Cape Peninsula University of Technology

Poly(γ-glutamic) acid (PGA) production from confectionery waste using *Bacillus* species

by

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DECLARATION

I, Sharon Rademeyer, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

December 2018

Signed **Date**

ABSTRACT

Approximately 9 million tonnes of food waste is generated annually in South Africa. Its treatment, including treatment of confectionery waste, is costly because of the high chemical oxygen demand (COD) loads; as a result much of this waste is sent to landfill. South Africa's confectionery industry contributes to a significant proportion of the country's economy. Among the confectionery waste entering landfills are defective material, expired sweets and returns. This high COD waste can create breeding grounds for pathogenic microorganisms and anaerobic methanogens, causing negative environmental impacts. Part of the Department of Science and Technology (DST) Waste Research, Development and Innovation (RD&I) roadmap initiative is to minimise waste entering landfills by identifying waste sources from which to produce value that will contribute to social and economic growth.

Confectionery waste has a high sugar content which can be used for feedstock to bioprocesses. By placing this bioproduction into a waste biorefinery framework, bio-based raw materials can be used to produce competitively priced products with low environmental impact, thereby optimising remediation and value generation simultaneously. Ongoing research at the Centre for Bioprocess Engineering Research (CeBER) at the University of Cape Town has shown that a wastewater biorefinery approach can use wastewater as feedstock for the generation of products of value. Previous studies have investigated potential products of value based on nutrient loads found in wastewater as well as the nature of the product. Among the organisms selected was the *Bacillus* species, producing the potential product poly-γ-glutamic acid (PGA), an extracellular poly-amino acid when there is an excess of nutrients. Similarly, this product could potentially be produced from sugar-rich waste candy.

The aim of this study was to explore the use of hard candy waste as a feedstock for PGA, and *Bacillus licheniformis* JCM 2505 was selected as it was characterised in terms of the nutrients needed. The most attractive attribute of this strain was that it did not need L-glutamic acid to synthesise PGA but could do so from sugar. L-glutamic acid is costly. Using a cheaper nitrogen alternative would make the process more cost effective. To investigate this potential, the confectionery waste was characterised to identify the nutrients, namely, sugars, organic nitrogen and key trace elements needed for cell function and PGA production. Results showed that the nitrogen content and trace element concentrations were insignificant, as it was determined that the waste consisted mostly of sucrose. This therefore had to be supplemented with a basal medium containing the supplementation needed for cell function and PGA production. The growth of *B. licheniformis* was profiled in Erlenmeyer shake flasks using candy waste supplemented with the basal medium, with sucrose supplemented with basal medium as a control. The results showed similar trends on candy waste and sucrose. These findings

were used to plan two batch experiments; one with sucrose supplemented with basal medium the other with candy waste supplemented with basal medium. Similar growth profiles were obtained on the two substrates with the exception of the longer lag phase seen on growth on the candy waste, which was attributed to the amount of Na^+ ions present in the alkaline buffer used to neutralise the acidic candy waste. Furthermore, PGA was found to be a growth-associated product, provided that there was an adequate amount of sucrose and nitrogen within the system. To increase the productivity and yield of PGA, duplicate fed-batch experiments were run by constantly feeding candy and NH_4Cl solution at 0.0833 L/h in the C:N ratio needed for optimal PGA production. The biomass concentration was increased from 5.86±0.68 g/L to 10.3±0.81 g/L, while the PGA productivity remained unchanged. Further investigation into the enhancement of PGA production is recommended. PGA production from solid confectionery waste will prevent the waste from going to landfill, thus reducing the environmental burden by preventing the breeding of harmful microorganisms that produce noxious gases.

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DEDICATION

This thesis is dedicated to my parents. God has blessed me with two wonderful souls that have equipped me with the tools to help me pursue my dreams, and for that I am eternally grateful.

“It always seems impossible until it’s done.” —Nelson Mandela

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GLOSSARY

Terms	Definition/Explanation
Acidulent	Acidic additive in confectionery contributing to tart taste (Sortwell, 2004).
Aerobic	A process that takes place in the presence of oxygen (Visser et al., 1990).
Anaerobic	The ability to perform cell function in the absence of oxygen (Visser et al., 1990).
Bio-based materials	Product produced from a renewable resource (Weber et al. 2002).
Biorefinery	A sustainable bio-based process where bio-based feedstock can be used to produce a variety of value-added products with minimal waste emissions (Fernando et al, 2006).
Flocculent	Substance that causes suspended particles to aggregate (Šulc and Ditl., 2017).
Gram-positive bacteria	Orientation of the cell wall that encapsulates the bacterium which can be identified when the cells are gram-stained. Gram-positive bacteria retain the blue dye within their thick single layered cell walls.
Humectant	A substance that retains moisture (Heck et al., 2002).
Hydrolysis	The breaking down of a bond in a molecule using water (Freeman, 2011).
Polysaccharide	A chain of many monosaccharides (monomers) joined together (Shuler & Kargi, 1992).
Sugar inversion	An inverted sugar is a syrupy mixture of glucose and fructose. Sugar inversion occurs when sucrose is split up into its monomers glucose and fructose, this process produces a sticky substance which is not ideal for hard candy (White and Cakebread, 1966).

ABBREVIATIONS

AC	Agitation control
AD	Anaerobic digestion
ADI-mbr	ADI-aerobic membrane bioreactor
BOD	Biological oxygen demand
B. licheniformis	*Bacillus licheniformis*
B. Subtilis	*Bacillus subtilis*
CAGR	Compound annual growth
CDW	Cell dry weight
CeBER	Centre for Bioprocess Engineering Research
CHP	Combined heat and power
COD	Chemical oxygen demand
CSIR	Council for Scientific and Industrial Research
DMB	Deoiled microbial biomass
DO	Dissolved oxygen
DOI	Dissolved oxygen indicator
DST	Department of Science and Technology
E. coli	Escherichia coli
EPS	Extrapolymeric substances
FAN	Free amino nitrogen
H_2	Hydrogen
HPLC	High-performance liquid chromatography
ICP-OES	Inductively coupled plasma – optical emission spectrometry
IEA	International Energy Agency
LCF	Lignocellulosic feedstock
MME	Modified medium E
NaOH	Sodium hydroxide
O_2	Oxygen
OD	Optical density
PGA	Polyglutamic acid
PgS	Polyglutamate synthase
PHB	Polyhydroxybutyrate
RDI	Roadmap initiative
rpm	Revolutions per minute
SA	South Africa
SDG	Sustainable development goals
SSF	Solid-state fermentation
TC	Total carbon
TCA	Tricarboxylic acid
TI	Temperature indicator
TIC	Total inorganic carbon
TKN	Total Kjeldahl nitrogen
TOC	Total organic carbon
UK	United Kingdom
USA	United States of America
vvm	volumes gas per volume liquid per minute, i.e. units of aeration

F	Feed rate	L/h
n	Number of repeats	
P	Product concentration	g/L
S	Substrate concentration	g/L
S_O	Substrate feed concentration	g/L
t	Time	h
V	Volume of medium	L
X	Biomass	g
x	Data point value	
$\bar{x}$	Mean	
$Y_{P/S}$	Yield of product with respect to substrate	g/g
$Y_{X/S}$	Yield of biomass with respect to substrate	g/g

Greek symbols

θ	Feeding time	h
Λ	Wavelength	nm
μ_{net}	Net specific growth rate	h^{-1}
μ_{max}	Maximum growth rate	h^{-1}

Subscripts

i	initial time at t=0
f	t value at t= θ

CHAPTER 1: INTRODUCTION

1.1 Background

With rapid population growth, there is a need for the development of processes and infrastructure to accommodate this growth without creating excess environmental burden. One such concern is the fate of wastes, including food waste, which is becoming a global concern (Ravindran & Jaiswan, 2015). Oelofse and Nahman (2013) did a study to estimate the magnitude of food waste generated in South Africa (SA). Results showed that an estimated 9.04 million tonnes of food waste are generated annually and one-third of food equivalent to the value of R60 billion is disposed of as waste annually (Olivier, 2015).

According to SA's *National Environmental Management: Waste Act No. 59 of 2008*, it has explicitly been stated that a hierarchy of waste management steps should be followed:

- Waste should be reduced at the source by minimising creation within the industrial process.
- Products should be re-used; this entails reusing them in their original form.
- Recycling of waste as raw material to produce other products should be used where possible.
- Waste should be minimised by removing valuable products from the stream through resource recovery before discarding.
- Waste should be treated before disposal.

Once all of the above steps have been taken, the remaining solid waste can be disposed of in a landfill in a controlled manner (Institute of Waste Management Southern Africa, 2007). Some of these wastes contain high biological oxygen demand (BOD) and chemical oxygen demand (COD) loads. Their treatment is costly and, as a result, they get dumped into landfills where they may support growth of pathogenic organisms or of methanogens, both of which are toxic to the environment (Digman & Kim, 2008). Global awareness of the accumulation of these large quantities of food waste in landfill has led to a need to find ways to divert waste from landfill by exploring waste valorisation options using waste as feedstock to produce products of value (Panesar & Kaur, 2015). Ideally, the waste should rather be remediated with the concurrent generation of value, using a value-generating biorefinery approach. Food waste has great potential for valorisation as a feedstock for biological processes as it contains a rich

source of organic components such as proteins, fats and polysaccharides (Arancon et al., 2013).

SA's well established and constantly growing confectionery industry has three main confectionery manufacturers: Mondelēz, Nestlé and Tiger brands (Peristeris et al., 2015). In 2016, the confectionery industry generated R5.6 billionof revenue, an increase of 6% from the previous year (Das Nair et al., 2017). With greater production comes greater responsibility to manage process waste streams which consist mainly of raw materials not fit for human consumption as well as production wastes in the form of dough, chocolate mass, fatty flavourings, and starch from jelly production (Rusín et al., 2015). According to one SA company, it generates 650 tonnes of confectionery waste annually. These organic, sugar-rich wastes could be used as feedstock for biological processes which can generate products of value. This could be made possible by using a biorefinery approach which can utilise waste streams as raw materials for biological processes to produce potential products of value while simultaneously treating the waste stream (Harrison et al., 2016). Part of the SA waste research, development and innovation (RD&I) roadmap initiative is to use waste from landfills as renewable resources to produce products of value which will contribute to social and economic development as well as reduce the negative environmental impact.

Polyglutamic acid (PGA) is a high-value product that can be produced by cultivating *Bacillus* species. In this study, it is proposed that it is viable to use confectionery waste, rich in C_6 sugars, from a local confectionery factory as a feedstock in a biorefinery using *Bacillus* species for the production of PGA. Among the many applications of PGA, this study selected it for its potential use in the agricultural industry as it has the ability to retain moisture and is a nitrogen source (Zhu et al., 2014).

1.2 Problem statement

SA is challenged by the rapid accumulation of solid food waste in landfill, and the confectionery industry is a significant contributor. The accumulation of food waste in landfill has a negative environmental impact owing to production of the greenhouse gas, methane, and uncontrolled support of microbial growth.

1.3 Research questions

- Which components in confectionery waste can be used as microbial feedstock to produce compounds such as PGA?
- Which *Bacillus* species will be suitable to produce PGA using a renewable resource such as confectionery waste?
- What is the productivity and yield of PGA?

1.4 Scope

The aim of this study was to determine the potential of using solid confectionery waste as afeedstock system to produce PGA.

1.5 The objectives

The objectives of the study were to:

- identify a *Bacillus* species that is able to produce PGA;
- characterise the confectionery waste from an identified factory in SA;
- identify key nutrients needed to supplement the waste to produce PGA;
- determine the sugar concentration range needed to produce PGA;
- commission and operate a bioreactor set-up that will produce PGA;
- determine the growth kinetics of *Bacillus* species for the production of PGA.

1.6 Significance

The findings in this report contribute to the “Value recovery from solid confectionery waste” project conducted at the Centre for Bioprocess Engineering Research (CeBER). This study will contribute to the Waste RDI Roadmap 10-year innovation plan coordinated by the Department of Science and Technology (DST) through the Council for Scientific and Industrial Research (CSIR).

1.7 Delineation

- All other products considered as potential products within the waste biorefinery will not be included in this dissertation.
- Only one *Bacillus* species was identified and tested.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to South Africa's confectionery industry

SA has a well-established confectionery industry where sugar commodity prices are at a constant low, making confectionery an affordable luxury. In 2016, the revenue of the confectionery market in SA was R5.6 billion, with a compound annual growth rate (CAGR) of 11% since 2011. In 2016, the highest revenue generators were pastilles and gums, jellies, boiled sweets and toffees (Das Nair et al., 2017).

There are approximately ten main confectionery producers in SA. Production quantities are site specific and are dependent on the variety of products produced. Information on the quantity of confectionery products produced cannot be readily found, as information is restricted and can only be obtained through interviews. Table 2-1 is a summary of production values sourced from Das Nair et al. (2017).

Table 2-1 Production quantities of confectionery manufacturers in SA (modified from Das Nair et al., 2017)

Producer	A	B	C	D	E
Products	Chews; lollipops	Creamy toffees; gums & jellies; panned sweets; speckled eggs.	Soft; hard candy	Hard candy; lollipops	Chews; gums; jellies; mallows
Production volumes (Ton/month)	650	1 200	8	1 300	4 000

2.2 Confectionary manufacturing

Confectionary manufacture predominantly involves sugar dissolving in water and concentrating it by boiling. This takes place in a steam-heated pan consisting of a series of pipes that circulate the steam (Edwards, 2000). Confectionery products are classified as sugar containing products combined with water and texturing agents such as fats, milk additives and syrups.

Table 2-2 is a summary of the compositions of different types of sweets. The confectionery industry manufactures a range of sugar-containing sweets, chocolates, chewing gum and gum drops, and its main raw materials are milk, flavouring agents, nuts and cereals (Ozgun et al., 2012). The sugar is supplied by local sugar mills as industrial-grade sugar since this grade is supplied to any industry partner needing sugar as a raw material for another process (Das Nair

et al., 2017). Confectionery manufacturing processes use six types of acidulents: acetic, fumaric, citric, lactic, malic and acetic acid. These acidulents are used as flavourants as well as preservatives to keep the pH of the candy low to prevent microbial contamination (Sortwell, 2004). Generally, there are three main colourants used in candy: (i) Brilliant blue FCF E 133; (ii) Allura red AC E129; and (iii) Tartazine E102. These colours are blended to produce various colours (Ozsoy & Van Leeuwen, 2010).

Candies can be divided into two groups based on their sugar structures: (i) non-crystalline or (ii) crystalline. Hard-boiled sweets are produced by concentrating a sugar solution containing glucose, sucrose, fructose and glycerol. During the boiling process, some of the sucrose is hydrolysed into glucose and fructose (Lees & Jackson, 1973). The acidulents used in hard candy are generally a blend of malic acid and lactic acid. Malic acid blends well and enhances fruity flavours, while lactic acid works well with creamy-flavoured confections (Sortwell, 2004).

Table 2-2 Composition of different sweets (adapted from Bussiere & Serpelloni, 1985)

Products	Moisture %	Total sugars %
Boiled sweets	2–5	35–60
Caramels, toffees and fudge	6–10	40–70
Chewy sweets	6–10	40–70
Nougat	5–10	30–60
Marshmallow	12–20	40–65
Gums, jellies and liquorices	8–22	30–75
Candied fruit	20–30	35–100
Fondants and creams	10–18	15–30
Chewing-gum	3–6	20–35
Soft coating	3–6	20–30
Hard coating	0–1	0–20
Lozenges, tablets	0–1	0–5

2.2.1 Confectionery waste as a renewable resource

The production of one week of confectionery manufacturing generates tonnes of waste and consists of raw materials that cannot be consumed by humans, as well as production wastes in the form of dough, chocolate mass, fatty flavourings, and starch from jelly production (Rusín et al., 2015). This includes products that do not meet specifications and are disposed of in landfill as food waste (Ozsoy & Van Leeuwen, 2010). This resourceful feedstock can be used as raw material for a myriad of biological processes. Table 2-3 is a summary of research done in the area of confectionery waste treatment.

Table 2-3 Products produced from confectionery waste found in literature.

