Selection of bacterial species from wastewater for potential production of poly (γ-glutamic acid): isolation, characterisation and growth kinetics

By

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SYNOPSIS

Wastewater treatment plants represent a source of nutrients for microbial growth and product formation. In an approach in which bioresource productivity is maximised, it is desirable to not only achieve water treatment to the desired standard, but at the same time to harness the value in these resources. Wastewaters are a source of nutrients such as carbon, nitrogen and phosphates. Macro-nutrients typically comprise the major share of the operating costs of commodity bioprocesses, such as the production of alcohols, organic acids and polymers. The nutrient loads in municipal wastewaters are dilute, but add up to significant daily loads because of the massive volumes generated in urban populations.

The effluent of most wastewater treatment plants in South Africa is released back into either rivers, lakes or the ocean without reuse. For many, there is a concomitant failure to comply with the country's effluent requirements. This has resulted in the need to move towards more sustainable water treatment systems, including more efficient and innovative solutions to treat wastewater. Additionally, by recovering value from waste, there is potential to improve plant operation; releasing water which is better treated, more compliant and can be reused, while simultaneously improving resource productivity and minimising environmental burden; thereby changing the economics of the wastewater treatment plant. This includes the recovery or the production of valuable resources, whilst in turn recovering clean water. Bioprocessing to reduce these nutrient loads in wastewater while producing a range of byproducts have conventionally included biogas and compost, produced with minimal modification of existing plants.

In extending the potential product range of these 'wastewater biorefineries', key design factors include using waste resources in a non-sterile environment, thus requiring a positive selection pressure for the product of interest, and ideally producing a product readily separated from the wastewater through a phase change such as precipitation. Stress and storage polymers satisfy both these requirements.

In this project, we explore such a wastewater biorefinery approach in which we aim to use the nutrient component of partially treated domestic wastewater for the production of poly (γ-glutamic acid) (γ-PGA).

γ-PGA, a polymer of D- and L-glutamic acid monomers connected by amide linkages, is a naturally occurring biopolymer, synthesized by a variety of micro-organisms. Most commonly, γ-PGA production has been studied in *Bacillus* species, such as *B. subtilis* and *B. licheniformis*. *Bacillus* species are ubiquitous in the environment, including an association with domestic wastewater treatment. Its enrichment has been associated with improved treatment processes. The favourable properties of this very promising polymer include its water solubility, anionic nature, biodegradability and non-toxicity towards humans, animals and the environment. Potential applications of γ-PGA are reported in the medical, food, cosmetic, wastewater treatment, plastic,agricultural and textile industries. In this project, we consider the production of γ-PGA by *Bacillus*-enriched species for the

partial treatment of domestic wastewater and concomitant production of the polymer for soil improvement and water treatment.

To design and optimise a process using wastewater for γ-PGA production, it is important to understand the growth kinetics of *Bacillus*-like microorganisms that can be found in a domestic wastewater treatment plant.

The base case for growth, substrate utilisation and biomass production has been presented for a *Bacillus licheniformis* type culture strain in this dissertation. In presenting this base case, a complete experimental methodology using both shake flasks and deep well plates is developed and appropriate analytical protocols selected.

The second objective of this project was to characterise the growth kinetics of the microorganisms isolated from the wastewater obtained from the Mitchell's Plain wastewater treatment plant. Following enrichment of wastewater samples collected, 18 isolates were obtained and characterised in terms of morphology. Further, using DNA sequence data for the enzymes involved in PGA synthesis, primers were designed to identify strains carrying γ-PGA synthesis potential through molecular biology studies. Two different media; namely Medium E, containing glutamic acid, citric acid and glycerol as carbon sources, as well as a modified Medium E, in which the glutamic acid was replaced with glucose, were used for these experiments. Isolates showing reproducible growth and evidence of polymer production were selected for detailed screening in terms of growth. Based on this, six isolates were chosen. These experiments were performed in both shake flasks as well as deep well plates with the growth kinetics and biomass production by the various strains carefully analysed. Biomass concentrations varied from 2 to 8 $g.L^{-1}$ while specific growth rates varied from 0.11 to 0.26 h^{-1} in these cultures. The results of the growth studies showed that the growth rates and biomass production of the different isolates varied greatly under identical cultivation conditions. Cultivation of the isolates in deep well plates generally yielded higher biomass concentrations in comparison to shake flask cultivation. A quantitative analysis of the data on carbon substrate utilisation in the media showed that glucose was the preferred of these, most consumed by the isolates for cell growth.

