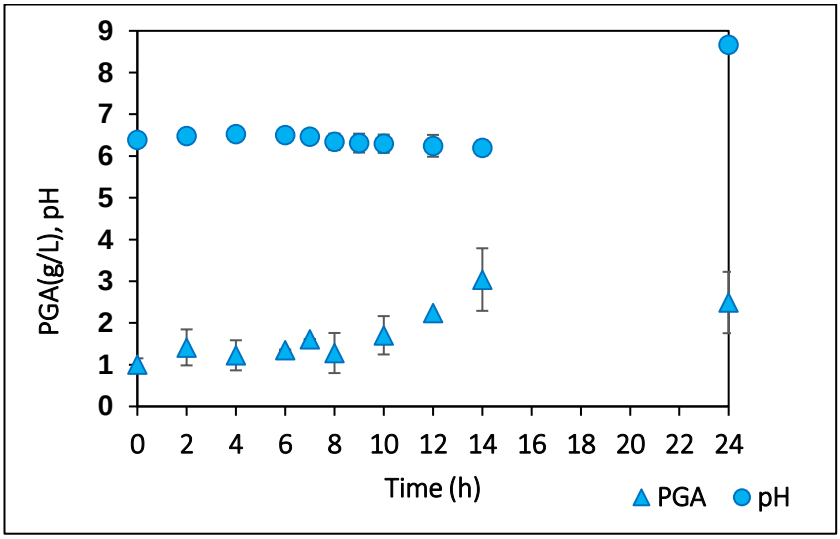


Figure 7-16 *pH profile and PGA formation on hard candy waste. (PGA concentration is given as (▲) while pH is given as (●))*

A comparative study between the kinetic parameters of *B. licheniformis* in batch mode using different substrates (sucrose and candy) was conducted and the results are tabulated in Table 7-12. More PGA was formed on sucrose than on hard candy. This could be for several reasons. Owing to the longer lag phase of the hard candy growth profile, an additional data point was needed at 16 and 18 h to quantify the PGA concentration, as *B. licheniformis* had not yet reached the stationary phase, and therefore maximum PGA concentration. The lower PGA may be offset against the higher biomass, should the difference be significant. Alternatively, there may have been compounds in the substrate matrix that activated different pathways or stress mechanisms, diverting C and N from PGA. The maximum specific growth rates were similar. The product yields with respect to substrates were similar. $Y_{x/s}$ values differed, with hard candy yielding more biomass overall, making candy a potential substrate for the production of PGA.

Table 7-12 Comparison between kinetic parameters of PGA production on sucrose and hard candy and sucrose

Substrate	Sucrose	Hard candy
CDW (g/L)	5.31 ± 0.68	5.86 ± 0.41
Maximum PGA concentration (g/L)	3.55 ± 0.63	3.04 ± 0.75
μ_{max} (h^{-1})	0.43 ± 0.06	0.44 ± 0.03
$Y_{P/S}$ (g PGA/g substrate)	0.14 ± 0.02	0.11 ± 0.05
$Y_{X/S}$ (g CDW/g substrate)	0.24 ± 0.02	0.30 ± 0.01

7.4.3 Fed-batch bioreactor studies

Based on the findings of the batch bioreactor runs, it was shown that PGA production is growth associated. Further, in the hard candy run, PGA production peaked at approximately 14 h; on subsequent depletion of the carbon source it began to decrease, suggesting it provided a nutrient source for cell maintenance. It was proposed that operation in fed-batch configuration would optimise PGA production by extending the biomass growth and associated PGA production phase, avoiding PGA consumption on deletion of the carbon source to achieve an enhanced PGA concentration and maintain the PGA productivity within the system at a high rate over an extended time period. Constant feeding of a mixture of hard candy and NH_4Cl into the reactor at a rate matching their consumption for PGA production was used from mid exponential phase.

7.4.4 Cultivation of *B. licheniformis* in fed-batch mode

The cultivation of *B. licheniformis* in fed batch took place in two stages. The first stage (12 h) was run as a normal batch process, where basal medium was supplemented with hard candy waste to a sugar concentration of 20 g/L. The cultivation took place at 37 °C, 1 vvm, an initial agitation of 500 rpm and pH 6.5. Feeding was initiated at 13 h at 0.083 L/h and continued for the next 9 h. The feed solution contained 60 g/L hard candy and 10.44 g/L NH_4Cl, providing a sugar feed rate of 4.98 g/h. The pH was maintained at pH 6.5, using 5 M NaOH for the first stage. No acid addition was used for pH correction. The growth profile is presented in Figure 7-17. During the first 12 h, the growth trend replicated the batch data. At 16 h, the cell concentration (CDW) continued to increase and there remained a low level of sugar within the system, as designed. During this feeding period, a consistent high biomass productivity was noted. The maximum dry cell biomass concentration at 23 h was 10.30 ± 0.81 g/L, a significant increase from the 5.86 ± 0.41 g/L produced in batch.

The pH and PGA profile can be found in Figure 7-18. For the first 12 h, the pH was maintained at 6.5. It began to increase at 16 h. The final pH value at the end of the cultivation was 7.60 ± 0.25. During the batch phase of cultivation, the PGA formation followed a similar trend to that of the previous batch experiments; concentration increased with the increase in cell concentration within the system. While an increase in PGA concentration was expected with increase in cell concentration during fed-batch operation, the PGA concentration remained steady. The ratio of the substrate added to the system that was converted to biomass increased and that to product decreased, resulting in a small mass-based increase in PGA. In other words, under the fed-batch conditions used, biomass growth was favoured over PGA production.

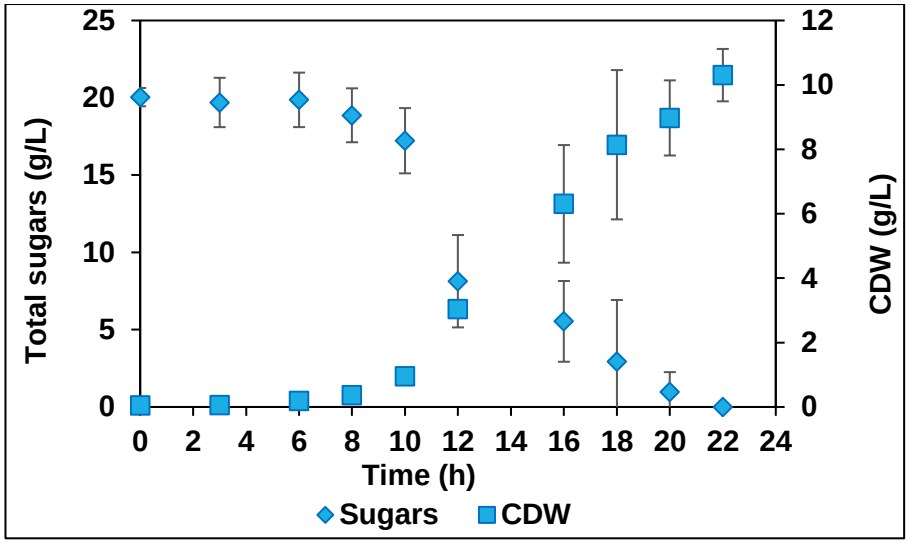


Figure 7-17 *Cultivation of B. licheniformis on hard candy-supplemented basal medium in fed-batch mode. Sucrose concentration is given as (◆), while biomass is given as (■).*

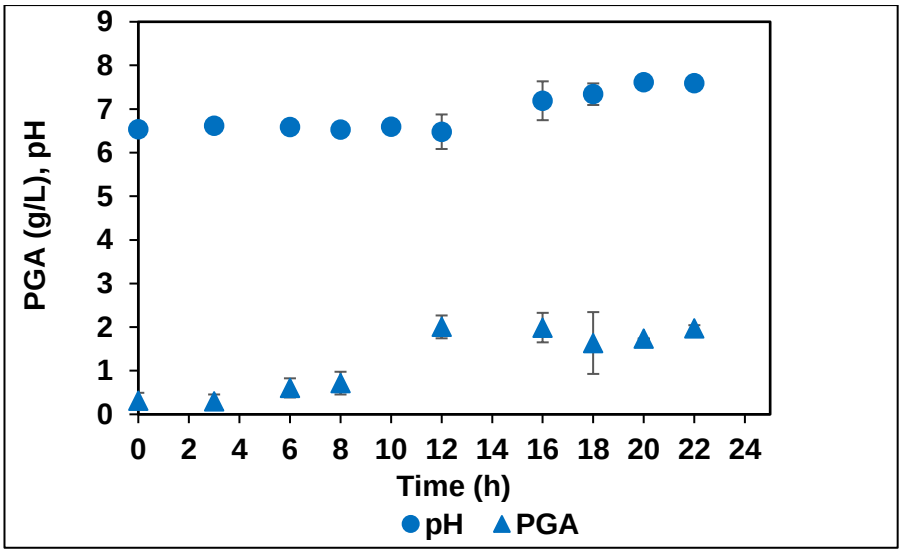


Figure 7-18 pH profile and PGA formation on hard candy waste in fed-batch mode. PGA concentration is given as (▲), while pH is given as (●)

7.4.5 The relationship between substrate consumption and biomass formation

A longer lag phase was seen when *B. licheniformis* was cultivated on hard candy waste than on sucrose. It was speculated that the unidentified compounds present in the waste caused inhibition. During the characterisation stage (Section 3.3.2), the approach was to identify the nutrients that could be used as nutrients for cell growth as well as PGA formation. Interestingly, insignificant amounts of trace elements were found. No nitrogen and fats were present in hard candy waste. The HPLC data showed that a large proportion of the waste comprised sucrose and invert sugars (a mixture of fructose and glucose). This information was used to plan an experimental approach; however, compounds like additional additives such as food colourants and acidulants were not quantified.