Confectionery source	Treatment	Micro-organism used	Product	References
Confectionery waste	Cultivation		Single-cell protein (SCP)	Hacking (1987)
Confectionery waste	Biogas integrated gasification fuel cell	Not mentioned (nm)	Electricity	Lunghi and Burzacca (2004)
Nougat and caramel waste	Fuel cell	*Escherichia coli & Rhodobacter sphaeroides*	Bacterial hydrogen and electricity	Green Car Congress (2006)
Confectionery waste products	Two-stage AD	Digester sludge	Acetic acid, lactic acid, ethanol and carbon dioxide	Magnusson (2010)
Flour-rich waste stream	Batch fermentation	*Cupriavidus necator*	PHB	Garcia et al. (2011)
Flour-rich waste	Fed-batch fermentation	*Lipomyces starkeyi*	Microbial lipid	Tsakona et al. (2014)
Flour waste streams	Batch fermentation	*Komagataeibacter sucrofermentans*	Bacterial cellulose	Tsouko et al. (2015)
Waste wafer material	AD	Digestate	Biogas and digestate	Rusín et al. (2015)

López et al. (2011) effectively produced polyhydroxybutyrate (PHB) from flour. The flour was pre-treated by hydrolising the starch to glucose by using crude extracts from another solid-state fermentation using a fungus which was then fed to a bioreactor to produce PHB. The feasibility of producing electricity from nougat and caramel waste in solution to produce hydrogen gas was investigated (Garcia et al., 2011). The reactor was inoculated with an *Escherichia coli* (*E. coli)* strain used to produce a mixture of hydrogen (H_2) gas and organic acids. The solution was transferred to a second reactor containing *Rhodobacter sphaeroides*, which had the ability to produce more H_2 gas from the organic acids produced from the previous step. The gas was collected and fed into a fuel cell which facilitated the reaction of hydrogen (H_2) and oxygen (O_2) to produce electricity (Green Car Congress, 2006). Bacterial cellulose, a polysaccharide comparable to plant cellulose that has a range of applications in the medical, food and electrical industries (Esa et al., 2014) can be synthesised with by using *Komagataeibacter sucrofermentans* using a flour-rich hydrolysate consisting of sucrose, glucose, and a small proportion of free amino nitrogen (FAN). This process is cost effective as the waste does not need to be supplemented with any additional nutrients (Tsouko et al., 2015).

Anaerobic digestion (AD) is a simple process that is used to produce biogas which is a renewable bioenergy with many potential applications. A study based in Opava, Czech Republic, loaded defective waste wafer material into a semi-continuous horizontal fermenter. The AD reactor comprised of a three-layered bag made up of Sioen B6070 with a 0.7 m^3 total volume and 0.5 m^3 working volume. The bag was attached to the fermenter nozzle with a diameter of 0.5 m. This system had a 500 kg capacity which produced 8.2 m^3/d biogas. The waste was sourced from a nearby biscuit-production site. This particular manufacturing site generates approximately 350 ton of waste per month. This process is advantageous as no trace elements are needed to supplement the waste and the technology used can accommodate heavy loads (Rusín et al., 2015).

2.2.2 Confectionery waste treatment in industry

Globally, there has been a paradigm shift in respect of waste generated by processes. This waste is seen as a resource that can be used in another process to produce energy or products of value. The latest phrase coined by industry is “zero waste”, which means that waste generated in the manufacturing process is treated, valorised and recycled into the process and zero waste is sent to landfill (Matete & Trois, 2008; Mia et al., 2015).

International companies have started putting systems into place where the confectionery waste is converted to energy in the form of biogas through AD. Nestlé United Kingdom (UK) and Ireland’s Fawdon factory in Newcastle upon Tyne managed to achieve “zero waste” by constructing an AD plant on site. 200 000 L of effluent from the factory in the form of “chocolate soup” is produced into biogas. The biogas produced by this process is able to generate approximately 200 kW of electricity by feeding the gas to a combined heat and power engine (CHP) (Kane, 2015). The Mars chocolate factory in Veghel, the Netherlands, constructed a Memthane® wastewater treatment plant to treat the chocolate wastewater effluent generated by the factory. This plant has the ability to 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 to 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 by installing a biogas engine with the capacity to power 400 homes with electricity (Food and Drink Federation, 2012).

Independent companies have been selling technologies that treat confectionery waste by reducing COD and BOD concentrations. ADI Systems did a case study at an unnamed confectionery-manufacturing plant in Pennsylvania, United States of America (USA) (ADI system 2015). The factory had an on-site anaerobic wastewater treatment system which was retrofitted to an anaerobic membrane bioreactor (ADI-mBR) which improved effluent quality. Water treatment reduced COD from 12 000 to 350 mg/L before discharge to a public-owned treatment works.

2.3 Biorefinery concept

In the quest to find alternative energy sources, the biorefinery concept has proved to be a solution and can be defined as a bio-based process where bio-based feedstock can be used to produce a variety of value-added products with minimal waste emissions (Fernando et al., 2006). A biorefinery should be able to produce bio-based products able to be sold for profit or to produce energy. The products produced may be intermediate products;

ultimately the products produced should be marketable and pricing should be 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 process can only be economically viable if the feedstock and processing costs are low (IEA, 2009).

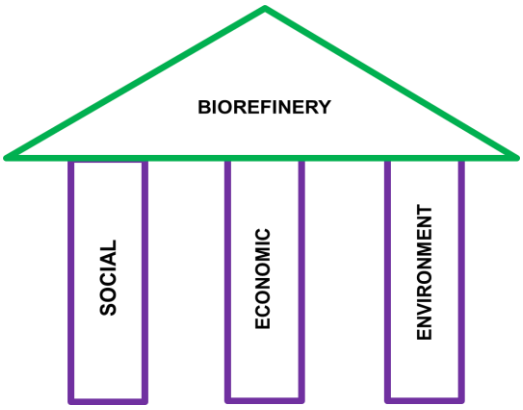

Figure 2-1 Biorefinery pillars

Existing studies in research focus on four main biorefinery systems: (i) the “lignocellulosic feedstock” (LCF) biorefinery uses dry cellulose containing waste, (ii) the “green biorefinery” uses wet green biomass, (iii) the “whole crop biorefinery” uses crop as feedstock, and (iv) the “two platforms concept” encompasses the use of sugar fermentation and gasification using microorganisms (Kamm, 2012). Luo et al. (2010) investigated the feasibility of producing the products presented in Table 2-4 by technically designing a Lignocellulose Feedstock (LCF) biorefinery, doing a techno-economic analysis, and comparing results to a single output ethanol manufacturing plant. They found it economically feasible to produce many products as opposed to one. An example of a “green biorefinery” is a pilot plant in Upper Austria where grass silage juice is fed to a membrane and displacement chromatography process to produce lactic acid and amino acids. This plant was able to generate yields of 27% and 73% of lactic acid and amino acid respectively (Ecker et al., 2012). Maurya et al. (2016) investigated the feasibility of using the deoiled microbial biomass (DMB), a bioproduct of biodiesel production to produce biogas, bioethanol, animal feed and fertilizer. Navarro-Pineda et al. (2016) proposed using *Jatropha curcas*, an oilseed plant originating from Mexico, as feedstock to a biorefinery, by combining existing studies on the plant as feedstock for processes.

Table 2-4 Types of biorefineries

Type of biorefinery	Feedstock	Products	Reference
LCF	Corn stover	Succinic acid, acetic acid and electricity	Luo et al., 2010
Green biorefinery	Grass silage	Lactic and amino acids	Ecker et al., 2012
Whole crop	*Jatropa curcas*	Bioethanol, glycerol, biodiesel, heat and power, activated carbon	Navarro-Pineda et al., 2016
Two platform	DMB	Biogas, bioethanol, animal feed and fertilizer	Maurya et al., 2016

Ongoing research at the University of Cape Town (UCT), Centre for Bioprocess Engineering Research (CeBER), Department of Chemical Engineering, has investigated a wastewater biorefinery approach. Verster et al. (2014) addressed the issue of poor-quality effluent leaving domestic wastewater treatment plants. Currently, domestic wastewater treatment plants are only able to treat biological waste in wastewater. With the increase in population and growth in industry over the years, these facilities are unable to treat dissolved contaminants in the form of heavy metal ions and phosphates. This water is not suitable for human consumption and effluent is pumped into rivers or oceans, which contributes to water pollution. A wastewater biorefinery approach was proposed after the nutrient loads were evaluated and potential products of value were investigated based on the nutrient loads.

Harrison et al. (2016) recently has shown that this approach can be a potential solution by using confectionery waste to produce products of value. Case studies were shown to use wastewater to produce biogas from municipal wastewater (Naidoo, 2013), and fertiliser from vinasse and PHA from chocolate wastewater (Tamis et al., 2014). A wastewater biorefinery can be defined as a process design that uses waste water streams as raw materials for biological processes to produce potential products while simultaneously purifying the water (Harrison et al., 2016). This study considered all of the waste streams (solid and liquid) leaving the confectionary manufacturing site, as well as the raw materials needed to produce the end product. Figure 2-2 is a basic overview of the confectionery-manufacturing process and the waste streams generated. The confectionery waste biorefinery should produce products needed that will contribute to the value chain of confectionery manufacture. The wastewater could potentially act as solvent for many biological processes, creating the opportunity to produce high-value products such as bioplastics that would contribute to the production of packaging material Garcia 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 will 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, is full of nutrients such as nitrogen, phosphate (P_2O_5), potash (K_2O) and trace elements. Food-based digestate contains approximately 80% total nitrogen (N). Digestate comes in three forms: (i) whole, a slurry containing approximately 5% dry matter; (ii) liquor, where most of the solid content has been removed, and (iii) fibre, which is a dry solid material which is typically used as a soil conditioner (Wrap, 2012). The advantage of using digestate is that no waste or wastewater is generated as it is seen as a nutrient source and can be used in all three forms in the agricultural sector. This additional step would close the waste loop, making the "zero waste to landfill" model possible.

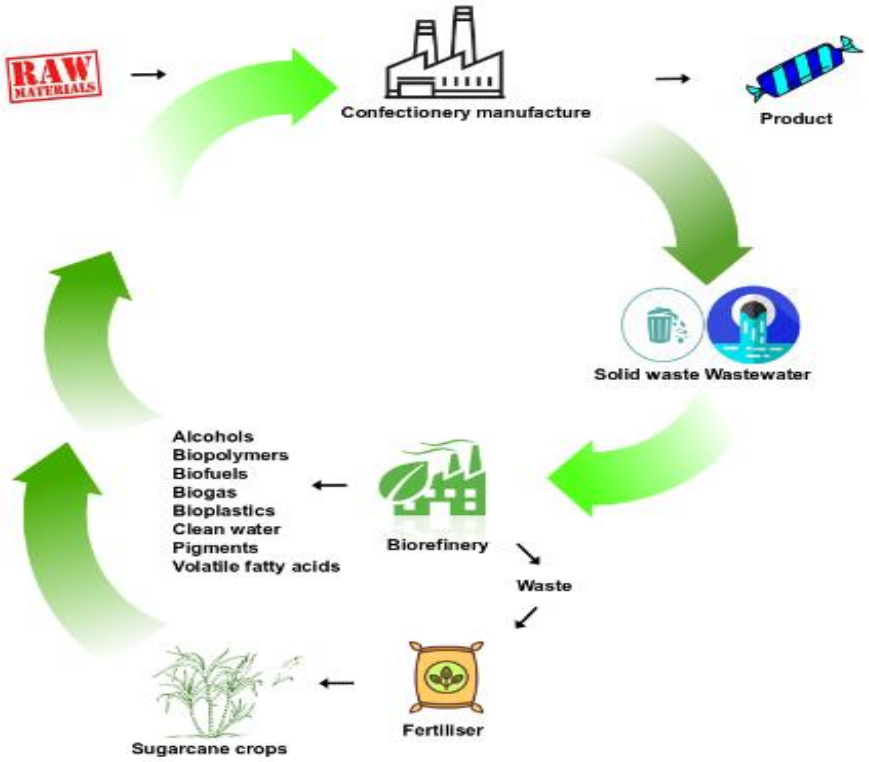

Figure 2-2 Confectionery waste biorefinery concept

2.4 Properties and uses of poly(γ-glutamic acid)

PGA is an extracellular polypeptide produced by many as a product of fermentation by *Bacillus* species (Goto & Kunioka, 1992). It is a biodegradable anionic substance that consists of D- and L-glutamic monomers held together by γ-amide linkages between the carboxylic groups (Xu et al, 2014), as shown in Figure 2-3.

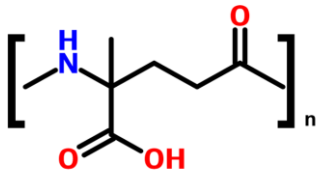

Figure 2-3 Chemical structure of PGA (image redrawn from Ogunleye et al., 2015)

This water-soluble, non-toxic polyamino acid has been used successfully in the food (Chen et al., 2010; Lim et al., 2012), wastewater (Bajaj & Singhal, 2011) and medical industries (Ogunleye, et al., 2015). It has potential for many industrial applications such as heavy metal ion removal in the wastewater industry, as it has the ability to bind to ions such as Ni^{2+}, Cu^{2+}, Mn^{2+} and Al^{3+} (Zhu et al., 2014). It is used in the cosmetics industry as a humectant, as a soil conditioner in the agricultural industry, and as a thickener in the food industry (Zhu et al., 2014).

2.4.1 Biosynthesis of PGA

Bacillus species is a renowned robust workhorse that is used in many industrial applications such as production of heterologous proteins, antibiotics, nucleotides, biosurfactants, biofuels and biopolymers (Meissner et al., 2015). It produces PGA when there is an excess of nutrients as a nitrogen and carbon source (Ogunleye et al., 2015). The industrial production of PGA is traditionally produced by running fermentation in a classic continuous stirred tank reactor (CSTR) with a steady nitrogen source supply (Bending et al., 2015). Table 2-5 provides a summary of a few PGA-producing *Bacillus* strains found in literature.

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 media 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 these 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).

Table 2-5 PGA-producing strains

Strain	PGA (g/L)	Reference
Bacillus subtilis TAM-4	22.1	Ito et al., 1996
Bacillus licheniformis	16.7	Du et al., 2005
Bacillus subtilis C1	21.4	Shih & Wu, 2009
Bacillus licheniformis SAB-26	33.5	Soliman et al., 2005
Bacillus subtilis CGMCC1251	101.1	Huang et al., 2011
Bacillus licheniformis A13	28.8	Mabrouk et al., 2012
Bacillus velezensis NRRL-23189	4.82	Moraes et al., 2012
Bacillus subtilis NX-2	33.6	Zhang et al., 2012
Bacillus subtilis HB-1	28.15	Zhu et al., 2014
Bacillus subtilis GX-28	19.92	Zeng et al., 2014
Bacillus methylotrophicus	35.34	Peng et al., 2015
*Bacillus TIST*R 1010	27.5	Kongklom et al., 2015
Bacillus licheniformis NCIM 2324	36.5	Kumar & Pal, 2015

For the production of PGA, nutrients such as C_6 sugars and NH^{4+} found in the culture medium are transferred into the cytoplasm via the cell membrane where the C_6 sugars enter the tricarboxylic acid (TCA) cycle as shown in Figure 2-4. The C_6 sugars are then metabolised into citric acid, then to isocritic acid, and then to α-ketoglutaric acid, a glutamate precursor (Moraes et al., 2012). The ammonium and α-ketoglutaric acid form L-glutamic acid; while pyruvic and α-ketoglutaric acid form D-glutamic acid. The presence of enzymes such as polyglutamate synthase (PgS) are responsible for the polymerisation of PGA. PgS B and C form the active site and PgS A is responsible for the removal of the chain of the PGA out to the culture medium via the cell membrane (Luo et al., 2016). The TCA cycle is active during exponential and stationary phases, provided that the cells are still alive. The rate at which PGA is produced increases during the exponential phase, and decreases as the cells enter the stationary phase. Inhibitors such as pH and dissolved oxygen (D.O) need to be controlled during this process. Although oxygen is not a precursor to the production of PGA, it is a crucial element that is responsible for the cycling of electrons within the TCA cycle (Kongklom et al., 2015). An alteration in pH, or if the dissolved oxygen is below the critical limit, hydrolysis of PGA will occur (Zhang et al., 2012).