Preliminary media optimisation was undertaken to identify optimal C:N:P ratios in the modified growth Medium E and to assess the impact of these medium components by using two-level Plackett-Burman factorial design. The dependence of biomass accumulation on C levels and maximum specific growth rate on N is illustrated. Response surfaces revealed the insignificant effect on the microorganism growth of varying P concentrations. Influence of P was attributed when P acted in tandem with C and N.

Identification of the isolates selected for detailed screening was confirmed using *16S rRNA* sequencing. There were two major families present – Proteobacteriaceae and Enterobacteriaceae – which grouped accordingly when analysed phylogenetically. The *Bacillus*, and opportunistically pathogenic enteric bacteria *Klebsiella* and *Enterobacter*, were reflective of genuses which have a high

probability of being present in a treatment plant which predominantly treats domestic wastewater containing high sewage loads, such as Mitchell's Plain.

A suitable reactor configuration, capable of treating wastewater and simultaneously producing γ-PGA, was proposed for integration into Mitchell's Plain wastewater treatment plant after studying the selected *Bacillus* isolate in a laboratory-scale bioreactor in optimal medium. The sustained biomass production and growth rate, even at lower medium carbon inputs, shows a great potential for application by enrichment, growth and product production of this *Bacillus subtilis* strain in a wastewater treatment plant. Although this work was able to make a substantial contribution to the current knowledge of microbial growth kinetics in various media and bioreactor systems, the study limitations related to growth profiling in more dilute and variable nutrient concentrations, non-sterile environments and mixed culture dynamics are acknowledged. These present a scope and opportunity for further research in this exciting field.

Analysis of extracted and purified γ-PGA showed a polymer composed of a combination of polysaccharides and protein (in roughly equal proportions in three of four of the isolates) in which histidine, homoserine and glutamic acid were typically the most dominant amino acids. The impure nature of this γ-PGA remains suitable for wastewater applications, but not for areas which require a product composed of specific, high molecular weight stereoisomers such as the medical industry. This study illustrates the importance of developing new experimental techniques for more γ-PGA-specific purification and improved analysis. Further it demonstrates the shortcoming of the gravimetric analysis of the crude extract typically reported in the literature.

The findings of this project are intended to be used in a system which applies wastewater as an economical and sustainable source of nutrients with the aim of producing valuable products through bioprocessing applications.

DECLARATION

I know the meaning of plagiarism and declare that all of the work in the document, save for that which is properly acknowledged, is my own.