Confectionery waste can be metabolised by *B. licheniformis*, provided that the correct nutrient supplementation is used. For example, candy waste contains acidulants that lower the pH to 3; this low pH acts as an inhibitor to microbes hence pH correction is required. After the adaptation phase, the organisms began to break down the sugars at 10 h as depicted in Figure 7-19. There was a sharp decrease in residual sugar concentration during the first 2 h (prior to feeding), and then a gradual decrease in sugar concentration with time for the rest of the cultivation. The rate at which the feed was introduced was not sufficient for PGA production as the sugar concentration was depleted at 20 h.

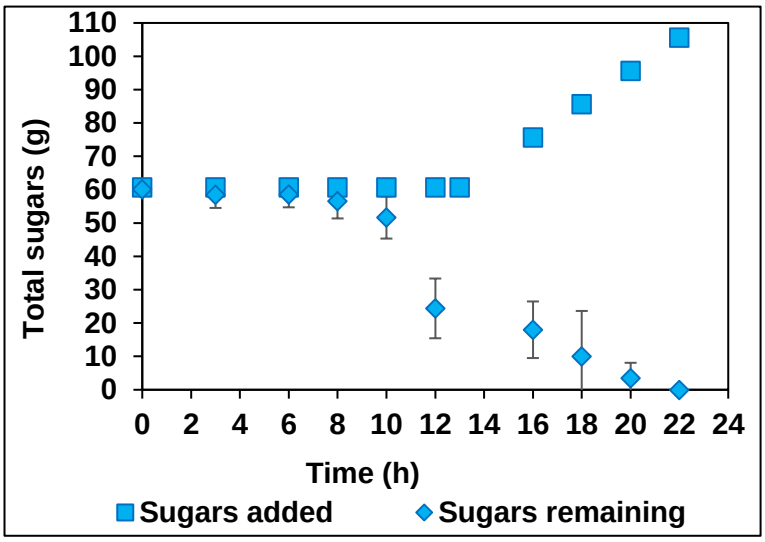


Figure 7-19 Relationship between sugar consumption and sugars added. (Sugars added is given as (■), while sugars remaining is given as (◆)

The accumulation of biomass and PGA on a mass basis is presented in Figure 7-20. Interestingly, the growth of *B. licheniformis* followed the same trend as the feeding, a final CDW of 38.64 ± 3.05 g. As expected, the PGA concentration increased with growth during the batch phase of the cultivation and peaked at 6.02 ± 0.79 g. The concentration remained the same, and began to increase slightly with a final mass of 7.62 ± 0.06 g. The feeding rate of 5 g/h of candy waste was not sufficient to produce PGA, and most of the carbon fed went to biomass. The challenge with fed-batch systems is implementing a feeding strategy that ensures that the system is fed with a C:N ratio adequate for biomass formation and PGA formation. Researchers Kongklom et al. (2015) maintained a sugar concentration of 6 to 20 g/L within the system. This feeding strategy increased the PGA concentration from 5.03 ± 0.04 to 27.54 ± 0.20 g/L. To further this study, it is recommended that a feeding strategy should be developed that will ensure that the sugar concentration within the system is maintained above 6 g/L to avoid sugar limitation and associated reduced rates. An indication of the onset of the stationary phase is the increase in pH. Since hard candy is acidic, it can be used in place of an acidic buffer, and this approach could potentially be used as a feeding strategy to maintain pH as well as the sugar concentration by using a more concentrated feed.

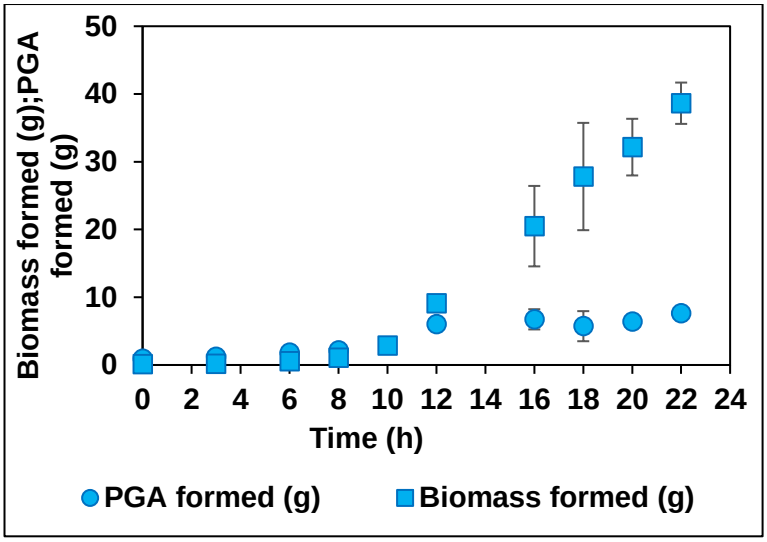


Figure 7-20 Relationship between biomass formed and PGA formed

7.4.6 Comparison kinetic parameters in batch and fed-batch reactors

The fed-batch experiment took place in two stages: (i) The batch stage lasted for the first 13 h; (ii) feeding of a mixture of 60 g/L hard candy and 10. 44 g/L NH_4Cl continued for 9 h, at 0.0833 L/h, which equated to the total of 45 g of hard candy waste added during the second phase. The feed had the same C:N as that of the batch feed.

From this additional substrate, 1.37 ± 0.27 g of PGA and 29.54 ± 3.0 g of biomass were formed. The $Y_{X/S}$ and the $Y_{P/S}$ for the batch phase at 13 h were 0.15 g biomass/g substrate and 0.10 g PGA/g substrate, respectively. In contrast, during the fed-batch stage a 4.4 fold higher $Y_{X/S}$ of 0.66 g biomass/g substrate was seen with a 3.3 fold lower $Y_{P/S}$ of 0.03 g PGA/g substrate, indicating that most of the nutrients fed went to biomass production and not PGA formation. This suggests that the feed rate may have been too low or that the nutrient balance was not conducive to PGA formation.

Table 7-13 Comparison between kinetic parameters in batch and fed batch

Parameter	Batch (Phase I) – 13 h	Fed batch (Phase II) – 9 h
Hard candy added (g)	60.12 ± 1.78	105.00 ± 0.49
DCW (g) formed	9.11 ± 0.11	29.54 ± 3.0
PGA formed (g) formed	6.02 ± 0.78	1.37 ± 0.27
$Y_{P/S}$ (g PGA/g substrate)	0.10	0.03
$Y_{X/S}$ (g biomass/g substrate)	0.15	0.66

7.5 Conclusions from Study of PGA Production on Confectionery Waste

The selected species *B. licheniformis* JCM2505 was able to produce PGA using a cheaper alternative nitrogen source (e.g. NH^{4+} ions or proteins) to glutamate, making it a potential organism of interest for PGA synthesis from waste organic products in the waste arena.

7.5.1 The performance of *B. licheniformis* in the production of PGA from confectionery waste

B. licheniformis JCM 2505 was able to grow on all three wastes (candy, chocolate and marshmallow), including a mixture of the three with the supplementation of a basal medium. This study focused on hard candy waste as a carbon source.

The growth of *B. licheniformis* on sucrose and sucrose supplied through hard candy waste was similar, except for the increased lag phase when using hard candy. This was attributed to the large addition of Na^{+} on neutralising the acidic candy waste with NaOH or to the presence of an acidulant, preservative or stabiliser with an inhibitory effect. The growth kinetics of *B. licheniformis* on candy and sucrose are comparable with slight differences.

More PGA was formed on sucrose with a yield coefficient of 0.14 ± 0.02 g-PGA/g-substrate and 0.11 ± 0.05 g-PGA/g-substrate for candy in batch. Interestingly, more biomass was formed on candy with a maximum DCW of 5.86 ± 0.41 g/L, making it a suitable substrate for the production of PGA.

The fed-batch configuration was used to maintain the exponential growth phase by feeding the system with a constant supply of carbon. However, during the feeding stage of the fed-batch experiment, a large majority of the substrate was used for biomass formation and very little PGA was formed. *B. licheniformis* favoured a specific C:N ratio for PGA production, and this was not achieved. It can be concluded that the feed rate was too slow or the C:N ratio inappropriate; hence the process should be optimised by increasing the feed concentration. Further work into investigating the required C:N ratio on production of PGA relative to biomass is recommended.