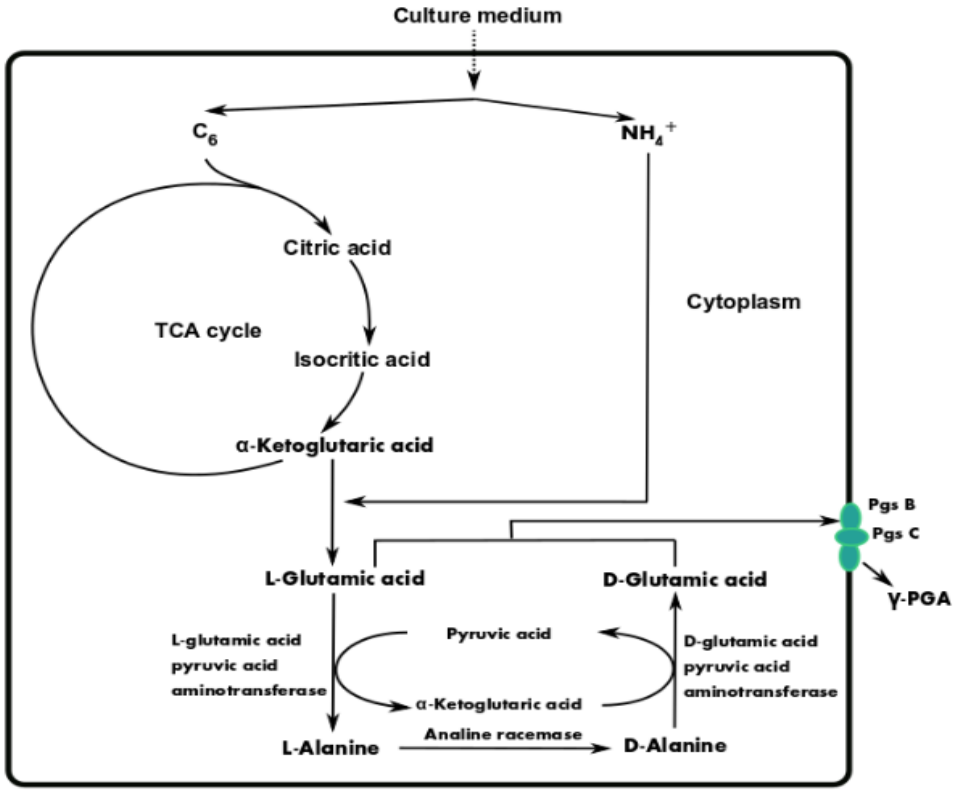

Figure 2-4 Biosynthesis of PGA (image redrawn from Kongklom et al., 2015 and Luo et al., 2016)

Numerous studies (Goto & Kunioka, 1992; Xu et al., 2005; Zhang et al., 2012) have been reported on the nutrient requirements for the production of PGA in *Bacillus* species. Goto and Kunioka (1992) isolated *Bacillus subtilis* IFO 3335 and cultivated the isolate in a range of carbon sources, namely, glucose, acetic acid, citric acid, L-malic acid, succinic acid, and fumaric acid to produce high-quality PGA without polysaccharide formation. They found by adding a citric acid medium containing L-glutamic acid, the medium yielded the highest concentration of high purity of 30 g/L of PGA. When glucose was added to the medium, a polysaccharide byproduct was formed and it was speculated that the addition of glucose to the medium produced a polysaccharide through glyconeogenesis, therefore preventing the rise in concentration of L-glutamic acid. Their study then concluded that large amounts of PGA could be produced from sugar and a nitrogen source without adding citric acid/glycerol to the medium. Zhang et al. (2012) investigated the effect of change in concentration of glycerol for the production of PGA and found that glycerol aids with the secretion of PGA. Xu et al. (2005) did a similar study, where newly isolated *Bacillus subtilis* NX-2 was cultivated in a series of shake flasks containing five sugars paired with organic acids for the production of PGA; it was reported that no PGA was produced without the presence of L-glutamic acid in the medium. When the simple sugars (sucrose, lactose, maltose, starch, and glycerol) were used, no polysaccharide was formed as a byproduct. It was speculated that *Bacillus NX-2* does not have

the enzymes of the glyconeogenesis pathway; therefore it could not use citric acid in the TCA cycle for cell growth and a PGA synthesis system cannot be formed.

2.4.2 Key nutrients needed for cell growth

The nutrients essential for microbial growth are a combination of carbon, nitrogen, oxygen and phosphate sources, as well as key trace elements, namely, magnesium, potassium, and iron (Forage, 1979). Biomass production from confectionery waste can be represented as:

$$C_6H_{12}O_6 + O_2 + N, P, K, Mg, S \rightarrow BIOMASS + CO_2 + H_2O + HEAT, \quad \textbf{Eq 2.1}$$

where simple C_6 sugars such as glucose/fructose, with the combination of oxygen and nitrogen, as well as trace elements, produce biomass, carbon dioxide, water and heat (Forage, 1979).

The type of nutrients needed are dependent on the organism used. Madonsela (2013) optimised the growth medium for *B. licheniformis* JCM 2505 called modified medium E (MME), which consists of 20 g/L glucose 1 g/L glycerol, 12 g/L citric acid, 3.48 g/L NH_4Cl, 2.99 g/L K_2HPO_4 and mineral salts (0.5 g/L $MgSO_4.7H_2O$, 0.104 g/L $MnSO_4.H_2O$, 0.04 g/L $FeCl_3.6H_2O$ and 0.15 g/L $CaCl_2.2H_2O$). It is imperative that these trace elements be added in the correct proportions as an oversupply of metal ions can hinder growth of microorganisms by causing cation toxicity, as an increase in cation concentration has been shown to diminish growth performance Sibanda (2009).

2.4.3 The different stages of cell growth

There are four main stages of cell growth as shown in Figure 2-5: (i) The **lag phase** is the stage at which cells adapt to their environment. This phase is dependent on a number of factors, such as low cell concentration, where no apparent changes to cell concentration can be seen, and the change in the metabolism of the microorganism to be able to break down compounds found in the medium. This phase is known as the transitional phase into the **exponential phase** (Rolfe et al., 2012). In a nutrient-rich medium, this phase is relatively short, as nutrients needed for growth are in excess. A minimal medium contains all of the nutrients needed for cell function, but the lag phase may be longer as the metabolism needs time to adjust to consume the nutrients. Another factor that affects the lag phase is the age of the inoculum; cells need to be viable in mid-exponential phase to shorten the lag phase (Brooks et al., 2011). (ii) During the **exponential phase**, balanced growth is seen and a rapid increase in cell concentration is observed. The rate at which cells grow is uniform at any given time throughout this phase. Furthermore, nutrient consumption rate is constant (Maier et al., 2009). This stage continues until the limiting substrate is depleted; the growth rate slows down as the

cells enter the (iii) **stationary phase**. During this stage, nutrient supply is limited and the rate at which cells die equates to the rate at which cells are formed; no apparent growth is seen. (iv) The **death phase** is the stage at which cells begin to die off and degrade. During the lag phase, the specific growth rate (μ) equates to 0. The exponential phase, μ, increases to its maximum (μ_{MAX}); and remains constant and approaches 0 as it enters the stationary phase, where it remains 0 (Shuler & Kargi, 1992).

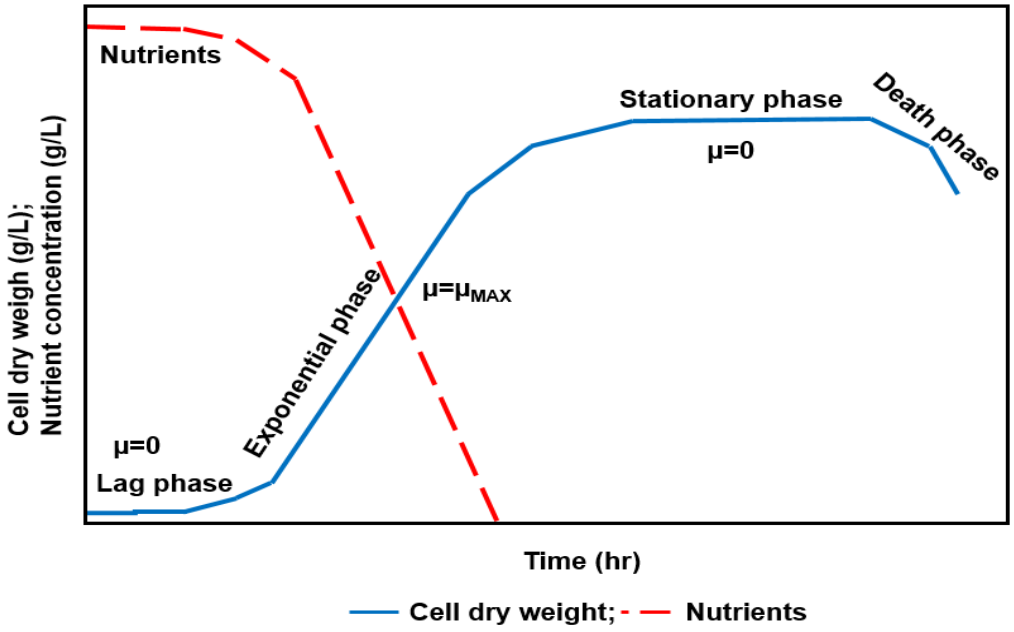

Figure 2-5 Stages of growth of a microorganism (Redrawn from Maier et al., 2009)

2.4.4 PGA production from renewable resources

To the author's knowledge, no literature exists that uses confectionery waste for the production of PGA; however researchers have been looking for renewable resources as feedstock to produce PGA as shown in Table 2-6. Zhu et al. (2014) investigated the possibility of using corncob hydrolysate containing glucose, xylose and arabinose as an alternative carbon source for the production of PGA using *Bacillus subtillis* HB-1. They found that xylose produces more PGA than glucose does. They concluded that corncob fibre hydrolysate can be used as a suitable carbon source in place of glucose. The use of waste streams as a nutrient source has also been explored by Zhang et al. (2012), using untreated cane molasses, which contains a combination of 24% (w/w) glucose and 28.3% (w/w) fructose. It was found that this was a suitable carbon source for the production of PGA using *Bacillus subtilis* NX-2. They found 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).

Researchers (Tang et al., 2015; Zang et al., 2015; Feng et al., 2016) used a “basal medium”, which contained several micronutrients, as well as glutamate as a nitrogen source.

The common nutrients across all of the media used for PGA cultivation were Mn^{2+}; $Mg^{2+}$and PO_4^{3-} source. Citric acid was commonly used, with the exception of *B. subtilis* NX-2, as this particular strain does not need it to produce PGA (Xu et al., 2005), and *Bacillus subtilis* HB-1, which was cultivated on corncob hydrolysate supplemented with 40 g/L of yeast extract, which is a nutrient-rich source containing a variety of nutrients needed for cell function (Zhu et al., 2014).

The glutamate-dependent strains need an organic nitrogen source in the form of glutamate for PGA synthesis, making the production medium costly as glutamate is expensive. 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(s), 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*.

Table 2-6 Medium selecion for PGA synthesis using renewable resources

Reference	Birrer et al. (1994)	Moraes et al. (2012)	Zhang et al. (2012)	Madonsela (2013)	Zhu et al. (2014)	Feng et al. (2016)	Kumar & Pal (2015)	Tang et al. (2015)
Organism	***Bacillus licheniformis***	***Bacillus velezensis* NRRL B-23189**	***Bacillus subtilis* NX-2**	***Bacillus licheniformis* JCM2505**	***Bacillus subtilis* HB-1**	***Bacillus subtilis* NX-2**	***Bacillus licheniformis* NCIM 2324**	***Bacillus* subtilis NX-2**
Carbon source	Glutamic acid	Molasses	Cane molasses	Glucose	Corncob hydrolysate (xylose)	Cane molasses	Diluted sugarcane juice	Rice straw hydrolysate
	Concentration (g/L)							
Carbon source		140	40	20	50	40		40
Citric Acid	12	12.5		12			10	
Glutamate	20		50		30	50	20	50
Glycerol	80			1				
$CaCl_2$					1			
$CaCl_2.2h_2O$	0.15	0.2		0.15			0.2	
$FeCl_3.7h_2O$	0.04			0.04			0.05	
K_2HPO_4	1.5	1		2.99			1	
$K_2HPO_4.3H_2O$			2			2		2
$MgSO_4$			0.1		1	0.1		0.1
$MgSO_4.7H_2O$	0.5	0.5		0.5			0.5	
$MnSO_4$			0.03			0.003		0.03
$MnSO_4.7H_2O$	0.104	0.05		0.104				
NaCl					10			
NH_4Cl				3.48			6	
$(NH_4)_2SO_4$		4						

2.5 Operation mode for the production of biopolymers and growth kinetics

The operation mode used for the production of biopolymers is dependent on the nature of the product as well as the organism. The main function of a microorganism is to respond to its direct environment. Depending on factors such as chemical and nutritional conditions, the microbe is able to produce an array of products. The relationship between available nutrients and cells is related to the products formed and cells grown (Shuler & Kargi, 1992).

Traditionally, many biopolymers are produced in a stirred tank reactor (Sukan et al., 2015). This system is able to create conditions that are conducive to growth as well as to product formation, and this is made possible by controlling parameters such as oxygen transfer via agitation and aeration, pH, and temperature. This control mode is useful when producing high-value products with high quality. In addition, this system is able to overcome variance in the feed composition as it controls its parameters effectively (Rani & Rao, 1999).

A batch system consists of a stirred tank reactor containing a sterilised cultivation medium that is inoculated by the seed culture at the beginning of the cultivation. The system is closed, and no additional components are added for the duration of the cultivation. The concentration of the nutrients in the medium as well as the cell concentration varies with time (Maier et al., 2009). To ensure that there is adequate oxygen in the system, the medium is aerated, mixing by impeller promotes bubble dispersion. The heat transfer and mass transfer of this system are adequate, and pH control is accurate. The advantage of the system is that sterility is maintained; the drawback of using a batch system is that resources are finite, and will ultimately lead to substrate limitation (Singh et al., 2014).

The fed-batch system consists of a stirred tank reactor with a pump that feeds a concentrated solution of the substrate. During the fermentation, the feed is introduced and no components are removed until the cultivation is complete, while maintaining sterility of the system. This system is highly favoured in the fermentation of many biological products such as amino acids, enzymes and vitamins (Qu et al., 2013). There is accurate control of parameters such as pH and temperature, heat, and mass transfer. The controlled feed enables the maintenance of a substrate concentration favourable for optimum growth of microorganisms, without overfeeding and inhibiting growth (Rani & Rao, 1999). This operation mode is used to obtain better yields than the batch system. The challenge with this mode is implementing a feeding strategy that is able to maintain a required substrate concentration within the system (Singh et al., 2014).

The moving-bed biofilm reactor (MBBR) system is advantageous as it has carriers containing immobilised cells (biofilm attached to the surface). The system is aerated and the carriers are

in constant suspension, allowing a continuous supply of nutrients into the system without the concern of potentially washing cells out. High biomass concentration in the form of biofilm accumulation combined with good oxygen mass transfer (Jing et al., 2009) has shown to increase PGA yields (Jiang et al., 2016). The technology has gained popularity in the wastewater treatment industry as it has a small footprint and is able to rapidly reduce the COD and BOD loads. This system has continuous flow, and is robust and simple to operate (Kamstra et al., 2017).

2.5.1 Operation modes for PGA production

Huang et al. (2011); Zhang et al. (2012) and Jiang et al. (2016) all reported that substrate limitation has a negative impact on the biosynthesis of PGA. The relationship between fed-batch and batch reactors was investigated in a 7.5 L batch bioreactor. As shown in Table 2-7, it was demonstrated that there is a considerable increase in PGA yield. Glucose as a carbon source produced the highest concentration of PGA at 53.0 g/L, while the renewable carbon source untreated cane molasses produced 52.1 g/L, which is fairly close to the glucose-containing medium. Furthermore, a relationship between biomass concentration and product formation can be seen; an increase in biomass yields more PGA. This was demonstrated by a system which used biofilm on carriers. When cultivating *Bacillus subtilis* NX-2, a comparison study between free cells in batch and immobilised cells increased from 32.86 ± 0.58 g/L to 34.74±0.61 g/L, respectively. Immobilised cells in fed- batch mode further increased the maximum PGA concentration to 66.38 ± 0.67g/L. The advantage of immobilised cells in the system shortens the lag phase and could turn into a semi-continuous operation without having to sterilise the system in between runs (Xu et al., 2014). Subsequent studies tested PGA productivity in an MBBR; a significant productivity of1.24 g/Lh was noted, the highest found in literature.

Since *Bacillus subtilis* NX-2 is a glutamate-dependent strain; the basal medium contained 50 g/L glutamate (Zhang et al., 2012), therefore higher yields were expected as glutamate is a PGA precursor (Sirisansaneeyakul et al., 2017). *B. licheniformis* TISTR 1010 is a glutamate-independent strainand research and development in the area of glutamate-independent strains is underway (Kongklom et al. 2015; Kongklom et al., 2017). Kongklom et al. (2015) compared different feeding strategies by investigating the effects of pH, dissolved oxygen (DO), nitrogen depletion and substrate inhibition of PGA production. Their initial fed-batch bioreactor study involved constantly feeding glucose (500 g/L) into a bioreactor, so that the concentration medium was maintained at 6 to 20 g/L, using improvised software. There were three different feeding stages: Phase A (8 h) when pH was not controlled; during the second phase B, pH was maintained at 7.3 by 3M NaOH, and the last stage did not have an acidic buffer to maintain

pH. DO was not controlled, aeration was 1 vvm, agitation 300 rpm. These conditions did not change throughout the experiments. After 44 h, PGA began to degrade as the nitrogen source was depleted. They speculated that pH should be controlled by feeding both carbon and nitrogen sources. Their subsequent work controlled the pH during phase (C) using an acidic solution of 20% citric acid and 20% NH_4Cl with DO control (>20% air saturation). This alteration increased the PGA concentration within the system from 5.03±0.04 to 27.54±0.20 g/L. The main conclusions of their study were: (i) oxygen is essential for the recycling of NaDH and $FaDH_2$ which are used by the enzymes in the TCA cycle; (ii) the TCA cycle is active in live and resting cells – this means that PGA can be produced during the stationary phase; and (iii) maintaining the carbon and nitrogen within the system prevents PGA degradation. Their successive study focused on maintaining the DO >50% saturation (sparged with oxygen-enriched air) in a fed-batch system by raising the agitation by 50 rpm increments when the DO dropped below the threshold. This increased the PGA concentration to 39.9 ±0.28 g/L (Kongklom et al., 2017).

Table 2-7 Operation mode for PGA production

	Bioreactor parameters				Polyglutamic acid				
Strain	**Sugar**	**Sugar (g/L)**	**Max CDW (g/L)**	**Mode**	**Productivity (g/Lh)**	**PGA (g/L)**	**Yield (g/g) sugar**	**μ (h^{-1})**	**Reference**
B. subtilis NX-2	Sugar mixture	60	6.02	Batch	0.44	32	0.53	0.17	Zhang et al., 2012
B. subtilis NX-2	Cane molasses	60	6.92	Batch	0.46	33.6	0.56	0.17	Zhang et al., 2012
B. subtilis NX-2	Cane molasses	90	7.91	Fed-batch	0.52	50.2	0.59	0.18	Zhang et al., 2012
B. subtilis NX-2	Molasses hydrolysate	90	7.95	Fed-batch	0.53	51.1	0.58	0.21	Zhang et al., 2012
B. subtilis HB-1	Xylose	60	11.1	Batch	0.74	23.6	0.38	0.09	Zhu et al., 2014
B. subtilis HB-1	Glucose	60	13.94	Batch	0.60	21.6	0.33	0.14	Zhu et al., 2014
B. subtilis HB-1	Xylose	60	13.64	Fed-batch	0.44	28.2	0.97	0.11	Zhu et al., 2014
B. subtilis NX-2	Glucose	60	4.87	Batch	0.46	32.9	0.55	-	Xu et al., 2014
B. subtilis NX-2	Glucose	60	7.43	Batch (immobilised cells)	0.72	34.7	0.58	-	Xu et al., 2014
B. subtilis NX-2	Glucose	90	10.58	Fed-batch (immobilised cells)	0.98	66.4	0.74	-	Xu et al., 2014
B. subtilis NX-2	Glucose		-	MBBR	1.24	74.2			Jiang et al., 2016
B. licheniformis TISTR 1010	Glucose	20	2.5	Batch	0.03	1	0.05	0.05	Kongklom et al., 2015
B. licheniformis TISTR 1010	Glucose	500	5.63	Fed-batch	0.09	5	0.05	0.08	Kongklom et al., 2015
B. licheniformis TISTR 1010	Glucose	500	6.36	Fed-batch	0.29	27.5	0.1	0.06	Kongklom et al., 2015
B. licheniformis TISTR 1010	Glucose	20	1.8	Batch	0.11	3.4	0.09	0.06	Kongklom et al., 2017
B. licheniformis TISTR 1010	Glucose	500	4	Fed-batch	0.93	39.9		0.11	Kongklom et al., 2017

The production of PGA has three major requirements: (i) A constant supply of carbon and nitrogen nutrients; (ii) pH control (Kongklom et al., 2015) and (iii) sufficient oxygen supply to the system (Kongklom et al., 2017). Confectionery waste was a suitable carbon source as it contains a large proportion of sucrose (Ozgun et al., 2012). To the author's knowledge, no literature has noted the use of confectionery waste for the production of PGA; however the key nutrients needed for cell function and PGA synthesis were sourced through similar studies that used renewable resources to produce PGA (Kumar & Pal, 2015; Tang et al., 2015; Zang et al., 2015; Feng et al., 2016). PGA has a range of potential applications; however owing to the nature of the feedstock, the application will be of value in the agricultural industry (Zhu et al., 2014).

Hypothesis 1

Confectionery waste dissolved in water supplemented with citric acid, a nitrogen, phosphate source, and trace elements can be used for the production of PGA using *Bacillus*, as *Bacillus* species are able to hydrolyse sugars like sucrose and solid candy waste.

Key questions

1. What sugar concentration should be used for the production of PGA?
2. How long will it take for the *Bacillus* species to hydrolyse the sugar before using it to produce PGA?
3. Does feedstock media need to be supplemented with other nutrients to aid in the production of PGA?

Hypothesis 2

Fed-batch operation will produce more biomass, which in turn will produce more PGA. A constant concentration of sugars within the medium promotes PGA production.

Key question

How does sucrose as a substrate affect growth of *Bacillus?*

CHAPTER 3: MATERIALS AND METHODS

This chapter details the research approach and methods used to achieve the objectives stated in Section 1.5. The selection of *Bacillus* sp; the cultivation of *B. licheniformis* in shake flasks using a nutrient medium and adaptation to a chemically defined medium; the preliminary waste characterisation; as well as proof of concept and the conditions used in the batch bioreactor studies are presented.

3.1 Selection of *Bacillu*s and cultivation of species

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

- A well-characterised microorganism was preferred.
- The focus was on the biosynthesis of PGA in L-glutamic acid independent organisms, able to produce PGA, and used as an alternative nitrogen source to glutamate, as glutamate is costly.

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 by determining the ideal proportions needed to facilitate growth and PGA production. The strain was originally obtained from Riken, the Japanese research institute.

3.1.1 Cultivation of *B. licheniformis* JCM2505 on nutrient-rich medium

A glycerol stock containing *B. licheniformis* JCM 2505, stored at −50 °C, was obtained from the CeBER master stock culture collection. An aliquot of this stock was suspended in a sterile tryptone-soy medium (30 g/L tryptone soy in deionised water) and incubated for 24 h at 150 rpm on an orbital shaker (MRC) in a constant temperature room set at 37 °C. The culture was then streak-plated onto sterile tryptone-soy agar (30 g/L tryptone soy, 15 g/L bacteriological agar in deionised water) and incubated at 37 °C for 24 h, as shown in Figure 3-1. For pre-inoculum preparation, a single colony was scraped off the agar and transferred to a 250 mL flask containing 50 mL sterile tryptone-soy medium. This was incubated with agitation at 150 rpm for 24 h. An aliquot of the pre-inoculum was transferred to a 500 mL shaking flask containing 100 mL sterile tryptone-soy medium to achieve an optical density (OD) of 0.1. The value of 0.1 is a standardised concentration. With all experimental data starting at an OD of 0.1, data analysis is uncomplicated as it is easier to compare reproducible data sets under different conditions. Furthermore, the addition of a more concentrated culture will shorten the exponential phase, depleting the nutrients in a shorter space of time and giving insufficient

data points to determine growth kinetics. Unbalanced growth will be seen as the cells enter the stationary phase, owing to a rapid depletion of the limiting nutrient (Hall et al., 2014). The inoculum was cultivated at a shaking frequency of 150 rpm and a temperature of 37 °C. After 12 h, an aliquot of the inoculum was transferred to 1 L flasks containing 250 mL tryptone-soy medium to make up an OD of 0.1.

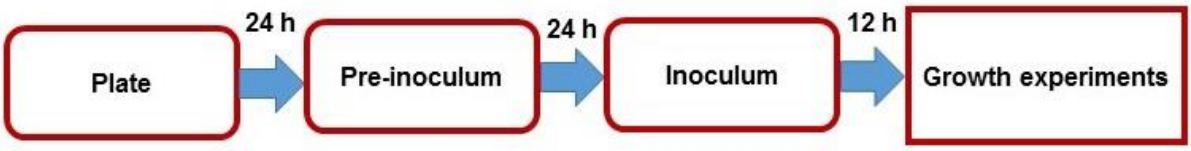

Figure 3-1 Inoculum train for the cultivation of *B. licheniformis* on nutrient-rich medium

3.1.2 Adaptation of *B. licheniformis* to a minimal medium

An aliquot of the glycerol stock containing *B. licheniformis* JCM 2505 was suspended in a 250 mL flask containing 50 mL sterile tryptone-soy medium (complex medium) and cultivated for 24 h at 150 rpm on an orbital shaker in a constant temperature room set at 37 °C. An aliquot of the culture was transferred to a 500 mL shake flask containing 100 mL sterile production medium (20 g/L glucose supplemented with basal medium) to achieve an OD of 0.1. The culture was cultivated at a shaking frequency of 150 rpm and a temperature of 37 °C. After 24 h, an aliquot of the culture was transferred to the 1 L flasks containing 250 mL sterile 20 g/L glucose supplemented with basal medium (inoculum) to achieve an OD of 0.1. This inoculum train, shown in Figure 3-2, was used for the cultivation of *B. licheniformis* on confectionery-based medium in multi-well plates, shake flasks, and for the bioreactor studies carried out using a minimal medium supplemented with sucrose.

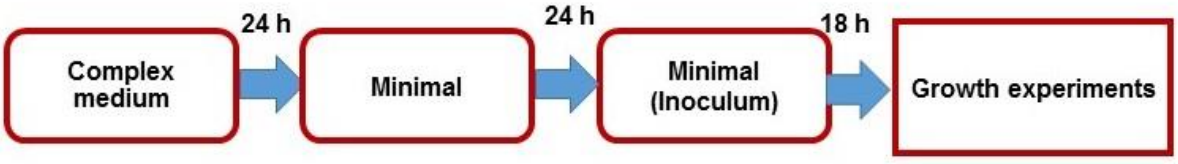

Figure 3-2 Inoculum train for the adaptation of *B. licheniformis* on a minimal medium

3.1.3 Cultivation of *B. licheniformis* in shake flasks using sucrose and candy waste

B. licheniformis was cultivated on 20 g/L solid hard candy waste suspended in deionised water, supplemented with basal medium. A medium containing 20 g/L sucrose in deionised water supplemented with basal medium was used as a control. The basal medium composition is summarised in Table 3-1. Experiments took place in three 1 L Erlenmeyer shake flasks containing 250 mL medium at a temperature of 37 °C 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 as described in Section 2.4.3. A final 24 h sample was taken to ensure that the cells were in the stationary phase.

Table 3-1 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

3.2 Characterisation of confectionery waste

The confectionery waste was obtained from an anonymous confectionery factory site, and contained defective chocolate, marshmallow, and hard candy. The macronutrients identified for cell growth as well as PGA production included a carbon, nitrogen and phosphate 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 3-2 is a summary of the analytical techniques used for the analyses and characterisation of the confectionery waste. Standard protocols of the analytical techniques can be found in Appendix A.

Table 3-2 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, i.e., C, H, O, N, P, K, Ca, Na, Mg.	ICPOES	Boss & Fredeen (2004)
Total organic carbon (TOC)	TOC analyser	HACH 10128

3.3 Identification of key nutrients needed for the cultivation of *B. licheniformis*

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, as discussed in Section 2.4.4 (Birrer et al., 1994; Moraes et al., 2012; Zhang et al., 2012; Madonsela, 2013; Kumar & Pal, 2015; Tang et al., 2015; Feng et al., 2016). The confectionery waste required supplementation with a “basal medium”, listed in Table 3-1. A screening

approach to determine whether *B. licheniformis* was able to grow on the different types of waste (candy, marshmallow, chocolate, and a homogenous mixture of the three) was used by cultivating the different types of waste supplemented with the basal medium. The control was MME, a medium composition optimised by Madonsela (2013). It contained 20 g/L glucose supplemented with basal medium. Solutions of 93.87 g/L marshmallow; 69.96 g/L chocolate; 89.49 g/L hard candy; and 84 g/L of a homogenous mixture of equal proportions of chocolate, marshmallow and hard candy waste were prepared in 100 mL deionised water, and autoclaved (Hirayama HG-50 autoclave) at 105 °C for 30 min. The basal medium was prepared separately in 50 mL deionised water; the quantities weighed out were 0.255 g NH_4Cl, 0.224 g K_2HPO_4 and mineral salts (0.0375 g $MgSO_4.7H_2O$, 0.0078 g $MnSO_4.H_2O$, 0.003 g $FeCl_3.6H_2O$ and 0.001 g $CaCl_2.2H_2O$. This solution was autoclaved at 121 °C for 20 min.

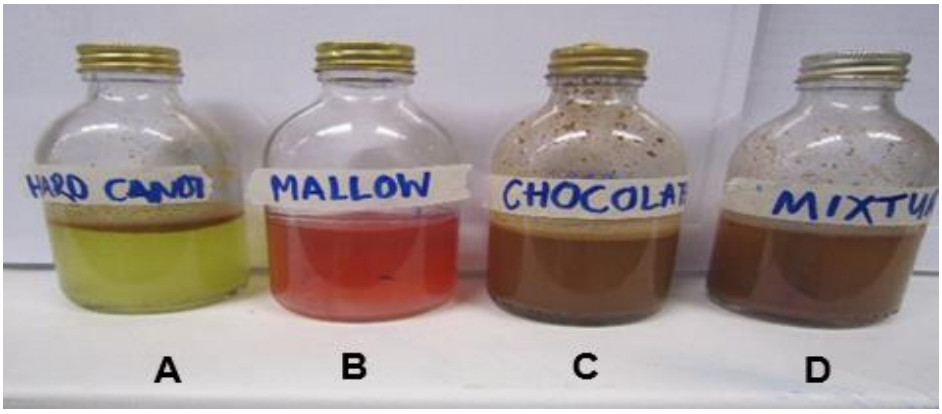

Figure 3-3 Confectionery waste solutions: (A) Hard candy; (B) Marshmallow; (C) Chocolate (D) Mixture of A, B and C

The orientation of the multiwell plates is indicated in Figure 3-4 and explained in Table 3-3. The rows are indicated as A, B and C and represent the rows of the wells. The columns are numbered as 1 to 5 and represent the rows of the wells. The 4 mL wells had a 3 mL working volume.

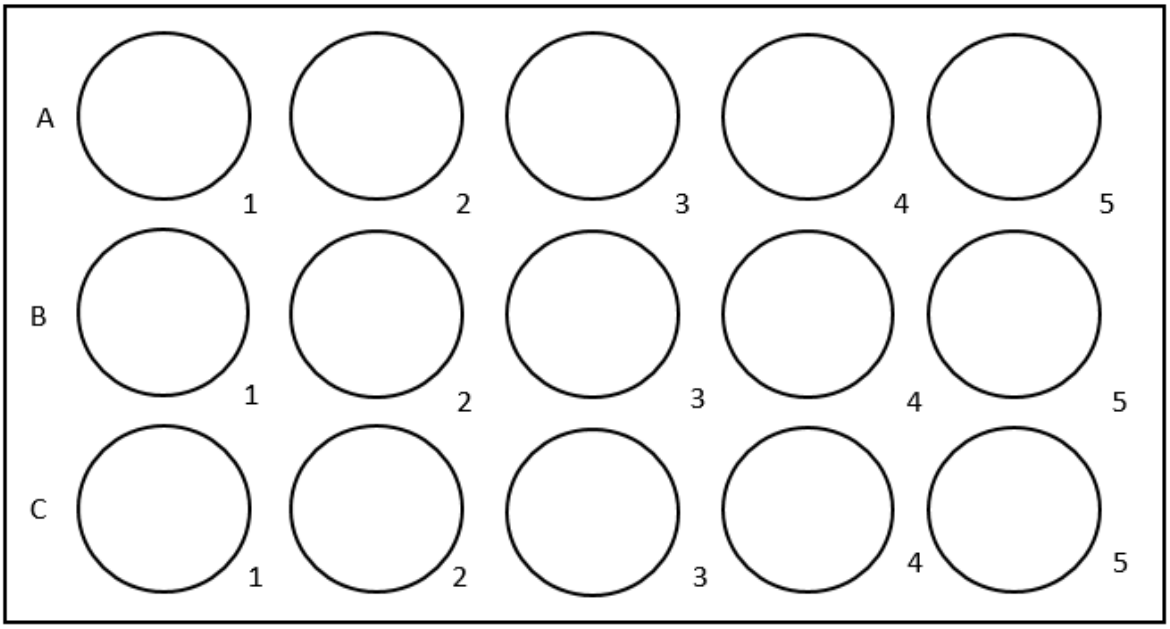

Figure 3-4 Orientation of the multi-well plates used in experiments

One mL of each of the different types of waste solutions was pipetted into separate wells, and 2 mL of basal medium was added to each well. The pH of the mixtures was recorded and not adjusted. The wells were inoculated with the inoculum from the inoculum train in Section 3.1.2 to make up an OD of 0.1. The plates were then sealed with a breathable sealing membrane and placed on an orbital shaker at 150 rpm and a temperature of 37 °C for 30 h. Table 3-3 is a summary of the conditions as well as the well identification.

Table 3-3 Orientation of mutiwell plates

Well ID	pH	Temperature (° C)	Volume (mL)	Carbon source	Carbon concentration (g/L)
A1,B1,C1	6.89	37	3	Glucose	20.00
A2,B2,C2	6.85	37	3	Chocolate	23.32
A3,B3,C3	6.77	37	3	Candy	29.83
A4,B4,C4	6.82	37	3	Mallow	31.29
A5,B5,C5	6.2	37	3	Mixture	28.00

3.4 Bioreactor studies

To understand the growth kinetics of *B. licheniformis* on confectionery waste, a base case experiment using sucrose supplemented with basal medium was conducted. Subsequently, *B. licheniformis* was cultivated on hard candy waste. All experiments were run in duplicate to ensure reproducibility using the parameters summarised in Table 3-4.