7.5.2 Recommendations for further investigation of PGA from confectionery waste

- Further characterisation studies are recommended to identify the additional nutrient compounds found in candy waste, as it is a complex source. This will facilitate minimisation of nutrients added to supplement the waste product.
- Optimisation experiments are needed to enhance PGA production on confectionery waste, particularly in fed-batch culture. This must include determination of the minimum supplementary nutrients needed, as well as ascertaining the optimal C:N ratio for PGA production.
- Further investigation into finding a wastewater stream high in nitrogen, i.e., corn steep liquor, micro-nutrients, and phosphates such as domestic wastewater, will aid in supplementing the candy waste and act as a solvent. It is highly recommended that further work is combined with wastewater treatment. This approach will minimise the process costs, as candy waste is needed in solution for PGA production. Combining effluents will contribute to knowledge in the wastewater biorefinery space.
- Furthermore, an appropriate downstream process for extraction of PGA must be further developed, as precipitation with cold ethanol is costly.

8 Pigment from Confectionery Waste

8.1 Introduction to Biopigments

Currently, there is a move towards the use of natural colourant alternatives in food, cosmetics, nutraceuticals and other related products. This is as a result of consumer preference for products which are perceived to be natural and healthy, the negative environmental and health implications of synthetic colourants, and government legislation limiting the application of certain synthetic pigments (Downham and Collins, 2000; Dufossé, 2006; Sigurdson et al., 2017). The majority of natural colourants in use are obtained from plant and insect sources and are affected by limitations including natural variation, low yield of product from the source and seasonal availability (Gunasekaran and Poorniammal, 2008; Mapari et al., 2009; Rao et al., 2017).

Microorganisms have garnered interest as potential sources of natural colourant alternatives to those obtained from plants and insects. They are able to produce pigments of similar chemical structure and colour, while overcoming some of the limitations of other natural pigment sources (Malik et al., 2012; Mapari et al., 2010; Torres et al., 2016). Microbial sources include bacteria, algae, yeast and filamentous fungi, each producing a range of potential pigment products, while also having associated limitations.

Filamentous fungi produce a diverse range of pigments while also having great potential for large-scale cultivation under controlled conditions (Dufossé et al., 2014). This group of organisms therefore represents an interesting alternative source of natural colourants. The pigments produced by filamentous fungi can be divided into two main groups, namely the carotenoids and polyketides (Mapari et al., 2008). Carotenoid pigments are further divided into the carotenes such as β-carotene and lycopene, and the xanthophylls which include lutein and zeaxanthin. Carotenoid pigments are oil-soluble, range in colour from yellow to red and exhibit sensitivity to heat, light and oxidation, limiting their application potential (Mortensen, 2006; Schmidt et al., 2005). Polyketides are a far more diverse group of compounds, which translate into a greater observed colour range and variable stability and solubility.

Well-known fungal polyketide pigment producers belong to the *Monascus* genus. The fermented rice product ‘angkak’ or red mould rice is the oldest example of a fungal colourant applied in a food product (Dufossé et al., 2005; Torres et al., 2016). A limitation associated with *Monascus* pigments, however, is the co-production of the mycotoxin citrinin (Liu et al., 2005; Shimizu et al., 2005). The filamentous fungus *Penicillium purpurogenum* was selected for investigation in this study as it is reported to produce *Monascus* pigment homologues, without mycotoxin co-production (Mapari et al., 2010; Ogihara et al., 2000). Previous experimental work completed in this study confirmed red pigment production, using *P. purpurogenum* DSM 62866 in a medium composed of malt extract and soya peptone, with a temperature of 30 °C and application of a 50 mM citrate buffer at pH 5 shown to have a beneficial effect on pigment production. This study investigated the ability of *P. purpurogenum* DSM 62866 to produce red pigmentation when cultivated on a medium composed of a selected confectionery waste product.

8.2 Experimental Study of Pigment Production from Confectionery Waste

The growth medium represents a major cost in the production of natural colourants by microbial fermentation. The use of a waste stream as an alternative growth substrate therefore offers a potential means of reducing process costs, contributing to making the product more economically feasible. In addition, any means of valorising waste contributes towards waste reduction and the move towards a biobased economy.

The assessment of whether *P. purpurogenum* could be cultivated on a confectionery waste substrate to produce red pigments was performed over a number of growth scales. Agar plate cultivation was used for initial evaluation of marshmallow and chocolate waste. Marshmallow confectionery was then selected for further investigation using submerged liquid cultivation. This progressed from multiwell plates into shake flasks and finally to 5 L cultivation in the New Brunswick BioFlo 110 bioreactor system. Supplementation of the marshmallow confectionery with either an organic or inorganic nitrogen source was also considered. Growth and pigment production achieved in the confectionery-based medium was compared to that obtained when using a malt extract-based medium previously applied during cultivation of *P. purpurogenum* DSM 62866.

8.2.1 Agar plate cultivation using confectionery waste substrates

Two waste streams, namely chocolate and marshmallow confectionery, were considered for cultivation of *P. purpurogenum*. The confectionery substrates were applied at 15 g/L concentration with bacteriological agar (15 g/L) used to solidify the medium. No supplementation of the confectionery substrate was considered. *P. purpurogenum* was spot inoculated onto the agar plate surface and was incubated at 30 °C for 10 days.

Both substrates supported limited growth of the organism, with pigmentation only observed when growing on the marshmallow plates. An image of the chocolate confectionery plate is provided in Figure 8-1, with top and bottom view of the plate shown. Development of growth and pigmentation on the marshmallow confectionery plate is represented in Figure 8-2.

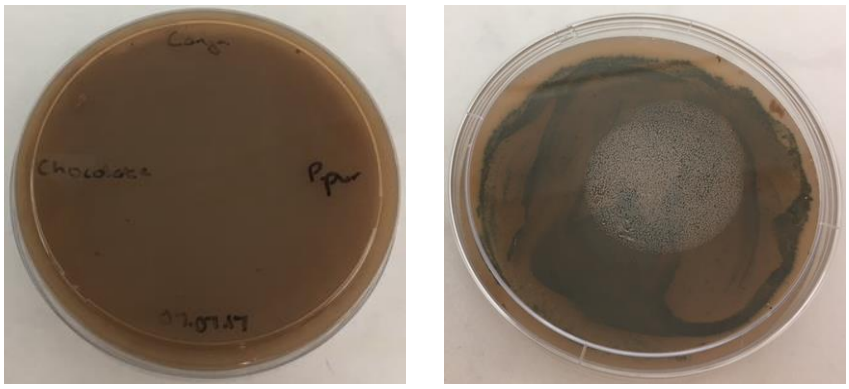


Figure 8-1 Growth of P. purpurogenum on chocolate confectionery-based agar plate. Incubated at 30 °C for 10 days.

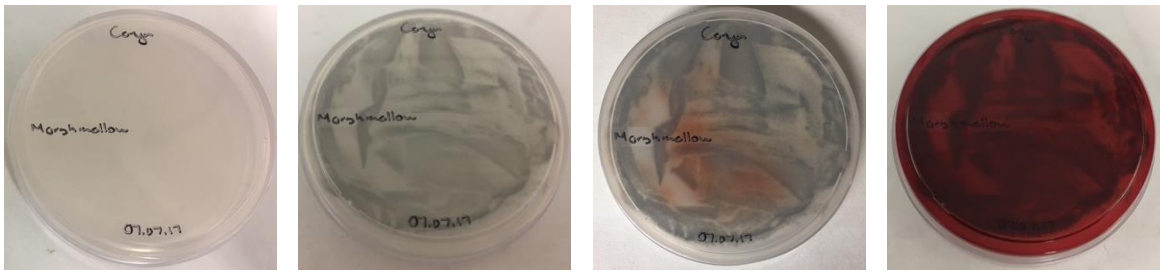


Figure 8-2 Growth and pigmentation of P. purpurogenum on marshmallow confectionery-based agar plate. Various points during incubation at 30 °C.

Red pigmentation, which had diffused into the surrounding agar, was observed on the marshmallow medium after 10 days of incubation at 30 °C. The marshmallow confectionery waste was therefore selected for further investigation through submerged liquid cultivation.

8.2.2 Small-scale liquid cultivation on marshmallow waste

Cultivation in 12-well multiwell plates was selected as a screening method to allow evaluation of a number of parameters, including supplementation of the marshmallow substrate with a nitrogen source and use of the citrate buffer shown to improve reproducibility of pigment production by *P. purpurogenum* in a malt-extract based medium.

The marshmallow substrate, applied at 15 g/L, was evaluated for liquid cultivation with and without the addition of a supplementary nitrogen source. Two nitrogen sources considered were soya peptone, a complex organic nitrogen source used in the malt extract based medium for *P. purpurogenum* cultivation, and sodium nitrate, an inorganic nitrogen source. These components were added at concentrations of 3, 1.5 or 0.75 g/L, with each medium composition evaluated with and without the application of the 50 mM citrate buffer at pH 5. MESP medium (30 g/L malt extract, 3 g/L soya peptone) and Half MESP medium (15 g/L malt extract, 3 g/L soya peptone), both with buffer addition, were included as controls. The 2 mL cultivation volume was inoculated at $1x10^5$ spores/mL using direct spore inoculation, before incubation at 30 °C with shaking for 14 days. The culture pH was monitored daily, while CDW was evaluated at the end of the cultivation, by sacrificing the entire well volume. The average CDW for each condition is plotted in Figure 8-3, with the colour of the bars representing the pigmentation achieved. The medium supplemented with sodium nitrate along with citrate buffer addition is excluded from these results as this medium composition was completely inhibitory to growth of *P. purpurogenum*.

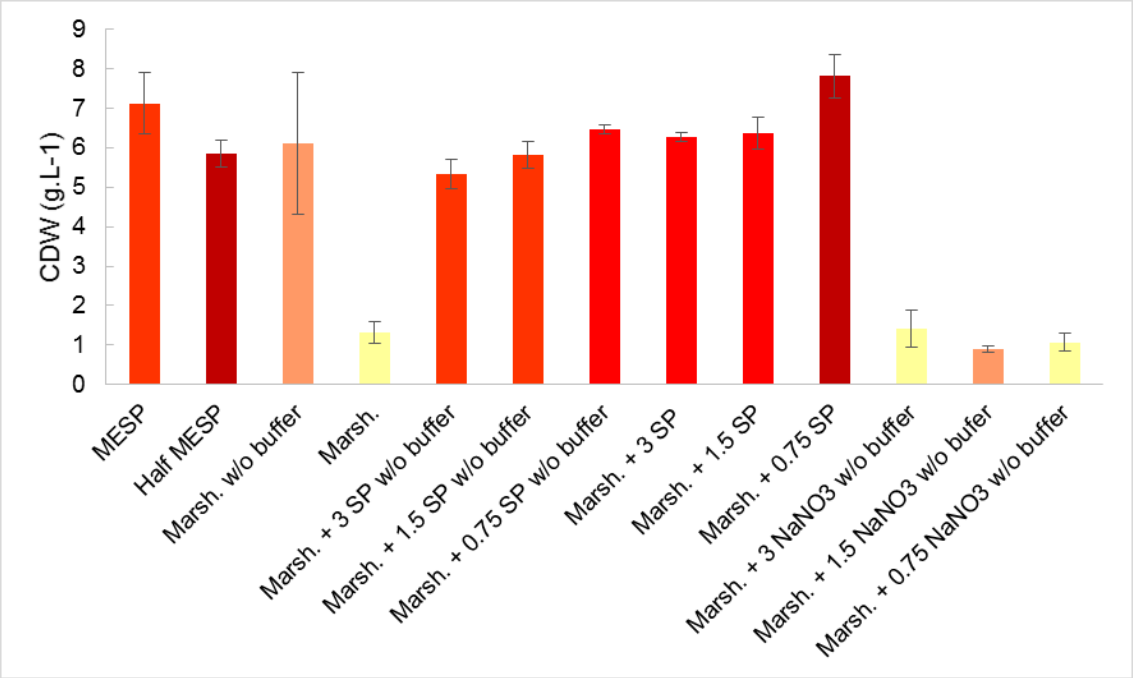


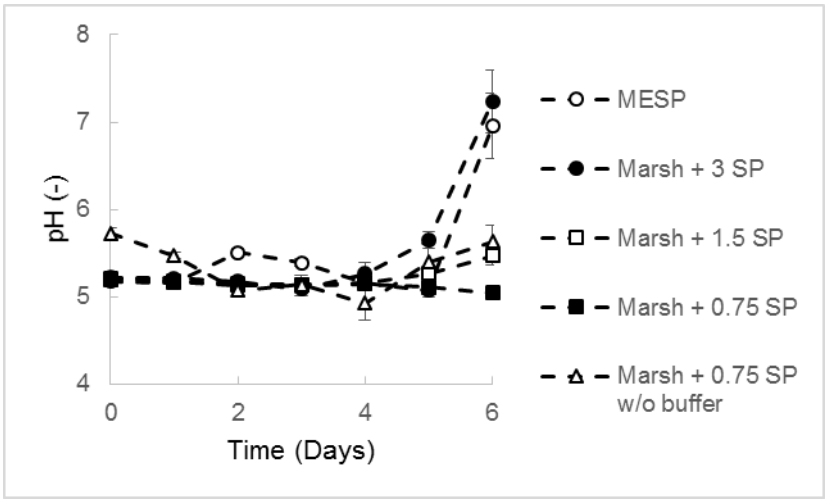
Figure 8-3 *Average cell dry weight achieved during multiwell plate liquid cultivation of P. purpurogenum in marshmallow confectionery-based media. MESP and Half MESP media are included as controls. Citrate buffer (50 mM), pH 5 is applied unless otherwise stated. SP denotes addition of soya peptone (g/L). Error bars represent the standard deviation across triplicate wells. Colour of the bars represents the pigmentation achieved.*

Marshmallow medium without nitrogen supplementation or buffer addition (Marsh. w/o buffer) supported growth comparable to that observed in the control media. Pigmentation was, however, significantly reduced with an A_{500} value below 1 OD unit. Use of the citrate buffer along with unsupplemented marshmallow confectionery (Marsh.) had a negative impact on growth, with a low biomass concentration of approximately 1 g/L recorded at the end of the cultivation. This medium also did not support pigment production. Similar trends were observed when supplementing marshmallow with sodium nitrate, as limited growth and pigmentation were observed.

Supplementation with soya peptone resulted in biomass concentrations in the range of 5.3 to 7.8 g/L and pigmentation was produced in all cases. Both growth and pigmentation were in the range of that observed in the control media, with buffer addition generally associated with increased growth and improved pigmentation. Highest growth and pigmentation were recorded for the medium supplemented with 0.75 g/L soya peptone along with buffer application.

Shake flask cultivation was then conducted using marshmallow media supplemented with soya peptone. Soya peptone was added at 3, 1.5 or 0.75 g/L along with buffer addition, while only 0.75 g/L supplementation without citrate buffer was considered. Cultivations were performed at 100 mL volume in 500 mL flasks, with MESP medium included as a control for the experiment. The flasks were inoculated by direct spore inoculation to yield a starting concentration of $1x10^5$ spores/mL and were incubated at 30 °C, with shaking, for 6 days. The pH, growth and pigmentation of triplicate flasks for each condition was monitored daily, with results provided in Figure 8-4.

a



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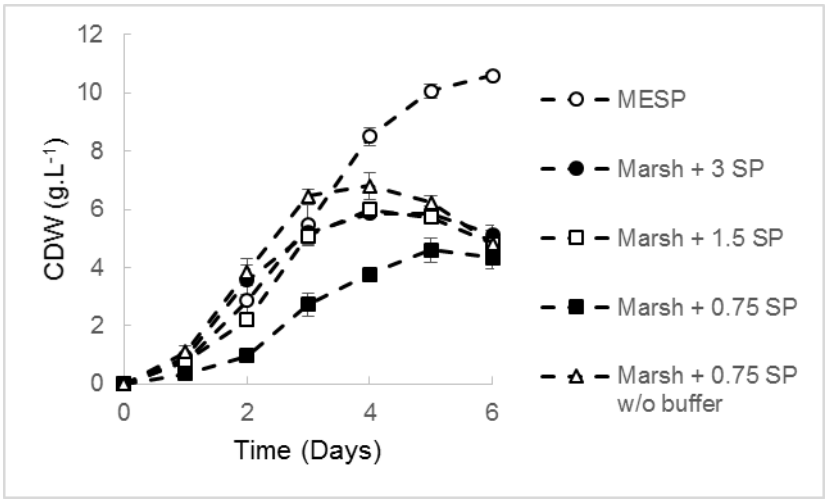

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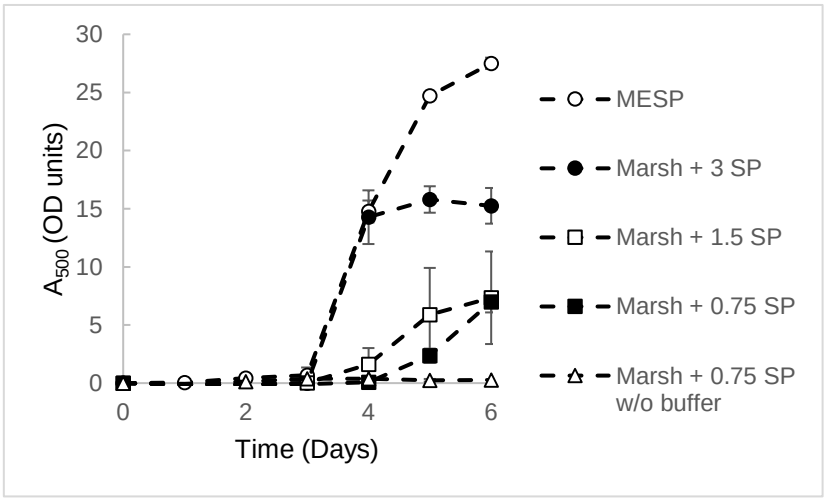


Figure 8-4 pH trends (a), growth (b), and pigmentation (c) during shake flask cultivation of P. purpurogenum at 30 °C in MESP and marshmallow confectionery-based media, buffered to a pH of 5 using 50 mM citrate buffer, unless otherwise stated. Results shown are the average values across triplicate flasks, with error bars representing the standard deviation of the data.

The three marshmallow-based media supplemented with soya peptone, along with buffer application, showed similar pH trends for the first 4 days of the cultivation. Beyond this point, a

higher concentration of soya peptone resulted in greater deviation from the desired pH of 5. Marshmallow media buffered to a pH of 5 and supplemented with 3 g/L or 1.5 g/L soya peptone supported similar growth. Supplementation with 0.75 g/L soya peptone, however, resulted in a reduction in maximum biomass achieved, reaching 4.6 g/L as opposed to 6 g/L. When comparing 0.75 g/L SP supplementation with and without buffer addition, it is observed that introducing the buffer has a negative impact on growth, which is in contrast to multiwell plate cultivation results.