Table 3-4 Parameters for batch bioreactor experiments

Parameter	Run 1	Run 2	Run 3	Run 4
Sugar	Sucrose	Sucrose	Candy	Candy
Sugar concentration (g/L)	20	20	20	20
DO (% sat)	≥20	≥20	≥20	≥20
pH	6.5	6.5	6.5	6.5
Temperature (° C)	37	37	37	37
Working volume (L)	5	5	5	5

3.4.1 Batch bioreactor studies

All batch experiments took place in a 7-L New Brunswick BioFlo 110 fermenter as shown in Figure 3-5. The seed culture preparation followed the same steps as the inoculum train used for the shake flask experiments outlined in Section 3.1.2. The starting OD of all experiments was 0.1 in 5 L of culture medium. Temperature was maintained at 37 °C, aeration was set at 1 vvm. The agitation started at 500 rpm; as the cells began to grow, more oxygen was required. The critical DO was set at 20%, and was controlled by adjusting the agitation speed. The pH was maintained at 6.5 with 5M NaOH for the first stage of the cultivation as well as during the exponential phase.

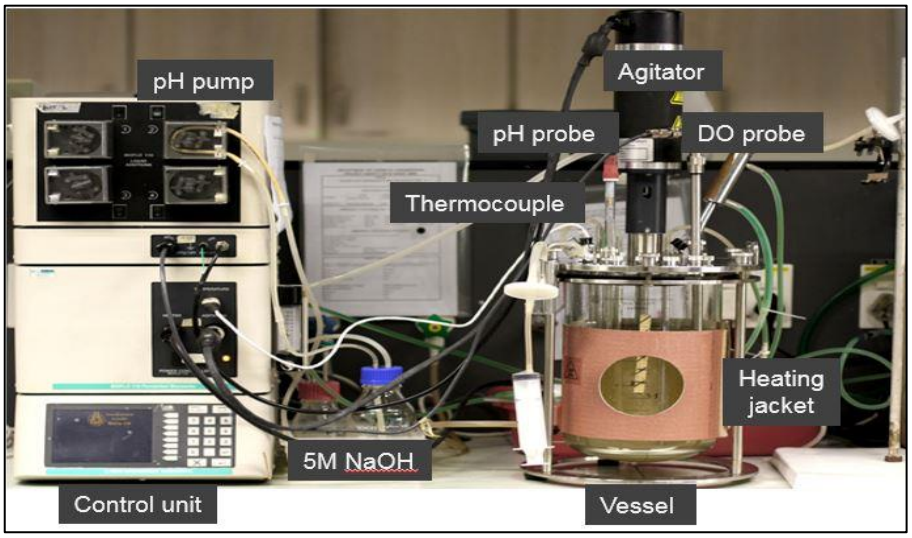

Figure 3-5 BioFlo 110 fermenter system

Sampling took place every 2 h and offline pH readings were taken. The cell concentration was measured using the Genesys 10S UV VIS spectrophotometer at a wavelength of 600 nm. 8 mL of culture medium was separated into 2 mL aliquots in centrifuge tubes and stored at −20 °C for HPLC sugars analysis.

3.4.2 Fed-batch bioreactor studies

All fed-batch experiments took place in a 7-L New Brunswick BioFlo 110 fermenter as shown in Figure 3-6. The parameters were controlled with pH indicator (pHI), when the pH within the system, signals transmitted from the controller turned the alkali peristaltic pump on to raise the pH. The system was supplied with compressed air, and flowrate was adjusted to 1 vvm by using a rotameter. DO was monitored by the dissolved oxygen indicator (DOI). When the DO% was below the critical amount, agitation rate was increased by agitation control (AC). Coolant was circulated through the system with a fixed speed pump, and temperature was controlled by the temperature indicator (TIC) transmitting signals to the control unit. A drop in temperature within the system prompted the controller to turn on the heating jacket to maintain the temperature set point. The concentrated feed was introduced into the system at a fixed speed using a peristaltic pump.

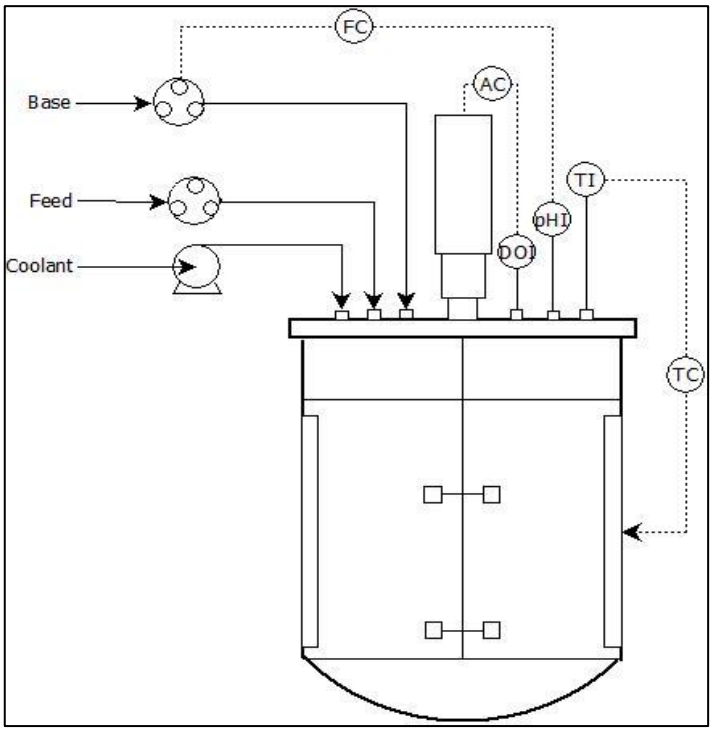

Figure 3-6 Fed batch fermentation in the BioFlo 110 fermenter system

The seed culture preparation followed the same steps as the inoculum train used for the shake flask experiments outlined in Section 3.1.2. The starting OD of all experiments was 0.1 in 5 L of culture medium. Bioreactor parameters are summarised in Table 3-5 and aeration was set at 1 vvm. The agitation started at 500 rpm and as the cells began to grow, more oxygen was required. The critical DO was set at 20%, and was controlled by adjusting the agitation speed. The pH was maintained at 6.5 with 5M NaOH for the first stage of the cultivation as well as during the exponential phase. The first stage of the cultivation was a normal batch process containing 20 g/L candy supplemented with basal medium. A peristaltic pump was used to

pump a feed containing a solution of 10.44 g/L NH_4Cl and 60 g/L candy. Feeding began at 13 h and continued for a duration of 9 h.

Table 3-5 Parameters for fed-batch experiments

Parameter	Run 5	Run 6
Sugar	Candy	Candy
Sugar concentration (g/L)	20	20
DO (% sat)	≥20	≥20
pH	6.5	6.5
Temperature (° C)	37	37
Working volume (L)	3–3.75	3–3.75

3.4.3 Calculation of kinetics

Equations 3.1 to 3.4 demonstrate the steps taken to quantify the growth kinetics. The exponential function was fitted to the biomass curve (change in cell dry weight (CDW) as a function time). This procedure was followed when determining the maximum specific growth rate (μ_{max}) values for all growth experiments.

$$\sum S + X \rightarrow \sum P + nX \qquad \textbf{Eq 3.1}$$

$$Substrate + cells \rightarrow extracellular\ products + more\ cells$$

The net specific growth rate (μ) is a function of the number of cells reproduced (X) and can mathematically be determined by (Maier et al., 2009):

$$\frac{dX}{dt} = \mu X \qquad \textbf{Eq 3.2}$$

Integrate:

$$\int_0^X \frac{1}{X} dx = \mu \int_0^t dt$$

$$lnX - lnX_0 = \mu t - t_0$$

$$\therefore ln\frac{X}{X_0} = \mu t$$

$$lnX = \mu t + lnX_0 \qquad \textbf{Eq 3.3}$$

$$X = X_0 e^{\mu t} \quad \textbf{Eq 3.4}$$

where:

$\mu = \ specific\ growth\ rate\ (h^{-1})$

$X_o = cell\ concentration\ (g/L) at\ t_0$

$X = cell\ concentration\ (g/L) at\ t$

3.4.4 Yield factors in batch

Yield factors indicate the relationship between biomass concentration (X) and substrate (S). Similarly, the relationship between product (P) concentration of PGA formed with regard to substrate consumed can be determined. These values indicate how much of the substrate went to biomass, and how much of the substrate was used for product formation. The yield coefficients for the batch were calculated as follows (Shuler & Kargi, 1992):

$$Y_{X/S} = \frac{V_f X_f - V_i X_i}{F\theta S_o + V_i S_i - V_f S_f} \quad \textbf{Eq 3.5}$$

$$Y_{P/S} = \frac{P_f - P_i}{S_i - S_f} \quad \textbf{Eq 3.6}$$

3.4.5 Yield factors in fed-batch

The yield factors differ within a fed-batch system as the volume of the system changes with time. To compensate for this change in volume, the product of the feeding rate (F), the feeding time (θ), as well as the feed concentration (S_O), are incorporated into the equation. The yield coefficients for the fed-batch were calculated as follows (Borzani, 2008):

$$Y_{X/S} = \frac{V_f X_f - V_i X_i}{F\theta S_o + V_i S_i - V_f S_f} \quad \textbf{Eq 3.7}$$

$$Y_{P/S} = \frac{V_f P_f - V_i P_i}{F\theta S_o + V_i S_i - V_f S_f} \quad \textbf{Eq 3.8}$$

where:

$F = Feed\ rate\ (L/\ h)$

$P = Product\ concentration\ (g\ /L)$

$S = substrate\ concentration\ (g\ /L)$

$S_O = substrate\ feed\ concentration\ (g\ /L)$

$V = volume\ of\ medium\ (L)$

$X = biomass\ (g)$

$Y_{X/S} = yield\ of\ biomass\ with\ respect\ to\ time\ (gbiomass/gsubstrate)$

$Y_{P/S} = yield\ of\ product\ with\ respect\ to\ time\ (gbiomass/gsubstrate)$

$i = initial\ time\ point\ at\ t = 0$

$f = t\ value\ at\ t = \theta$

$\theta = feeding\ time\ (h)$

3.5 Analytical techniques

3.5.1 Total organic carbon (TOC)

TOC is a measure of the total amount of organic substance in a solution. This is a familiar technique in wastewater treatment as it is a useful tool to compare the amount of organic contaminants in the influent and effluent of wastewater treatment facilities. TOC is able to measure the sum total of all organically bound carbon and is not the same as total carbon (TC). TC is the sum of TOC, and total inorganic carbon (TIC), which are carbonate containing compounds as shown below:

$$TC = TIC + TOC \qquad \textbf{Eq 3.10}$$

Samples were sent to A.L. Abbott & Associates for TOC analysis. The HACH 10128 method was used to quantify TOC.

3.5.2 Total Kjeldahl nitrogen (TKN)

Application of the TKN method is used in the food, beverage and wastewater treatment industry. This method involves three main steps: (i) digestion of samples to form ammonium ions (NH_4^+); (ii) liberation of ammonia NH_3 from NH_4^+ and (iii) the addition of Nessler's reagent responsible for indicating the presence of NH_3 (Spedding et al., 2012). Samples were sent to A.L. Abbott & Associates for TKN analysis.

3.5.3 Inductively coupled plasma – optical emission spectrometry (ICP-OES)

This technique determines the elemental composition of the confectionery waste by using a combination of a plasma and a spectrophotometer. Samples were digested according to the protocol outlined in Appendix A before ICP-OES tests were conducted (Boss & Fredeen, 2004). Sample preparation as well as ICP-OES analysis was performed by the analytical laboratory in the Department of Chemical Engineering, University of Cape Town.

3.5.4 Ninydrin assay

This method was developed by Lie (1973) as an alternative to the Kjeldahl method to test the free amino nitrogen (FAN) content in beer samples. When the sample combined with the reagents is heated, a colour change takes place and is measurable at 570 nm. Ninhydrin, the oxidant, removes the carboxylate group forming CO_2, NH_3 and an aldehyde. The unreduced ninhydrin reacts with the reduced ninhydrin and NH_3. This reaction is indicated by a blue/purple colour change. The presence of fructose acts as a reductant. Potassium iodate inhibits further reaction of the oxidised ninhydrin (Spedding et al., 2012).

3.5.5 High-performance liquid chromatography (HPLC) of carbohydrates

HPLC was used to quantify the different types of carbohydrates found in confectionery waste. This was achieved by using a carbohydrate analysis column (Aminex HPX-87H) on a Thermo Scientific Spectra system AS3000 autosampler and Thermo Scientific RefractoMax521 refractive index (RI) detector to profile the different types of sugars found in confectionery waste. Owing to the complexity of confectionery waste, optimisation of standard methods was done to obtain good peak resolution. Figure 3-7 is a chromatogram of the different sugars and their retention times. The mobile phase used was 5 mM sulphuric acid, a flowrate of 0.5 mL/min and a temperature of 40 °C and standards for citric acid, fructose, glucose, and sucrose were used.

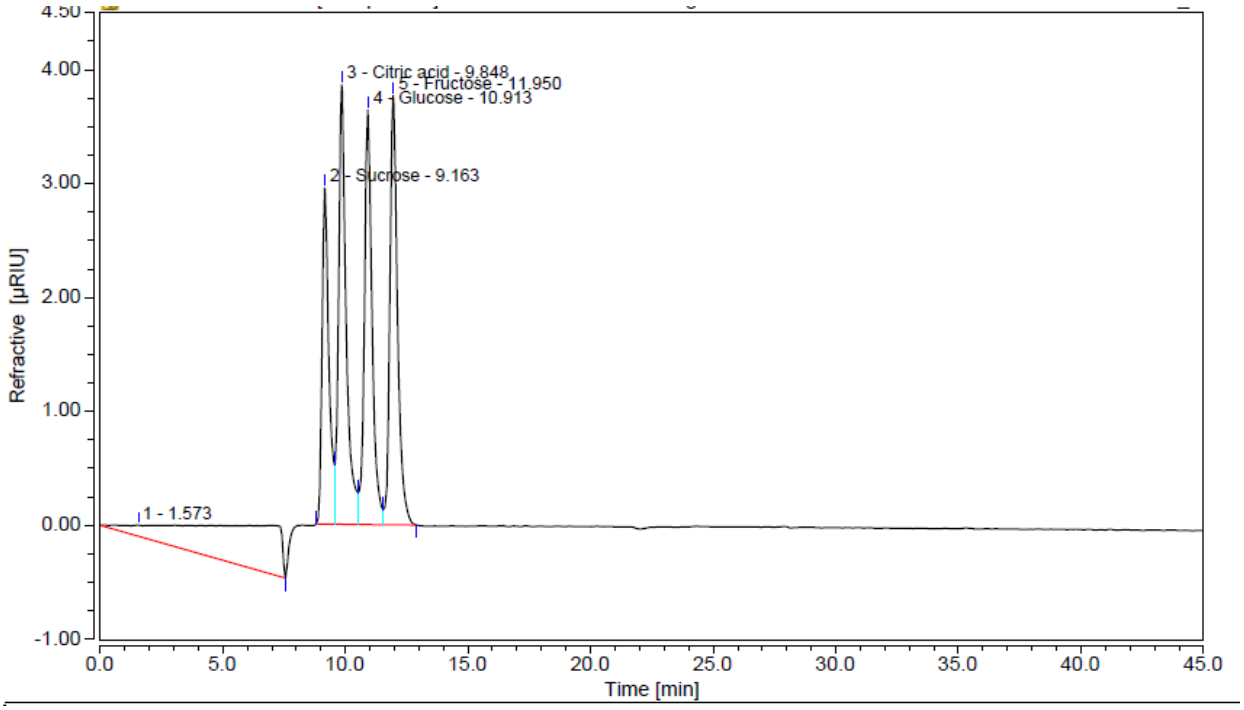

Figure 3-7 Sugar chromatograms and their retention times

3.5.6 PGA spectrophotometric assay

PGA was gravimetrically extracted from the culture medium and concentration was detected at an absorbance of 204 nm on a Thermo Helios UV spectrophotometer, a method developed by Zeng et al. (2012). The culture was diluted with 3 v/v distilled water. The dilute culture broth was centrifuged at 13 400 g for 20 minutes. An aliquot of the supernatant (0.3 mL) was combined to 3 parts cold ethanol (1.2 mL), where the polymer precipitates and is centrifuged at 13 400 g at 4 °C for 20 minutes. The precipitate was left to dry in the solvent's fume hood, and the dry pellet was re-suspended into pH 7 phosphate buffer. The solution was transferred to a quartz cuvette and measured at a wavelength of 204 nm (Zeng et al., 2012).

3.5.7 Optical density (OD)

The cell concentration can be spectrophotometrically quantified by measuring the turbidity of the cells in solution as a function of time by adding 2 mL of sample to a cuvette and reading it at a wavelength 600 nm (Maier et al., 2009). There is a direct correlation between cell turbidity and the cell dry weight (CDW). This relationship can be obtained from experimental data by plotting CDW as a function of OD. The correlation coefficient used for calculations was 0.56 as shown in Equation 3.11 (Link et al., 2008).

$$CDW = 0.56 \times OD_{600} \qquad \textbf{Eq 3.11}$$

3.5.8 Statistical analysis

Deviation between data sets was determined by using the standard deviation (Harvey,2011):

$$Standard\ deviation = \sqrt{\frac{\sum(x - \bar{x})^2}{(n-1)}}$$

Eq 3.12

where:

$n = number\ of\ repeats$

$x = data\ point\ value$

$\bar{x} = mean$

The above analytical techniques were used to characterise the confectionery waste, quantification of the product, and substrate concentration. The PGA analytical method was used to profile product formation throughout the cultivations. These values were used to determine the growth kinetics of *B. licheniformis* in the different growth experiments.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Introduction

Microorganisms, including *B. licheniformis,* need a sufficient nutrient supply to ensure proper cell function (Birrer et al., 1994; Moraes et al., 2012; Zang et al., 2012; Madonsela, 2013; Kumar & Pal, 2015; Tang et al., 2015; Feng et al., 2016). PGA production is growth associated. The amino acid monomer is produced from intermediates of the TCA cycle; hence it needs a supply of C_6 sugars and NH_4^+ ions (Kongklom et al., 2015; Kongklom et al., 2017). This chapter contains the results from the characterisation of confectionary waste and the cultivation of *B. licheniformis* on a sucrose-based minimal medium as base case and on confectionery waste supplemented with basal medium. The shake flask data in Section 4.4 was used to determine the growth behaviour of *B. licheniformis* in order 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 was able to control parameters at the optimum determined for growth and provided enhanced oxygen transfer to ensure that the cells within the system were not deprived of oxygen.

4.2 Characterisation of 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 carbohydrates (Bussiere & Serpelloni, 1985), and acidulents (Sortwell, 2004) which can form the carbon source for the cultivation of *B. licheniformis*. 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 PGA production, such as free amino nitrogen, fermentable sugars, and trace elements, identified in Section 2.4.4

The N, TOC and trace element results obtained for confectionery waste are tabulated in Table 4-1. The results show that chocolate, marshmallow and hard candy 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 (calculations can be found in Appendix B). 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 1.1 g of NH_3 was needed for PGA production. The C:N ratios of chocolate, marshmallow and hard candy were calculated and are presented in Table 4-1. 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 and it had to be supplemented with additional nutrients for the cultivation of *B. licheniformis.*

Table 4-1 Confectionery waste characterisation

Parameter	Chocolate	Marshallow	Hard Candy
Total Kjedahl nitrogen (g/g as N)	0.005	0.003	0.001
Total organic carbon (g/g as C)	0.551	0.41	0.43
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

4.3 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) (as discussed in Section 2.4.4) which consists of 20 g/L glucose, 1 g/L glycerol, 12 g/L citric acid, 3.48 g/L NH_4Cl, 2.99 g/L K_2HPO_4 and mineral salts (0.5 g/L $MgSO_4.7H_2O$, 0.104 g/L $MnSO_4.H_2O$, 0.04 g/L $FeCl_3.6H_2O$ and 0.15 g/L $CaCl_2.2H_2O$), 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 4-2). From Table 3.2, the TOC values were multiplied by 12.83 g C/L to calculate the respective amount of waste required (Table 4-3) to ensure that the amount of carbon added from the confectionery waste was equal to the amount of carbon added from the MME.

Table 4-2 The individual carbon requirements of glucose, citric acid and glycerol

· Carbon source	· Stoichiometric ratio of C	· C required (g/L)
Glucose (20 g/L)	0.4	8
Citric acid (12 g/L)	0.37	4.44
Glycerol (1 g/L)	0.39	0.39
Total C required		12,83

Table 4-3 Respective carbon amounts added from various confectionery waste

· Waste source	· TOC (g Carbon /g waste)	· Waste required (g)
Mallow	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

Multiwell plate experiments were conducted using confectionery waste supplemented with basal medium inoculated with *B. licheniformis.* Figure 4-1 shows the plates prior to inoculation, while Figure 4-2 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 4-1, it is seen that the chocolate imparts a brown colour and some turbidity to the medium. Figure 4-2 shows that glucose, hard candy as well as marshmallow displayed growth, monitored by OD at 600 nm blanked against media, as well as colour change.

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

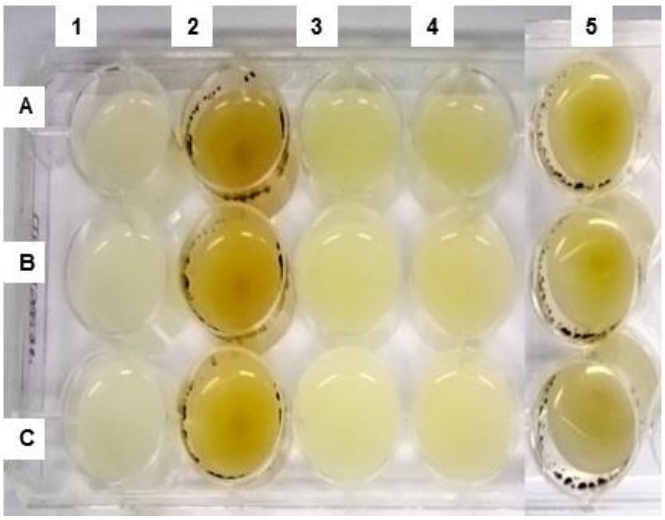

Figure 4-2 Multiwell plates containing 1) glucose, 2) chocolate, 3) hard candy, 4) marshmallow, and 5) the mixture after 30 h cultivation

The screening of confectionery waste for growth showed promising results. Optical densities of the multiwell plate experiments at 30 h as well as the starting pH and the pH after 30 h of cultivation are shown in Table 4-4. Marshmallow waste showed the best growth over all substrates, achieving an OD of 5.62±0.36. The glucose and hard candy produced similar cell concentrations with final ODs of 4.29±0.34 and 4.23±0.24 respectively. Chocolate, which was the most complex substrate, produced the second highest cell concentration with a final OD of 5.2±0.31. The starting ODs was determined by blanking each type of waste against their respective media. The mixture which contained equal proportions of the three had a final OD of 5.07±0.12. The starting pHs 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.

OD measurement 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. In terms of upscaling and producing PGA, it is preferred to use a carbon source that is able to produce PGA in the shortest duration possible. The hard candy dissolved completely into the medium with a starting pH of 6.77, which is the closest to the chosen pH 6.5. This acidic property is advantageous as this reduces production costs as less acidic buffer is needed to lower the pH.

Table 4-4 Growth of *B. licheniformis* in multiwell plates after 30 h cultivation

	OD			pH		
Carbon source	**Starting**	**Final**	**STDEV**	**Starting**	**Final**	**STDEV**
Chocolate	0.1	5.2	0.31	6.85	4.93	0.08
Glucose	0.1	4.29	0.34	6.89	4.46	0.03
Hard candy	0.1	4.23	0.24	6.77	4.47	0.06
Marshmallow	0.1	5.62	0.36	6.82	4.2	0.02
Mixture	0.1	5.07	0.12	6.8	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 4-3 represents the sugar profile of hard candy used in this study. A large proportion of the sugars consisted of sucrose. Since sucrose is a disaccharide consisting of glucose and fructose molecules, the remainder of the sugars were the simpler sugars, glucose and fructose. *Bacillus* species are able to break down sucrose by utilising their internal sucrose utilisation system (Zhang et al., 2012).

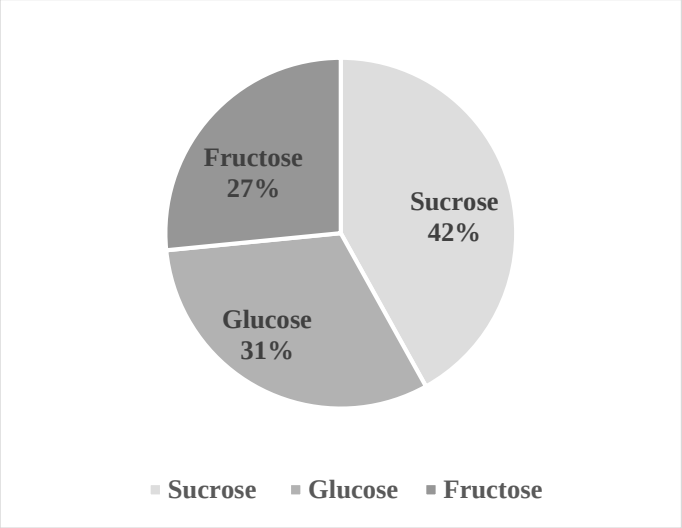

Figure 4-3 Sugar profile of hard candy per gram of waste

4.3.1 Cultivation 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 4-4.

The starting cell concentration within the system was a CDW of 0.056 g/L. The cells entered the exponential phase and 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 remained constant between 10 and 24 h and was 1.40 g/L±0.70. The pH decreased from a starting pH of 6.9 to a minimum pH of 6.2 at the end of the exponential phase, whereafter the pH increased to pH 6.6 in the stationary phase.

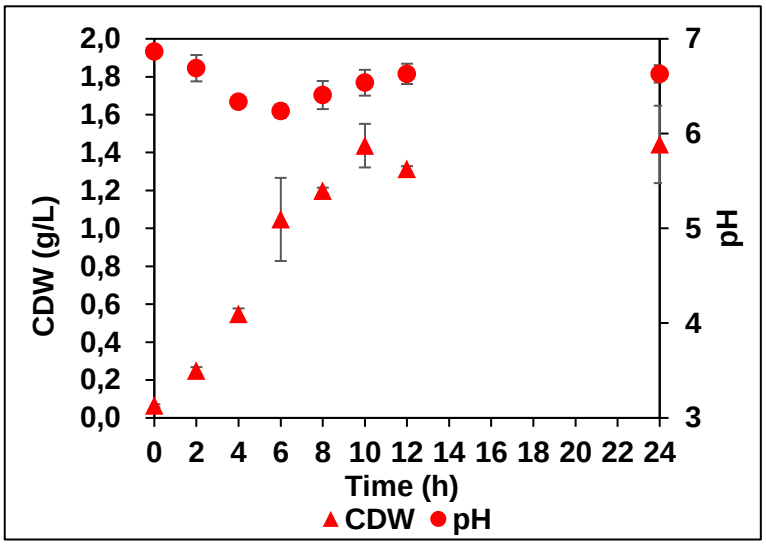

Figure 4-4 Growth of *B. licheniformis* on nutrient-rich medium

4.3.2 Growth of *B. licheniformis* on sucrose

Since candy consisted mostly of sucrose, *B. licheniformis* growth was profiled in shake flasks containing sucrose-supplemented minimal medium ("basal medium" described in Section 3). Sampling was done at 2 h intervals for the first 14 h to monitor growth and physicochemical conditions.

Prior to autoclaving, the basal medium pH was adjusted to a pH of 6.5 using 5 M NaOH. 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 4-5. A steady drop in pH was seen for the next 10 h, where after it remained constant at 5.6 ± 0.2 between 12 and 14 h and increased to 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 ± 0.002, but 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 simple sugars, namely, fructose and glucose (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.

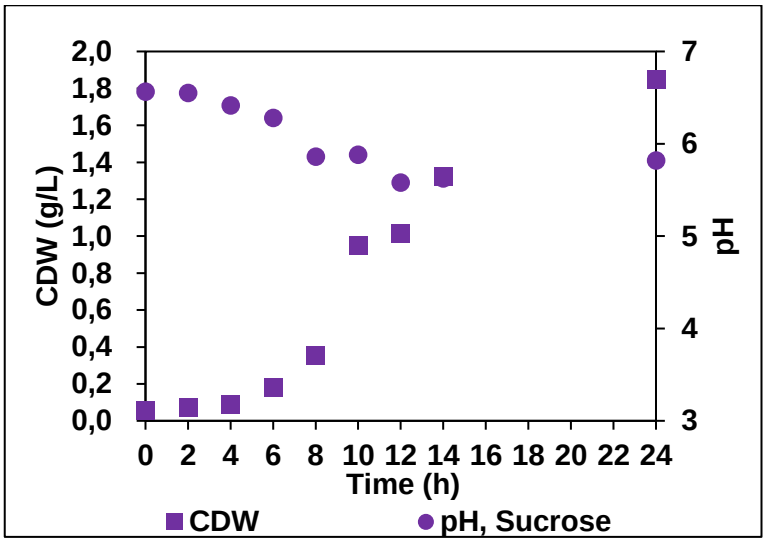

Figure 4-5 Growth *of B. licheniformis* on sucrose in shake flasks (pH is given as (●) while biomass concentration is given as (■))

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

The cultivation of *B. licheniformis* on candy waste followed the preparation procedure stated in Section 4.4.2. Hard candy waste contains acidulents added as flavourants; hence in solution it is 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. 2001). In Figure 4-6, the pH profile followed a similar trend to that of the sucrose shake flask experiments in Figure 4-5. 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.

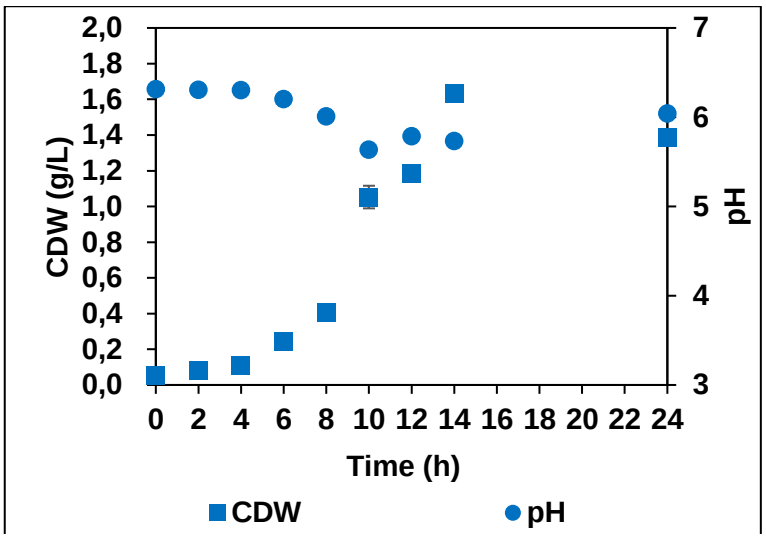

Figure 4-6 Growth of *B. licheniformis* on candy waste in shake flasks (pH is given as (●) while biomass concentration is given as (■))

The comparison of growth kinetics of *B. licheniformis* in shake flasks using different substrates is summarised in Table 4-5. Sucrose yielded the highest biomass with a maximum CDW of 1.85 g/L after 24 h of cultivation, followed by tryptone soy with a CDW of 1.44 g/L. Interestingly, 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±0.01.

Table 4-5 Kinetic parameters for shake flasks

Substrate	Tryptone Soy	Sucrose	Hard candy
CDW (g/L)	1.44±0.20	1.85±0.00	1.38±0.02
μ_{max} (h^{-1})	0.48±0.01	0.32±0.01	0.32±0.02

4.4 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.

4.4.1 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 4-7. 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 (Malthusian growth) is seen under balanced nutrient conditions, after which a decrease in growth rate occurs owing to exhausted nutrients (Shuler & Kargi, 1992). 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 bars 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. The challenge with biological systems is the unpredictability of microorganisms; although parameters were identical during runs, *Bacillus* consumed the sugars a lot faster in one run than the other. Between 10 h and 14 h, the sugars within the system were completely exhausted. During this phase, *B. licheniformis* may start to break down extrapolymeric substances (EPS) such as PGA for growth and maintenance.

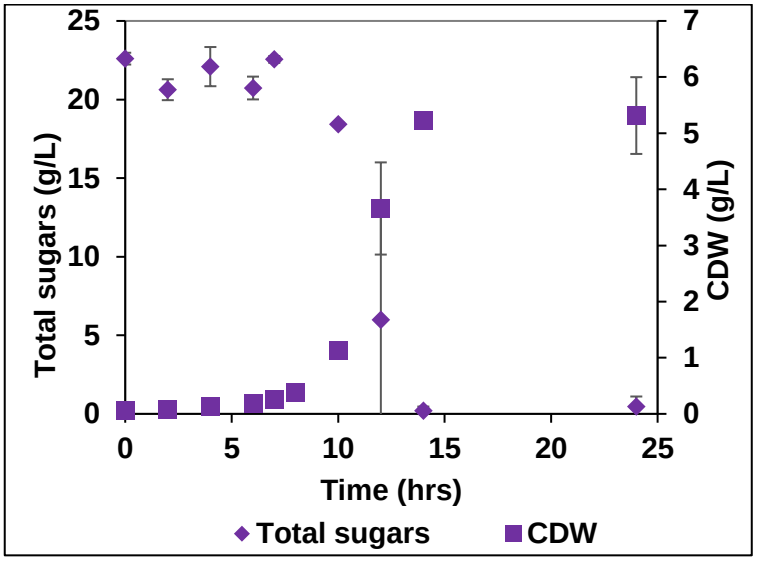

Figure 4-7 Sugar utilisation of *B. licheniformis* on sucrose at pH 6.5, aeration at 1 vvm and 37 °C with agitation starting at 500 rpm. (Biomass concentration is given as (■) and total sugars given as (◆))

In Figure 4-8, 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 14 h, the pH began to increase as the cells entered the stationary phase and organic acid production was complete. There was no pH control to reduce pH in the reactor. 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.