The three buffered marshmallow media all supported pigment production, with the medium supplemented with 3 g/L soya peptone pigmentation measured at approximately 15 OD units, while the other concentrations resulting in pigmentation at less than half that value. The medium without buffer application exhibited no colouration. This is in contrast to results observed during multiwell plate cultivation, where 0.75 g/L SP supplementation was shown to result in highest pigment concentrations and the unbuffered medium supported pigmentation.

Differences in mixing and oxygen transfer between multiwell plate and shake flask systems could be responsible for the observed variation in growth and pigmentation. The good correlation observed between shake flask and BioFlo 110 reactor cultivation with the MESP and Half MESP media (data not shown), however, supports the selection of marshmallow + 3 g/L soya peptone, with 50 mM citrate buffer at pH 5, for further experimentation.

8.2.3 BioFlo 110 reactor cultivation on marshmallow waste

8.2.3.1 Biomass, pigment and residual sugar profiles on marshmallow-based growth medium

The marshmallow confectionery medium supplemented with 3 g/L soya peptone was scaled up into the New Brunswick Scientific BioFlo 110 bioreactor with a 5 L cultivation volume. The system was aerated at 1 vvm, with agitation maintained in the range of 300-400 rpm using dual Rushton-type impellers. The culture pH was maintained at around 5 through the application of 50 mM citrate buffer, with no additional pH control. The system was inoculated using a *P. purpurogenum* spore solution to yield an initial concentration of $1x10^5$ spores/mL. Sampling was performed twice daily with pH, growth, pigmentation and residual sugar concentration evaluated for each time point. This data has been plotted in Figure 8-5.

The pH of the culture remained relatively stable over the first 4 days of the cultivation, beyond which it began to rise. This is consistent with results observed in shake flasks (Figure 8-4a), as well as previous reactor cultivations with malt-extract based medium (data not shown). The pH reached a maximum value of approximately 8 units by day 6 of the cultivation. A maximum biomass concentration of 4.7 g/L was achieved by day 3 of the cultivation but declined to approximately 3 g/L by the end of the cultivation. Pigmentation was first observed on day 3 of the cultivation, once growth had begun to slow. This is consistent with trends observed in Half MESP medium (data not shown). Maximum pigmentation was defined by an absorbance value of 13.9 OD units.

Residual sugar concentration at each sampling point was determined using HPLC analysis. This revealed a starting concentration of approximately 11.5 g/L total sugars. An approximately linear decrease in sugar concentration, from 11.2 g/L to 2.4 g/L, between 30 and 72 hours of cultivation corresponded to the period of maximal growth and the onset of pigmentation. The concentration continued to decline, with only 0.5 g/L total sugars remaining at the end of the cultivation. HPLC

analysis also allowed component sugars to be measured individually. The utilisation trends for sucrose, glucose and fructose are provided in Figure 8-6. The results indicate that *P. purpurogenum* DSM 62866 does not preferentially consume any of the sugars, utilising all measured sugars concurrently.

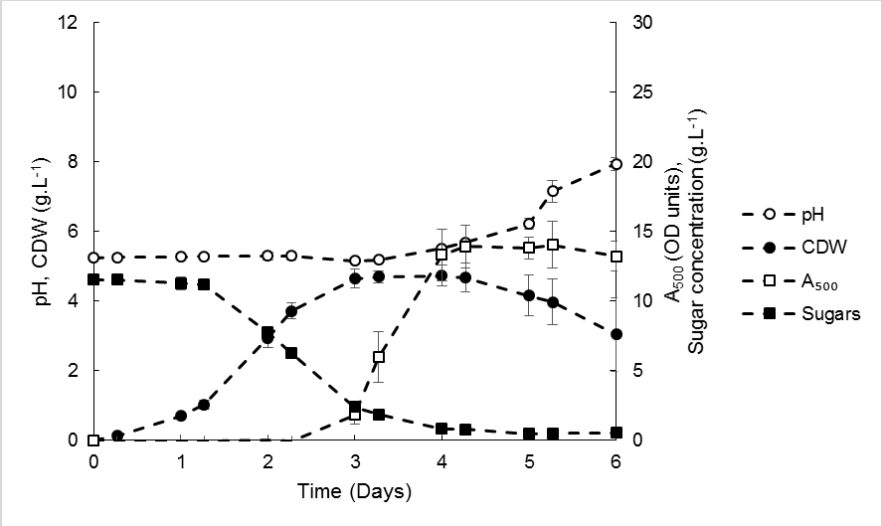


Figure 8-5 *Summary of cultivation of P. purpurogenum DSM 62866 on Marshmallow + 3 SP medium, pH 5 (50 mM citrate buffer), at 30 °C in the BioFlo 110 modular benchtop fermentor for 6 days. The average pH, growth, pigment production, and sugar concentration for triplicate measurements performed during duplicate cultivations are shown, with error bars representing the standard deviation of the data.*

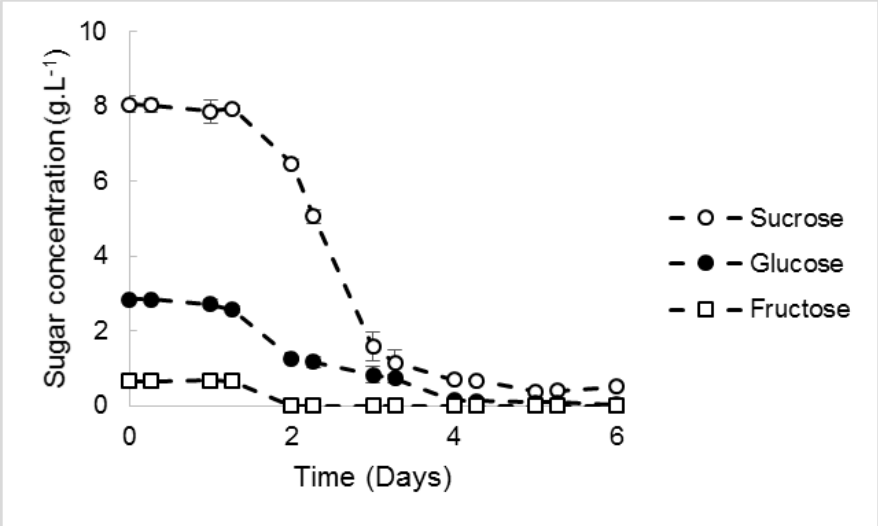


Figure 8-6 *Concentration of individual sugars during BioFlo 110 cultivation of P. purpurogenum on marshmallow medium supplemented with 3 g/L soya peptone, pH 5, 50 mM citrate buffer at 30 °C, determined using HPLC analysis. Error bars represent the standard deviation associated with triplicate measurements across duplicate cultivations.*

8.2.3.2 Growth kinetics and yields

The growth of the organism on marshmallow confectionery exhibited two periods of approximately linear increase, with an initial lag phase, followed by a period of increased growth rate between approximately 30 and 55 hours. The average biomass productivity defining these periods, determined by linear regression using the Microsoft Excel LINEST function, are given in Table 8-1. The maximum specific growth rate of 0.087 h^{-1} was determined in the early stage of the cultivation.

Table 8-1 Growth rate of P. purpurogenum DSM 62866 during different growth phases when cultivated in the BioFlo 110 bioreactor on Marshmallow + 3 SP medium, pH 5, 50 mM citrate buffer, at 30 °C.

Time (hours)	Average biomass productivity (g/L/h) [a]
0 to 30	0.034 ± 0.003
30 to 55	0.111 ± 0.002

a Results are presented as average ± standard deviation

The pigment was produced as a secondary, biomass associated product during the stationary phase. Yield of pigment on biomass was calculated using the time point of maximum recorded absorbance. This was the 102.5 h sampling point when growth was in a period of maintenance following maximum biomass achieved i.e. stationary phase, and pigmentation had reached maximum recorded value. The yield of pigment on biomass was calculated to be 2.98 OD units/g dry cell mass. These productivities and yields are compared to those observed using malt extract-based media in Section 8.2.3.3.

8.2.3.3 Comparison of BioFlo 110 bioreactor cultivations using marshmallow confectionery- and malt extract-based media

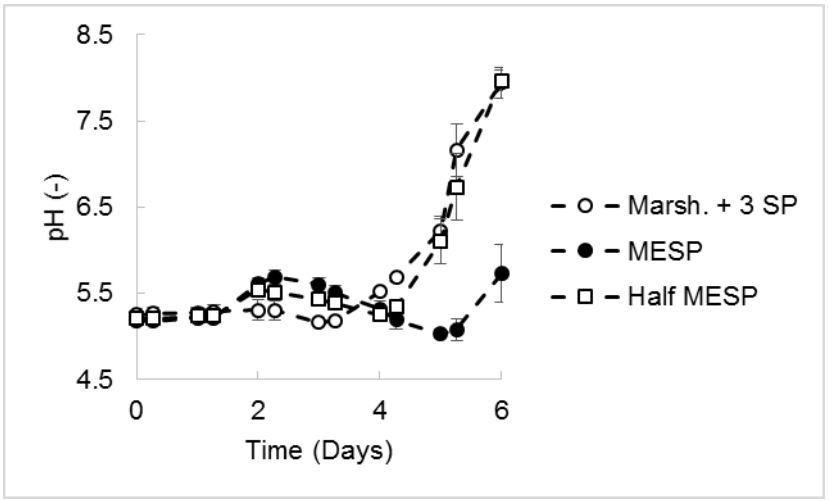
The BioFlo 110 reactor cultivations using marshmallow- and malt extract-based cultivation media were compared to evaluate the suitability of the confectionery waste as a substrate for growth and pigment production of *P. purpurogenum*. All pH, growth and pigment production trends have been plotted in Figure 8-7, with cultivation parameters summarised in Table 8-2.