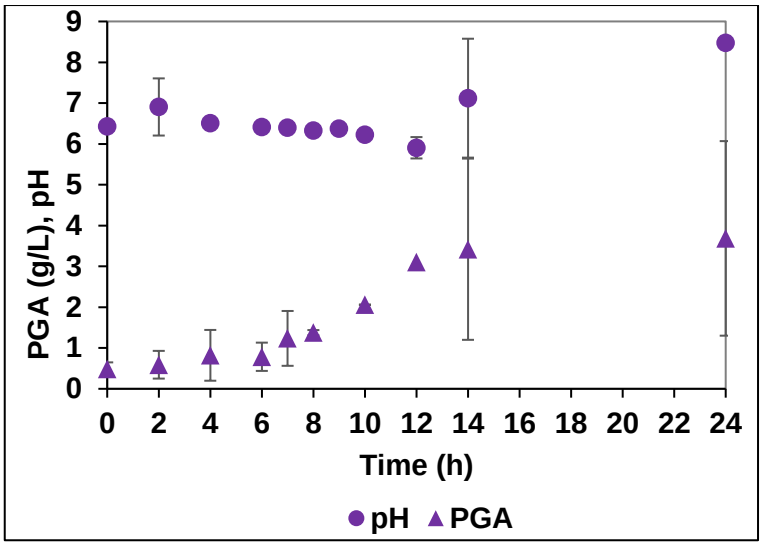

Figure 4-8 PGA formation and pH of a *B. licheniformis* culture grown in sucrose at pH 6.5, aeration at 1 vvm and 37 °C with agitation starting at 500 rpm. (PGA concentration is given as (▲), while pH is given as (●))

The relationship between biomass formation (CDW) and PGA formation during the first 12 h of the cultivation is shown in Figure 4-9. Sucrose as substrate was used to validate the growth-associated classification. A linear relationship was seen, and this suggested that the PGA product was formed in proportion to cell growth. The PGA trend indicated that PGA is growth associated, i.e., as the cell concentration increased in the system, so did the PGA.

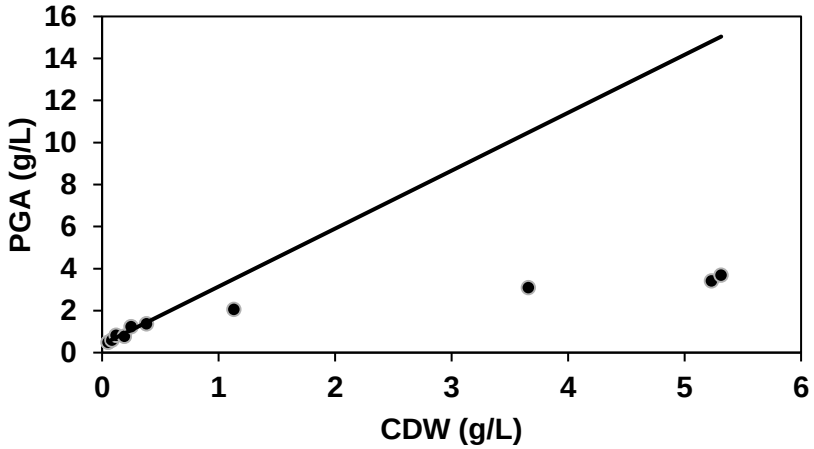

Figure 4-9 Correlation between CDW versus PGA

4.4.2 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. A slightly longer lag phase was seen in Figure 4-10. When the hard candy waste-based medium was prepared, it was very 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. 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 4-10. 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. Interestingly, 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.

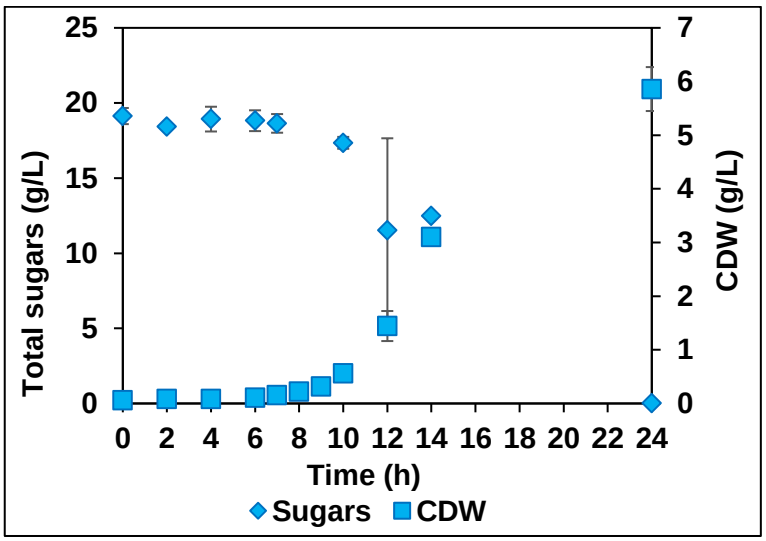

Figure 4-10 Growth of *B. licheniformis* and associated sucrose utilisation when grown on hard candy waste at pH 6.5, aeration 1 vvm and 37 °C with agitation starting at 500 rpm. Total sugar concentration is given as (◆), while biomass concentration is given as (■).

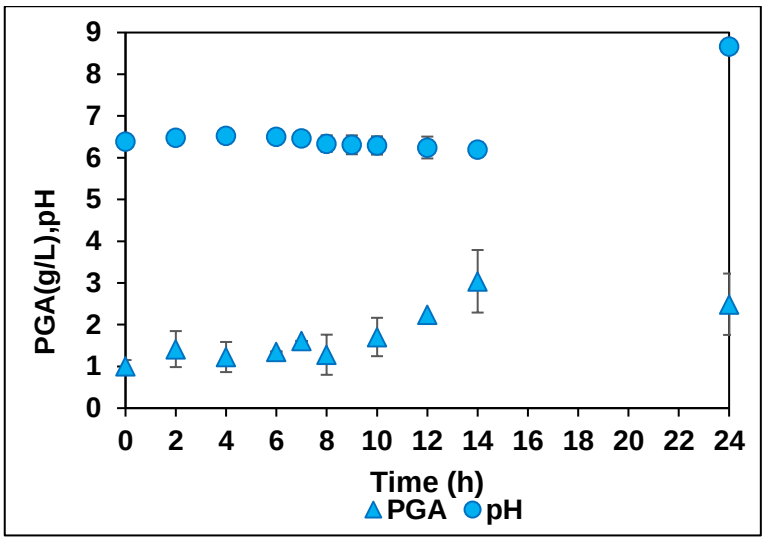

Figure 4-11 pH profile and PGA formation on hard candy waste. (PGA concentration is given as (▲) while pH is given as (●))

A comparitive 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 4-6. More PGA was formed on sucrose thanhard candy.This can be due to the following 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 thereby 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, and other compounds were formed in addition to the PGA. The maximum specific growth rates were similar, signifying that sucrose and hard candy have similar components to support growth and metabolism. 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 4-6 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	0.3±0.01

4.5 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, and 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, and by avoiding PGA consumption on deletion of the carbon source. The aim of the fed-batch was to enhance PGA concentration, and maintain the PGA productivity within the system at a high rate over an extended time period. This was attempted by constantly feeding a mixture of hard candy and NH_4Cl into the reactor at a rate matching their consumption for PGA production.

4.5.1 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 20 g/L of hard candy waste and added to the reactor prior to inoculation. 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 and contained a solution of 60 g/L hard candy and 10.44 g/L NH_4Cl. 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 can be found in Figure 4-12. As expected, 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.

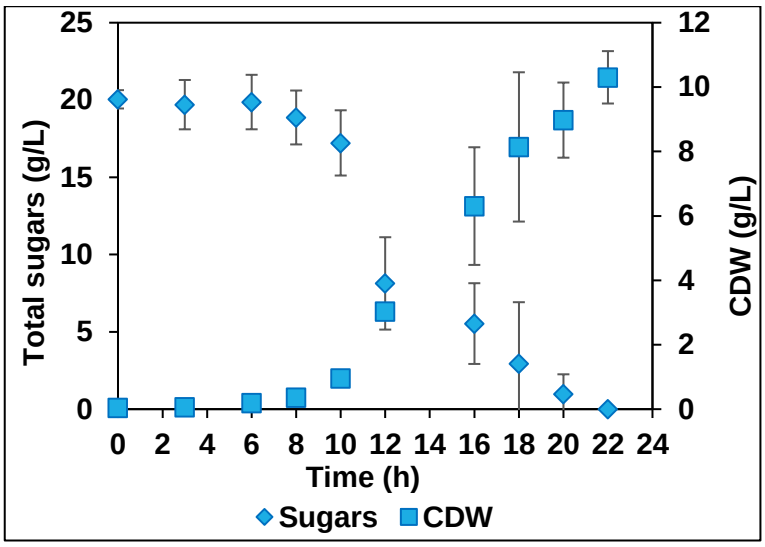

Figure 4-12 Cultivation of *B. licheniformis* on hard candy-supplemented basal medium in fed-batch mode. Sucrose concentration is given as (◆), while biomass is given as (■).

With the continuous feeding of both the carbon and nitrogen source, and a pH maintained at 6.5 using 5M NaOH, the aim of the experiment was to enhance PGA production over an extended period, and at least maintain the PGA concentration within the system of increasing volume. This was achieved by continuously pumping 0.0833 L/h of a mixture of 60 g/L of candy and 10. 44 g/L of NH_4Cl. The pH and PGA profile can be found in Figure 4-13. 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 of feeding, the PGA concentration neither increased nor degraded. The substrate added to the system was converted to biomass and not product. This means that under the fed-batch conditions used, biomass growth was favoured over PGA production. The concentrations in Figure 4-14 did not factor in dilution, owing to extra volume in the reactor, indicating that PGA formation continued at a rate compensating for the culture dilution. It is useful to consider total biomass production, PGA production and sugar utilisation to provide further insights.

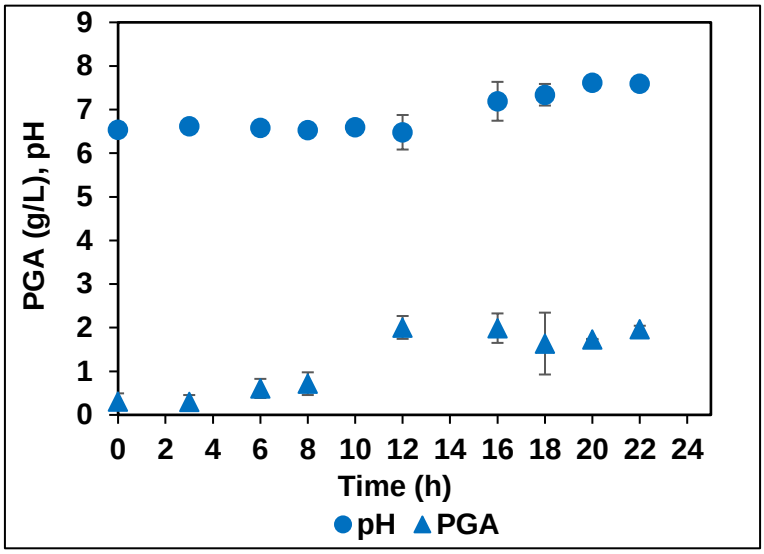

Figure 4-13 pH profile and PGA formation on hard candy waste in fed-batch mode. PGA concentration is given as (▲), while pH is given as (●).

4.6 Integrated discussion

4.6.1 The relationship between substrate consumption and biomass formation

As discussed in Section 4.4.2, a longer lag phase was seen when *B. licheniformis* was cultivated on hard candy waste. It was speculated that the other unidentified compounds in the waste, in addition to the sugar, were the cause of the inhibition. During the characterisation stage of the research, the approach was to identify the nutrients that could be used as nutrients for cell growth as well as PGA formation. Interestingly, a very insignificant amount of trace elements was found. No nitrogen and fats were found in hard candy waste. The HPLC data showed that a large proportion of the waste comprised mostly 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 acidulents (Section 2.1.1) were not quantified.

The general consensus was that confectionery waste can be metabolised by *B. licheniformis*, provided that the correct nutrient supplementation in solution is used. For example, candy waste contains acidulants that lower the pH to 3; this low pH acts as an inhibitor to microbial contamination. After the adaptation phase, the organisms began to break down the sugars at 10 h as depicted in Figure 4-14. 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.

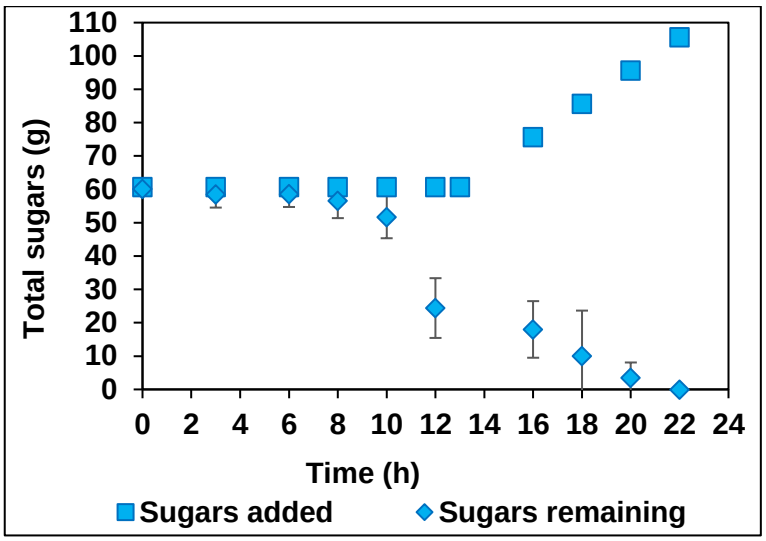

Figure 4-14 Relationship between sugar consumption and sugars added. (Sugars added is given as (■), while sugars remaining is given as (◆)

The accumulation of biomass and PGA on a mass basis is presented in Figure 4-15. 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 of 5 g/h of candy 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 sufficient C:N ratio 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 developing a feeding strategy that will ensure that the sugar concentration within the system is maintained above 6 g/L. 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.

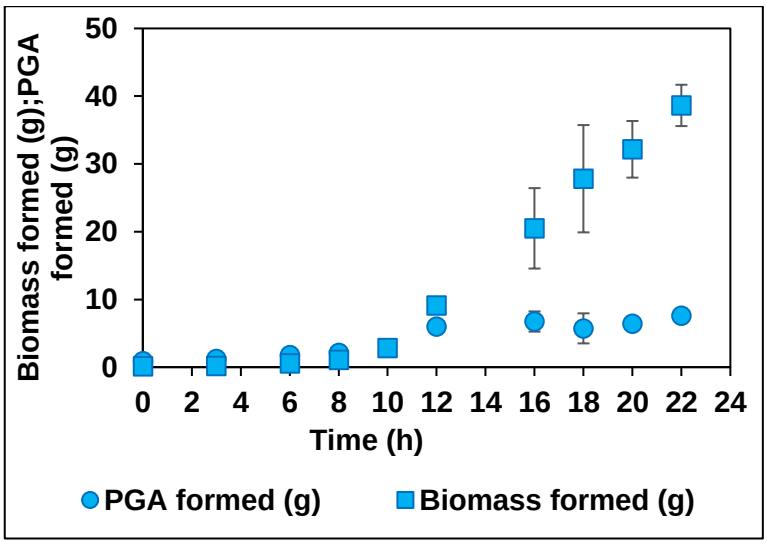

Figure 4-15 Relationship between biomass formed and PGA formed

4.6.2 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 at the end of the cultivation. The composition of the feed had the same C:N as that of the batch feed.

From this addition, 1.37±0.27 g of PGA was formed and an additional 29.54±3.0 g of biomass was formed. The $Y_{X/S}$ for the batch phase at 13 h was 0.15 g biomass/g substrate; and the $Y_{P/S}$ was 0.1 g PGA/g substrate. Interestingly, the dynamics changed during the fed-batch stage; a higher $Y_{X/S}$ of 0.66 g biomass/g substrate was seen, and a relatively 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 was too low and that PGA will be produced, provided the nutrient supply is in excess.

Table 4-7 Comparison between kinetic parameters in batch and fed batch

Parameter	Batch (PhaseI)	Fed batch (PhaseII)
Hard candy added (g)	60.12 ±1.78	105.00±0.49
DCW (g)	9.11±0.11	29.54±3.0
PGA formed (g)	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

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

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 that of glutamate, making it a potential organism of interest for PGA synthesis from waste organic products in the waste arena.

Following characterisation, *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, as candy waste consists mainly of sugars and can be used as a potential carbon source for PGA production when supplemented with a basal medium that contains all of the complementary nutrients needed for optimal cell function and PGA production.

The C_6 sugar is metabolised into two C_3 components, each losing a CO_2 molecule, and are activated to acetyl CoA, which entered the TCA cycle where α-ketoglutaric acid is a key intermediate. The presence of NH_4^+ in the cultivation medium drives the formation of L-glutamic acid, which combines with D-glutamic acid to produce polyglutamic acid. In addition to a carbon and nitrogen source, the key nutrients needed for cell function were a phosphate source and trace elements. 20 g/L of candy waste supplemented with “basal medium” which contained 12 g/L citric acid, 3.48 g/L NH_4Cl, 2.99 g/L K_2HPO_4 and mineral salts (0.5 g/L $MgSO_4.7H_2O$, 0.104°g/L $MnSO_4.H_2O$, 0.04 g/L $FeCl_3.6H_2O$ and 0.15 g/L $CaCl_2.2H_2O$) were selected as the cultivation medium for the production of PGA.