Table 8-2 Summary of the parameters defining the BioFlo 110 reactor cultivations of P. purpurogenum DSM 62866 using marshmallow confectionery waste- and malt extract-based cultivation media.

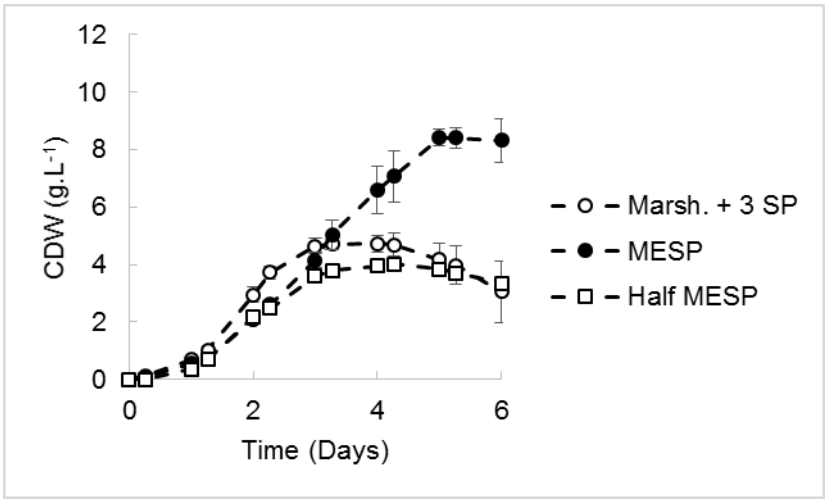
	Marshmallow medium	MESP medium	Half MESP medium
Maximum CDW (g/L) [a]	4.73 ± 0.29	8.42 ± 0.29	4.02 ± 0.10
Period of maximum growth (h)	30.5 – 54.5	30.5 – 120	24 – 72
Maximum avg productivity (g/L/h) [a]	0.111 ± 0.002	0.089 ± 0.002	0.069 ± 0.003
$Y_{P/X}$ (OD units/g_X)	2.98	2.86	6.12
Maximum A_{500} (OD units) [a]	13.94 ± 1.54	24.07 ± 1.97	24.61 ± 0.34
Period of maximum pigment production (h)	72 – 96	78.5 – 102.5	72 – 96
Maximum pigment productivity (OD units.h^{-1}) [a]	0.46 ± 0.05	0.72 ± 0.06	0.71 ± 0.04

a Results are presented as average ± standard deviation

a



b



c

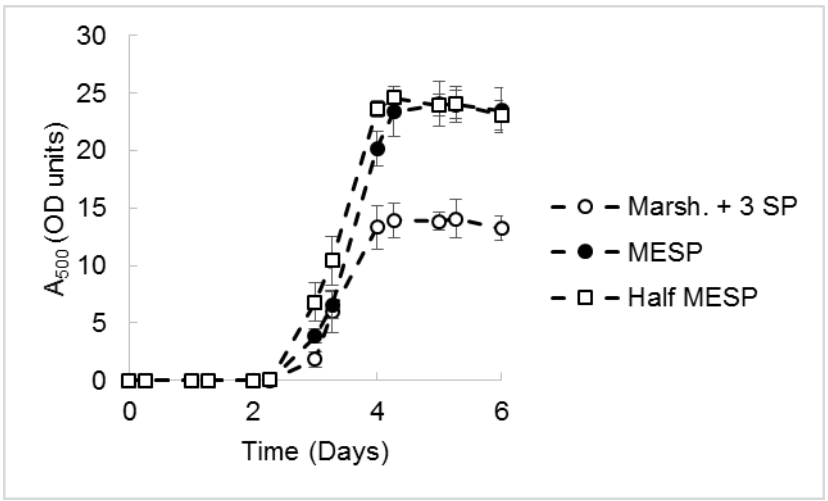


Figure 8-7 Comparison of the pH trends (a), growth (b) and pigment production (c) when cultivating P. purpurogenum in the BioFlo 110 fermentor at 30 °C, using Marsh. + 3 SP, MESP or Half MESP medium, all maintained at a pH of 5 through application of a 50 mM citrate buffer. Results show the average of triplicate measurements during duplicate cultivations, with error bars representing standard deviation of the data.

Over the first 3 days of the cultivation, the citrate buffer was able to maintain the pH of the Marsh. + 3 SP medium more effectively than that of the malt extract-based media, despite the higher growth rate observed in this medium at this early stage. Beyond day 3 the culture pH began to increase, as observed in the Half MESP medium. Overall, a similar growth trend to that achieved with Half MESP medium was also observed, reaching maximum recorded CDW by day 3 of the cultivation. The marshmallow and Half MESP media supported similar maximum biomass concentrations. As expected, this was significantly lower than that obtained when using MESP medium, given the lower concentration of available substrate, as shown in Figure 8-8.

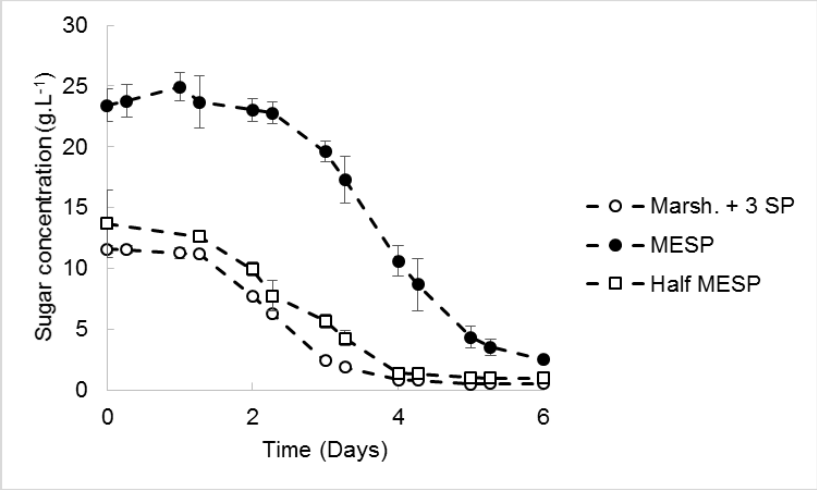


Figure 8-8 *Comparison of sugar utilization trends in the BioFlo 110 fermentor using either marshmallow- or malt extract-based cultivation media. Results represent the average of triplicate measurements during duplicate cultivations. Error bars show the standard deviation of the data.*

The volumetric pigment production supported by the Marsh. + 3 SP medium was, however, reduced in comparison to both the MESP and Half MESP media. This corresponded to a similar pigment yield with respect to biomass for the Marsh. + 3 SP medium and MESP cultures; however the yield was more than 2-fold higher in the half MESP culture with reduced protein concentration and available growth factors. This finding indicates that an additional factor to available sugar concentration plays a determining role in pigment production by *P. purpurogenum*.

8.3 Conclusions from Study of Pigment Production on Confectionery Waste

Although the BioFlo 110 bioreactor cultivations using Marsh. + 3 SP and Half MESP media showed similarities in terms of pH, growth and sugar utilisation, pigmentation differed significantly. Reduced substrate availability in the marshmallow-based medium in comparison to the MESP medium resulted in lower biomass concentrations as well as a reduction in volumetric pigment concentration. This is in contrast to the Half MESP medium cultivation, where only growth was affected by halving the malt extract concentration. An overall conclusion which can be drawn from these three sets of bioreactor cultivations is that total substrate availability, represented here as sugar concentration in the medium, is a determining factor for maximum biomass concentration, but not for volumetric pigment concentration. Given that all three medium types considered

contained the same concentration of soya peptone, it appears that additional protein content or other trace elements in malt extract, but not present in marshmallow, play a role in controlling maximum volumetric pigment concentration. This is, however, not concentration dependent, given the equivalent pigmentation observed in cultivations using MESP and Half MESP medium.

This study was able to demonstrate red pigment production by *P. purpurogenum* DSM 62866 on a medium based on marshmallow confectionery waste, across various growth scales, with soya peptone supplementation shown to have a beneficial impact on pigmentation. Marshmallow confectionery has been shown to be a suitable substrate for cultivation of *P. purpurogenum*, with further supplementation studies having the potential to address the reduction in pigmentation observed in comparison to malt extract-based media.

9 A Technoeconomic Sample Study: Valorisation of confectionery waste by bioconversion to poly glutamic acid (PGA)

9.1 The South African context for confectionery waste and PGA

The production of confectionery in South Africa results in a large quantity of waste, with approximately 5% of production going to landfill, equating to some 650 tons/ year from one factory (DST SA, 2016; Rademeyer, 2018) . If one third of this is hard candy waste, as opposed to chocolates, mallows or the entirely separate category of pastries, then the feed for this valorisation case study is 220 tons/ year of hard candy waste from a single factory. This was chosen as it is the simplest waste stream, primarily composed of sugar (42, 31, 27% sucrose, glucose and fructose respectively) with minimal nitrogen and some acidulants (citric, tartaric, etc acids), colourants and flavourings. It is assumed that a preliminary separation in the plant takes place to produce only hard candy waste.

Any form of use or remediation saves the cost and ecological impact of disposal to landfill.

Poly γ-glutamic acid (PGA) is an extracellular polypeptide produced by many *Bacillus* species as a product of fermentation. It is water-soluble, non-toxic and has uses in the food, cosmetic, medical and water treatment industries. The chosen use for this study is as food grade PGA due to its higher product value, however this requires higher purity standards which may be difficult to achieve when using a waste stream with its inherent variability.

The use of PGA as a soil conditioner due to its high nitrogen content and water retention was not an economically viable process due to the low product value (approximately $1/kg).

9.2 Simplified flowsheet for PGA production from confectionery waste

The process for production of PGA is shown in a simplified form in Figure 9-1. The hard candy stream is dissolved and has nutrients added to form a growth medium for *Bacillus lichenformis* TISTR1010, a glutamate-independent high PGA yielding bacterium (Kongklom et al., 2017). This is grown in one of three inoculum chains before a 48 hour cycle in one of three 8000 L fed-batch stirred tank fermenters with a concentrated sugar, NH_4CL and citric acid feed and supplemental oxygen.

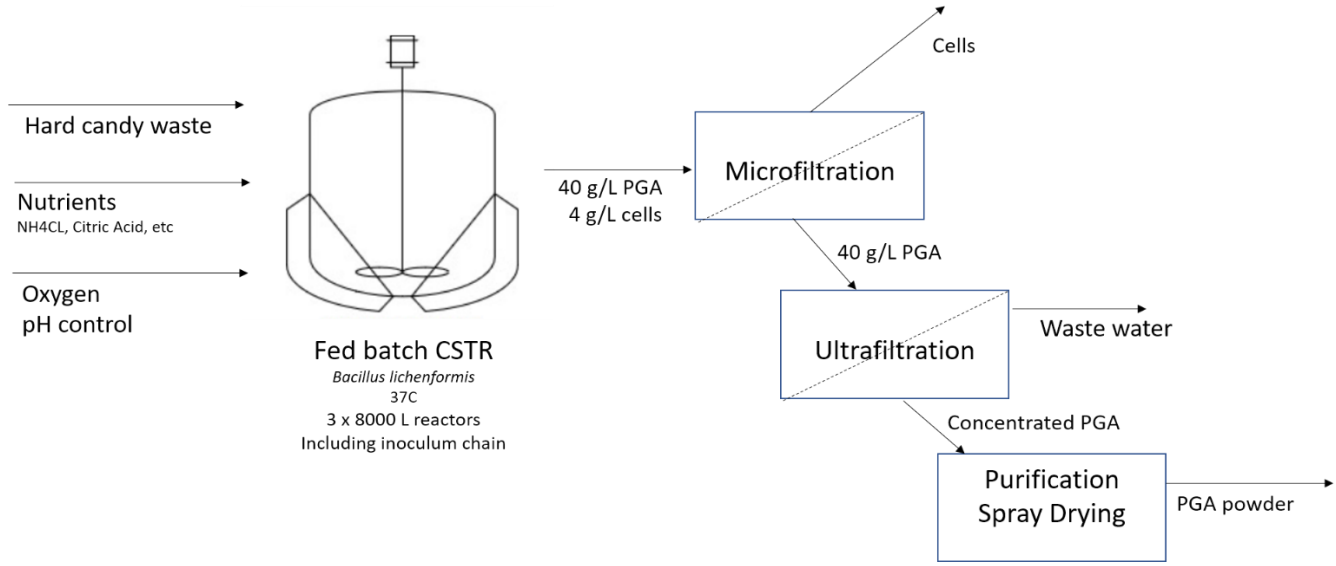


Figure 9-1 Simplified PGA production flowsheet

The downstream process comprises a microfiltration step to remove the *Bacillus* cells and endospores before a two stage ultrafiltration to concentrate the 40 g/L fermenter output to a PGA rich gel. This goes through a purification step before spray drying to produce a 5% moisture PGA food grade powder worth ± $100/kg (www.alibaba.com).

9.3 Upstream

The upstream process comprises several holding and mixing tanks to prepare and supply the chemical components needed in the fermentation. The medium for cell growth in the inoculum chain: 20 g/L sugar, 30 g/L citric acid, 7 g/L NH_4Cl, trace minerals and vitamins (for cell growth); and a concentrated feed for PGA production in the fed-batch process: 500g/L sugar, 40.5 g/L citric acid and 110 g/L NH_4Cl. Additionally, there is 3M NaOH and 3M HCL for pH control of the reactors and antifoam.

Three independent inoculum preparation and fermentation chains are used to optimise the interface between these batch processes and the continuous downstream stages (Petrides et al., 2014). Each chain comprises 4 L of roller bottles at 20°C, a 10 L lab scale bioreactor at 30°C and a 201 L bioreactor at 37°C to prepare sufficient cells to seed an 8000 L fermenter, all of which are only 70% full to allow headspace for aeration, bubble disengagement and foam breakage. After 17 hours of batch operation, the culture in the fermenter reaches ± 3 g/L dry cell weight (DCW) and sugar levels reach 5 g/L, at which point the concentrated sugar, citric acid and NH_4Cl feed is started. This fed- batch process continues for 26 hours till a DCW of ± 4 g/L is reached, with a PGA concentration of ± 40 g/L (Kongklom et al., 2017).

The selected microorganism is *Bacillus lichenformis* TISTR1010, having shown itself to be a glutamate-independent high PGA yielding strain (Kongklom et al., 2017, 2015). There is large variability in production rate and molecular weight of PGA produced, rate of PGA degradation, and

endospore formation (which complicates calculations of CDW and overall growth phase) between strains (Do et al., 2001).

The reactors require high levels of mixing and aeration in order to keep dO_2 >50% (Kongklom et al., 2017), which is higher than the standard 25-40% in bioreactors (Baltz et al., 2010). As cell concentration increases, O_2 demand increases, and PGA production increases viscosity (reducing the mass transfer coefficient k_la), creating the need for supplemental oxygen in the industrially sized reactor. Decreases in dO_2 or nutrient supply can have significant effects on PGA productivity.

Rapid mixing is also required for temperature control. Large amounts of heat are generated and must be removed by cooling jackets and coils. The pH is controlled to 7.4 by addition of 3M NaOH and 3M HCl. The flow rate of the concentrated feed is controlled to keep reactor sugar concentration within 12-15 g/L. Sugar uptake will be complicated by the mix of three sugars supplied: glucose, fructose and sucrose. The steam sterilisation step and presence of acidulants will help to invert some of the sucrose, but *B. lichenformis's* preference for each sugar and resulting PGA production will need to be verified. PGA concentration can be estimated online during the production phase through a correlation between its effect on culture viscosity and the resulting power/torque of the mixing impeller.

Table 9-1 Upstream Capital Costs

Capital Costs	***USD (2018)***
Inoculum chain[1]	440 000
8 000 L fermenter[1]	1 200 000
Total purchase cost (3 chains)	4 920 000
Lang factor	4.74
Fixed Capital Investment	23 320 800

[1] Most expensive reactor costing of sources found

Table 9-2 Upstream Operating Costs

Operating Costs	***USD (2018) /year***
NH_4Cl[2]	10 800
Citric acid[2]	70 800
Media components	3 500
O&M costs (5% of installed value)	1 200 000
Total	1 285 000

[2] Costing was of relatively crude grade sources. Cost may rise for a food grade product

It is clear from Table 9-1 and 10-2 that the main fermenters are the major capital cost, and source of operating costs through maintenance. The reactor costs are likely to decrease, but operating costs will rise with the inclusion of sterilisation costs (necessary for food grade product) and labour, and additional raw materials.

9.4 Downstream

The downstream process treats 8350 L/day of viscous fermenter outflow, containing 40 g/L PGA, 4 g/L DCW and moderate concentrations of nutrients (sugar, NH_4Cl, citric acid) to produce a high

purity, dry, food grade PGA powder. The production of a high PGA concentration gel as a soil conditioner was explored, but the low product value (± $1/kg) meant it was not economically viable using this production train.

The *Bacillus* cells and endospores are removed by cross flow microfiltration across a 0.8 µm pore ceramic membrane (Tomasula et al., 2011), removing a source of contamination and preventing any further biodegradation of the PGA by nutrient starved cells or released specific depolymerases (Regestein née Meissner et al., 2017). This creates the possibility of a cell recycle to reduce the required inoculum train or the length of the cell growth phase in the fermenter. Viscosity due to PGA is an issue, so the pH is lowered to pH 3 as at acidic pH the suspension shows lower viscosity due to a reduction of the isoelectric repulsion of the negatively charged PGA (Do et al., 2001), with possible useful effects on cell coagulation (Tomasula et al., 2011). High cross flow velocities are required to reduce cake formation, cake compression and gel polarisation, all factors that significantly increase membrane resistance. It is assumed that some (5%) of the PGA will be lost to the cell-containing retentate stream, again motivating for its recycle.