The similarity of growth of *B. licheniformis* on sucrose and sucrose supplied through hard candy waste was seen, with the exception of 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. Alternatively, it may have resulted from the inhibitory acidulants and additives present in the hard candy. 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 aim of the fed batch operation mode was to maintain the exponential growth phase by feeding the system with a constant supply of carbon. During the feeding stage of the fed- batch experiments, a large majority of the substrate fed went to 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 and that 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 needed.

5.2 Recommendations

- Further characterisation studies are recommended to identify the additional nutrient compounds found in candy waste, as it is a complex source. Optimisation experiments are needed to enhance PGA production on confectionery waste.
- A techno-economic feasibility study should be conducted to determine if it is economically viable to produce PGA from confectionery waste. Granted the waste was used as feedstock, a lot of supplementary nutrients were needed to ensure good growth of the organism and PGA production. Furthermore, determination of the minimum supplementary nutrients that need to be added needs investigation. When using waste, one needs to ensure that productivity remains constant.
- Further investigation into finding a wastewater stream high in nitrogen, i.e., corn steep liquor, metal ions, 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 in solution is needed for PGA production. An integration of these effluents will contribute to knowledge in the wastewater biorefinery space.
- Furthermore, the extraction of PGA can be further improved, as precipitation with cold ethanol is effective yet costly.

PGA is a highly versatile compound, and can be used in a range of applications. 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 the breeding of harmful anaerobic methanogens and pathogens responsible for causing harm to the environment. Owing to its hygroscopic nature, it can be used as a soil conditioner as well as a fertiliser. SA is a drought-stricken country and can benefit from the industrialisation of this process, and coupled with other processes in a biorefinery setting, could potentially produce high-value products. The biorefinery concept contributes to seven of the sustainable development goals (SDGs), as it tackles many of the issues relating to (i) infrastructure, (ii) renewable energy, (iii) pollution, (iv) responsible production, (v) bridging the gap between different industries, (vi) promoting a circular economy, and finally, addressing (vii) water and sanitation issues facing the manufacturing industry.

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APPENDIX A: STANDARD PROCEDURES

Table: A-1 Tryptone soy agar

Component	Concentration (g/L)
Tryptone soy	30
Agar bacteriological	15

Table A-2 Composition of 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

A.1 HPLC method for sugars

Standard

A stock solution of a mixture of 10 g/L citric acid, fructose, glucose and sucrose was prepared. The mobile phase consisted of 2 L of 5 mM H_2SO_4 prepared and vacuum filtered through a 0.22 µm filter and degassed in a sonication bath under vacuum for 1 h. The stock solution was diluted into a series of dilutions ranging between 200 mg/L and 1000 mg/L in 2 mL Eppendorf tubes. 1.5 mL of each sample was then syringe filtered into glass HPLC vials using 0.22 µm syringe filters.

Sample

All experimental samples were diluted five times with mobile phase and syringe filtered into HPLC vials ready for analysis.

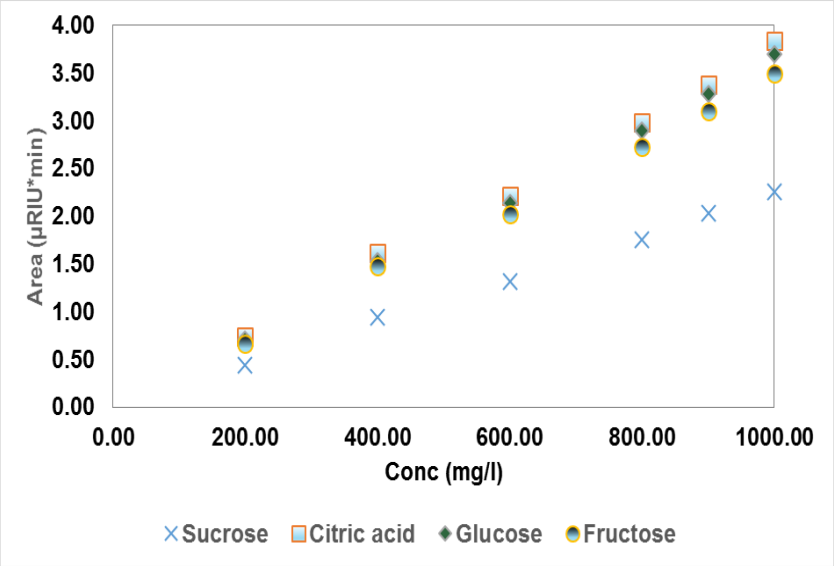


Figure: A-1 Sugars standard curve using HPLC

A.2 ICP-OES (Inductively coupled plasma – optical emission spectrometry)

Digestion of confectionery waste for ICP Place sample combined with 6 mL concentrated hydrochloric acid (HCl), 2 mL concentrated hydrofluoric acid (HF). Cover with express Teflon stopper and leave to digest overnight. Place the vessels inside the MARS-Xpress digestion unit.

A.3 Ninydrin method

Standard curve

A 10 g/L stock solution of glycine was prepared for the standard curve; a series of dilutions ranging between 100 and 1 000 ppm were prepared. 2 mL samples of each were added to test tubes. 1 mL of ninhydrin reagent was added to each test tube. The test tubes were then covered with test tube caps and transferred to a water bath containing water set at a temperature of 98°C for 16 minutes and then removed. The samples were left to cool at room temperature and 5 mL dilution solution were added to each test tube. The samples were transferred to cuvettes and read on a Thermo spectrophotometer at an absorbance of 575°nm.

A.4 PGA quantification

Standard

A 1000 mg/L stock solution of PGA was prepared in pH 7 phosphate buffer in a volumetric flask and refrigerated overnight. For the standard curve preparation, a series of dilutions ranging between 100 and 1 000 ppm were prepared for the standard curve below:

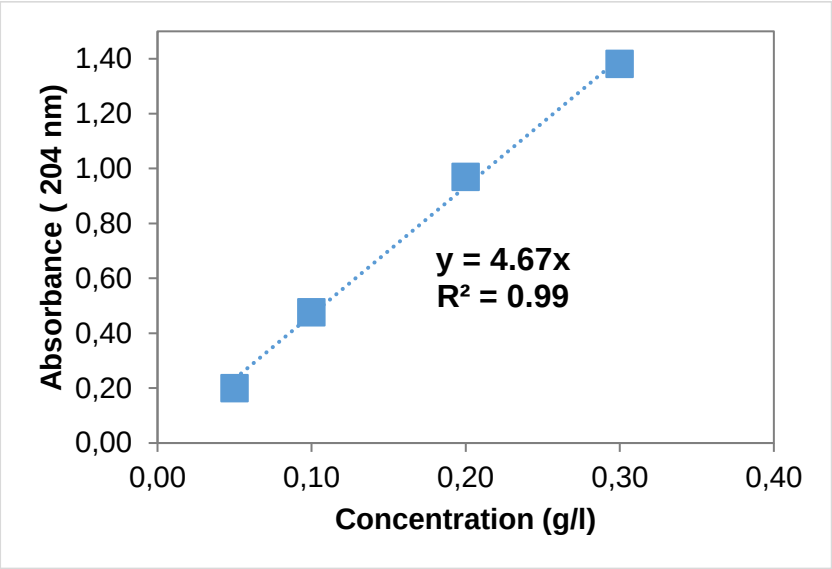


Figure 5-1 PGA standard curve

Sample

γ-PGA was gravimetrically extracted from the culture medium and concentration was read at an absorbance of 197 nm on a Thermo Helios UV spectrophotometer, a method developed by (Zeng et al., 2012). The culture was diluted with 5 v/v distilled water. The dilute culture broth was centrifuged at 13 400 g for 20 minutes. An aliquot of the supernatant (0.3 mL) was combined with 3 parts cold ethanol (1.2 mL), where the polymer precipitates and is centrifuged at 13 400 g for 20 minutes. The precipitate was left to dry in the solvents fume hood. After weighing the dry pellet, it was resuspended into phosphate buffer, pH 7 (Zeng et al., 2012).

APPENDIX B: CALCULATIONS

Multiwell calculations

Citric acid ($C_6H_8O_7$; mw = 192.1 g/ mol

(12 x 6)/ 192.1 = 0.37 g C/g citric acid

Carbon in 12 g/L citric acid = 12 x 0.37 = 4.44 g C/L

Glycerol ($C_3H_8O_3$; mw = 92.09 g/mol)

(12 x 3)/ 92.09 = 0.39 g C/ g glycerol

Carbon in 1 g/L glycerol = 1 x 0.39 = 0.39 g C/L

Glucose ($C_6H_{12}O_6$; mw = 180 g/mol)

(12 x 6)/ 180 = 0.4 g C/ g glycerol

Carbon in 20 g/L glucose = 0.4 x 20 = 8 g C/L

Total carbon = 8 + 4.44 + 0.39 = 12.83 g C/L

Ammonium chloride (NH_4Cl); mw=53,491g/mol

(14/53.491)=0.262 g N/g ammonium chloride

0.262x3.48=0.91 g N/L

NH_3

(17/53.491)=0.32 g NH_3/g ammonium chloride

0.32x3.48=1.1 g NH_3/L

Required C:N ratio: 12.83:0.91=14:1

***Note* Candy was assumed to be the limiting substrate when doing the calculations**

Batch calculations

Within a batch system the following equations are applicable

$$\sum S + X \rightarrow \sum P + nX$$

$$Substrate + cells \rightarrow extraellular\ products + more\ cells$$

The net specific growth rate (μ) is a function of the number of cells reproduced and can mathematically be determined by:

$$\frac{dX}{dt} = \mu_{net} X$$

$$\frac{dX}{dt} = \mu_{net} X$$

Integrate:

$$\int_0^X \frac{1}{X} dx = \mu \int_0^t dt$$

$$lnX - lnX_0 = \mu t - t_0$$

$$\therefore ln\frac{X}{X_0} = \mu_{net} t$$

$$lnX = \mu_{net} t + lnX_0$$

$$X = X_0 e^{\mu t}$$

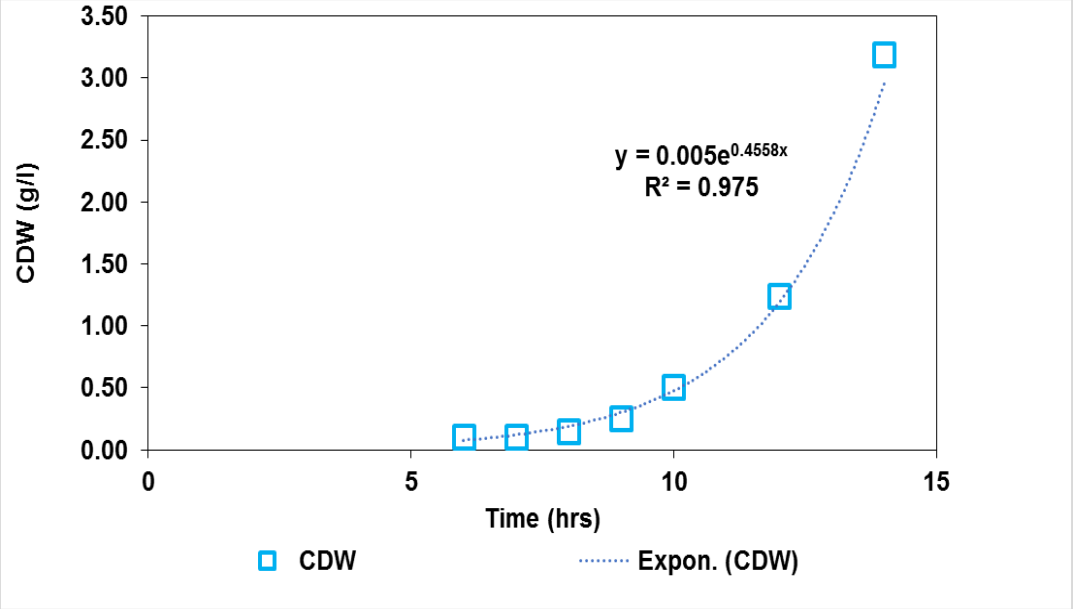
where:

$$\mu_{net} = net\ specific\ growth\ rate\ (hr^{-1})$$

$$X_o = cell\ concentration\ (g/l) at\ to$$

$$X = cell\ concentration\ (g/l) at\ to$$

The exponential function was fitted to the biomass curve as depicted below. This procedure was followed when determining the μ_{max} values for all growth experiments.



Yield

The yield coefficients were calculates as follows:

*Note: Yield coefficients were calculated based on the maximum PGA calculation (14 h).

	Candy		PGA		CDW	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
Time (h)	Concentration (g/L)		Concentration (g/L)		Concentration (g/L)	
0	19,51	19				
14	8,34	9,64	8,34	9,64		
24	0,04	0,00	0,04	0,00	5,8	6,15

Biomass

$$CDW\ formed\ at\ 24\ h\ (g/l) = \frac{(\ 5.80\ g/L + 6.15\ g/L)}{2} = 5.98 \pm 0.25$$

Candy

$$Candy\ concentration\ at\ 0\ h\ (g/L) = \frac{(19.51\ g/l + 19.00\ g/l)}{2} = 19.26 \pm 0.36\ g/L$$

$$Candy\ concentration\ at\ 14\ h\ (g/L) = \frac{(8.34\ g/L + 9.64\ g/L)}{2} = 8.99 \pm 0.92\ g/L$$

$$Ratio\ of\ biomass\ formed{:}\ candy\ = \frac{5.98}{19.26} = 0.31 \frac{g\ biomass}{g\ candy} = Y_{X/S}$$

PGA

$$PGA\ concentration\ at\ 14\ h\ (g/L) = \frac{(2.51\ g/L + 3.57\ g/L)}{2} = 3.04 \pm 0.75$$

$$PGA\ concentration\ at\ 24\ h\ (g/L) = \frac{(1.97\ g/L + 3.01\ g/L)}{2} = 2.49\ \pm 0.74$$

$$Ratio\ of\ PGA\ formed{:}\ candy\ \ in\ \ batch\ (14\ h) = \frac{3.04}{19.26 - 8.99} = 0.30 \frac{g\ PGA}{g\ candy} = Y_{P/S}$$

$$Ratio\ of\ PGA\ formed{:}\ candy\ \ in\ \ batch\ (24\ h) = \frac{2.49}{19.26} = 0.13 \frac{g\ PGA}{g\ candy} = Y_{P/S}$$

Fed batch calculations

The values below were obtained from experimental data:

Feed composition: 60 g/L candy and 10. 44 g/L NH_4Cl

Feed rate= 5 g/hr=0.083 L/hr

Feed continued for 9 hrs ∴ 0.75 L was fed

Starting reactor volume: 3 L

Reaction volume after cultivation: 3.75 L

	Candy		PGA		CDW	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
Time (h)	Concentration (g/L)		Concentration (g/L)		Concentration (g/L)	
0	19.62	20.46				
13	10.25	15.7	1.82	2.19	3.06	3.01
22	0.00	0.00	2.02	1.92	10.88	9.73

Batch:

Biomass

$$CDW\ formed\ at\ 13\ h\ (g) = \frac{(3.06 g/L + 3.01 g/L) * 3L}{2} = 9.11 \pm 0.11$$

PGA

At 13 h, the average PGA formed in g:

$$PGA\ formed\ at\ 13\ h\ (g) = \frac{(1.82 g/L + 2.19 g/L) * 3L}{2} = 6.02 \pm 0.78$$

$$Ratio\ of\ PGA\ formed: candy\ \ in\ \ batch = \frac{6.02}{60} = 0.1 \frac{g\ PGA}{g\ candy} = Y_{P/S}$$

$$Ratio\ of\ biomass\ formed: candy\ added\ in\ batch = \frac{9.11}{60} = 0.15\ \frac{g\ biomass}{g\ candy} = Y_{X/S}$$

Fed batch:

Biomass

$$CDW\ formed\ at\ 22\ h\ (g) = \frac{\left(10.88\ \frac{g}{L} + 9.73\ g/l\right) * 3.75\ \ L}{2} = 38.64 \pm 3.05$$

Sugar added $= 5\ \frac{g}{h} \times 9\ h = 45\ g$

PGA

$$PGA\ formed\ at\ 22\ h\ (g) = \frac{\left(1.92 \frac{g}{L} + 2.02\ g/L\right) * 3.75 L}{2} = 7.39\ \pm 0.27$$

$$PGA\ formed\ during\ the\ feeding\ stage\ (g) = 7.39 - 6.02 = 1.37\ g\ PGA$$

$$Biomass\ formed\ during\ the\ feeding\ stage\ (g): 38.64 - 9.11 = 29.54\ g\ biomass$$

$$Ratio\ of\ PGA\ formed: candy\ fed\ in\ fed\ batch = \frac{1.37}{45} = 0.03 \frac{g\ PGA}{g\ candy} = Y_{P/S}$$

$$Ratio\ of\ biomass\ formed: candy\ fed\ in\ fed\ batch = \frac{29.54}{45} = 0.66 \frac{g\ biomass}{g\ candy} = Y_{X/S}$$