The PGA rich filtrate from the microfiltration is concentrated by ultrafiltration using 500 000 MWCO (molecular weight cut off) membranes at pH 3 in a two stage process with a recycle and additional rinse/diafiltration to reduce impurities (Do et al., 2001). The lower pH is advantageous for ultrafiltration as for microfiltration due to lower viscosity, but the conformational change in PGA increases passage across the membrane and resulting PGA loss. The final product is a 600 g/L PGA gel. A 20% loss of the produced PGA is reported. Behaviour of such a concentrated PGA solution is unknown, so lower ultrafiltration performance and reduced PGA concentration may occur. Additionally, a less concentrated stream may be more appropriate for the following steps.

A purification step has been added to the process, with a generic crystallisation step costed. The specific purification step(s) will depend on the exact *Bacillus* strain, fermenter conditions, micro and ultrafiltration outcomes as well as market specifications for ‘food grade’ purity and allowed contaminants.

The final stage is to remove the remaining water and produce a consistent dry powder. This is done through spray drying (Domínguez-Niño et al., 2017). High temperatures are not a concern as PGA is stable to 190°C (Portilla-Arias et al., 2007). In other food grade spray drying processes, maltodextrin or silicates may be added to reduce stickiness of the powder.

Anaerobic digestion, explored as a unit operation in Chapter 5, is a well understood method for reducing COD in the wastewater, both from the PGA production process and the confectionery factory, along with any other solid confectionery waste. Benefits include reduced disposal costs and ecological effects, and an energy stream, either as biogas or as combined heat and power (CHP).

Table 9-3 Downstream Capital Costs

Capital Costs	***USD (2018)***
Microfiltration	14 600
Ultrafiltration	31 000
Purification[1]	73 000
Spray Dry	50 000
Lang factor	4.74
Fixed Capital Investment	799 000
[1] Generic crystallisation step	

Table 9-4 Downstream Operating Costs

Operating Costs	**USD (2018) /year**
Electricity[2]	15 100
O&M costs (5% of installed value + membranes)	118 600
Total	133 700
[2] Based on a heuristic of electricity use per mass of retentate in ultrafiltration (Cheremisinoff, 2002)	

Table 9-3 and Table 9-4 show that the capital and operating costs of this downstream process is much less expensive that of the upstream process, contrary to the suggestion of Harrison et al. (2016) that the main cost is purification. This results from the simplicity of the downstream process used, with recovery and purification based only on size reduction and crystallisation. Further, it results from the use of sophisticated bioreactor design as typically used for high value products, in place of the simpler bioreactor used in remediation processes, such as the moving bed bioreactor shown as highly suitable for PGA production (Raper, 2019). Full development of purification steps and ultrafiltration concentrating stages will likely increase both the capital costs and the pumping costs.

9.5 Process Economics

Table 9-5 Process Economics

	USD (2018)
Revenue: 83810 kg/year PGA sold at $100/kg	$ 8 381 000 / year
Total Operating Cost	$ 1 418 700 / year
Fixed Capital Cost	$24 702 700
Working Capital (15% of fixed)	$3 705 400
Total Capital Cost	$28 408 100
Taxation (%)	28,0
Escalation (%)	6,2
Years of depreciation	10
Scrap value (%)	5,0
WACC (discount rate, %)	15,0
ROI (%)	33,8
Payback Period (Years)	4,6
NPV ($ 2018 after 15 years)	$12 022 000
IRR (%)	23,0

Table 9-5 shows that the process is strongly profitable when producing food grade PGA. However, this is dependent on being able to reach the required grade of product, which is likely to increase the capital and operating costs of the downstream process and increase PGA losses in the process.

The main capital costs are the fermenters, with the associated large expenses of reactor maintenance. Here, alternatives are relevant with the potential to maintain productivity while reducing reactor cost. Further, selection of product use will inform sterilisation and aseptic requirements (essential for food-grade product). Citric acid and pumping costs (electricity) are the main 'day to day' expenses.

The process is sensitive to the productivity of the fermenters, where perfect scale-up from 7 L to 8 000 L vessels has been assumed. Aeration, temperature and nutrient control will be the primary design considerations, as well as the robustness of the *B. lichenformis* growth pattern to the varying hard candy waste stream.

9.6 Changes

- Membrane and pH of micro and ultrafiltration process refined to match PGA characteristics and optimise process economics. Each ultrafiltration stage to match the differing fluid characteristics.
- Defining and testing the 'purification' stage – currently just a generic crystallisation.
- Labour, laboratory and QA costs.

10 Conclusions

The food processing industry, of which the confectionery industry is part, produces organic-rich waste streams, many of which are sent to landfill for disposal. This has a variety of detrimental effects. These include placing load on ever-decreasing landfill sites. Being readily available to microbial action in the landfill, these compounds contribute to generating uncontrolled methane and organic acids in the landfill. The former contributes strongly to global warming if not collected while the latter aids leaching of toxic components, especially metals. Finally, the disposal of these organic-rich compounds, rather than their re-use, represents a loss of resource efficiency, particularly as the organics present are typically more bio-available than many other lignocellulosic feedstocks specially generated for production of bio- products and materials. Hence disposal f these organic wastes to landfill is increasingly unacceptable in our increasingly resource-constrained environment, impacted by the associated environmental burden of resource inefficiency.

A developing concept to counter these negative attributes with respect to environmental burden and to improve resource efficiency is that of the waste biorefinery. The waste biorefinery integrates one or more unit operations with the overall process, or further processes the waste generated by the core process to minimise the final waste footprint by generating products of value. These products may be applicable within the core process, creating a secure market and increasing resource efficiency, or may be sold on. Increasingly, over the past 10 years, energy generation units using e.g. anaerobic digestion have been added downstream to the food processing plant or confectionery plant for this end. While the implementation of anaerobic digestion downstream of food plants is finding traction in South Africa e.g. at Rainbow Chickens in Worcester and Appletiser in Elgin, such developments in confectionery have been restricted to international sites, outside of South Africa. To enhance this concept and improve the valorisation of the waste material, it is desirable to extend the valorisation of organic waste to the majority of sites and to enhance this through creating products of varied value, depending on the nature of the waste material, the scale of the process, the economic value proposition and its potential to have a positive effect on minimising environmental burden.

In this study, we have considered the potential for valorisation of waste from the confectionery industry in South Africa to eliminate the requirement for landfill. The major confectionery products in South Africa have been considered to provide insight into key waste materials and their volumes assessed. These materials are largely sugar-based, with some starchy products and small amounts of proteins and lipids present. Following the specification of product selection criteria, a group of six products were selected for further investigation to assess their amenability to manufacture from compounds typical in confectionery waste. These include the biopolymers polyglutamic acid as a flocculant, water retaining agent and soil conditioner, polyhydroxyalkanoates as biodegradable plastics, the purple Monascus-lie pigment, bioethanol and biogas. The focus on these products was informed by short paper-based investigations conducted but not reported here e.g. production of the organic acid and acidulant citric acid.

In each case, the production system was explored at laboratory scale, leading to kinetic data and yield data for its further assessment. The impact of the sugar type, glucose, fructose or sucrose, on

the performance was considered. In some cases, starch and fats were used as feedstock. The potential for conversion of confectionery feedstock to each of these products was demonstrated.

The production of PGA was considered in depth to the level of fed-batch production and both sucrose-based candy waste and starch base biscuit wastes were considered. The techno-economic analysis of the PGA process was conducted and showed great potential for high value food-grade product at a market price of $100 per kg, giving a payback period of 4.3 years and a return on investment of 33.8%. However, it was not cost-effective for a low value soil conditioner at a market price of $1 per kg. It is recommended that intermediate value products be assessed. Further, it is recommended that cheaper reactor configurations be explored, such as the moving bed bioreactor demonstrated as applicable in the study of Raper (2019).

Production of poly-β-alkanoates from chocolate wastewaters and starch-based confectioneries has been demonstrated abroad and is currently being assessed for commercial production. Our study showed the suitability of South African confectionery products and their waste for similar. In our analysis, we highlight both the importance of maintaining good process performance on the substituted nutrient sources and of accounting for reduced performance in the techno-economic studies, demonstrating the importance of good substrate-specific experimental data. Further, the offsetting of environmental burden and its cost of handling through the re-purposing of the waste carbon source, in place of disposal, impacts the process economics significantly.

While bioenergy products sit at the bottom of the valorisation pyramid, these are often easier to implement and readily integrate into the core plant providing immediate value. Both bioethanol and biogas production were demonstrated from sugar-rich confectionery-based solutions. Process economics remain to be assessed.

This study highlights the characteristics of confectionery waste streams being appropriate for valorisation through bioprocessing. It demonstrates that additives in the confectionery industry do not preclude this objective. It provides TRL 2-3 level data across the processes selected and provides techno-economic assessment of two processes, demonstrating their potential value.

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