Signed by candidate

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Ziningi Charity Madonsela

September 2013

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NOMENCLATURE

BOD	-	biological oxygen demand [$mg.L^{-1}$]
CCT	-	city of Cape Town
CDW	-	cell dry weight [$g.L^{-1}$]
COD	-	chemical oxygen demand [$mg.L^{-1}$]
D	-	dilution rate [hr^{-1}]
DF	-	degrees of freedom [-]
EDTA	-	Ethylenediaminetetraacetic acid
F	-	F- value [-]
HPLC	-	high performance liquid chromatography
K_La	-	oxygen mass transfer co-efficient [g O2.hr^{-1}]
K_s	-	substrate saturation constant [$g.L^{-1}$]
Mn	-	number average molecular weight [Daltons]
MS	-	mean of squares [-]
Mw	-	weight average molecular weight [Daltons]
OD	-	optical density [-]
OTR	-	oxygen transfer rate [$g.L^{-1}.hr^{-1}$]
OUR	-	oxygen utilisation rate [$g.L^{-1}.hr^{-1}$]
p	-	p-value [-]
P	-	product concentration [$g.L^{-1}$]
PD	-	polydispersity index [-]
Q	-	volumetric flowrate [$L.hr^{-1}$]
R^2	-	co-efficient of determination
S	-	substrate concentration [$g.L^{-1}$]
S_i	-	initial substrate concentration [$g.L^{-1}$]
SS	-	sum of squares [-]
t	-	time
TOC	-	total organic carbon [$mg.L^{-1}$]
X	-	biomass concentration [$g.L^{-1}$]
X’	-	extended biomass concentration [$g.L^{-1}$]
X_i	-	initial biomass concentration [$g.L^{-1}$]
X_{max}	-	maximum biomass concentration [$g.L^{-1}$]
μ	-	growth rate [hr^{-1}]
μ_{max}	-	maximum specific growth rate [hr^{-1}]
WWTW	-	wastewater treatment work
$Y_{X/S}$	-	biomass yield [$g.g^{-1}$]

1. INTRODUCTION

1.1 Project scope and context

South Africa is classified as a mostly semi-arid country by the United Nations Environmental Programme. Statistics provided by the Water Research Commission estimates South Africa's average annual rainfall to be 500 mm, with 21% of the country receiving less than 200 mm - a figure which falls significantly below the world average of 860 mm. The increasing demand for already strained water resources is due to population growth (7 million increase between censuses 2007 and 2011), improving living standards and the government's mandate to provide access to clean and safe water and sanitation for all citizens, further driven by growing unrest accompanied by widespread service delivery protests (StatsSA, 2012).

The City of Cape Town (CCT) operates more than 20 public wastewater treatment works (WWTW). These plants treat a combined total of more than 567 ML of bulk wastewater per day (CCT, 2009). According to the Department of Water Affairs (DWA), the Western Cape provincial average risk rating was 61.1% in the 2011 Green Drop Report, falling within the medium risk category as defined by the ministry. The Green Drop is an incentive-based certification programme for wastewater quality management regulation in South Africa. The analysis of these risk profiles are based on defined assessment areas which include: technology, the ratio of design capacity ($ML.day^{-1}$) to daily operation, effluent quality compliance and the technical skills available. Based on these assessments, the province was named the best performing and awarded first place overall in the country in June 2011. The score in the April 2012 progress report showed a score of 51.5%, an improvement of almost 10%. Cape Town's metropolitan municipality was classified as a low risk area with a cumulative risk rating of 49% in 2012. The municipality was also included amongst provincial and national leaders in terms of performance and progress in risk abatement. Whilst 20 out of the 27 plants listed under this municipality showed improvements between the 2011 and 2012 audits, the highest risk contributors were due to poor effluent quality or lack of monitoring (DWA, 2012).

The Mitchell's Plain WWTW is situated in the CCT. This WWTW was originally designed to be able to treat 45 $ML.day^{-1}$, but currently treats 37.5 $ML.day^{-1}$ bulk wastewater from Mitchell's Plain and the surrounding Mandalay and Merrydale catchment areas. In 2005, the plant used conventional activated sludge process with mechanical dewatering of sludges before anaerobic digestion, followed by a maturation pond system. The plant was also designed for partial denitrification and biological phosphorus removal using older technology (CCT, 2006) and the original UCT process for municipal wastewater treatment (Dold *et al.*, 1980; Van Haandel *et al.*, 1981). This plant had a risk rating of 55.6% in 2012, improving 8% from 2011 (DWA, 2012). The report highlighted effluent compliance as a high risk area as all the effluent from this plant is released back into the environment, not re-used. The same is true for 15 of the 26 plants in Cape Town (CCT, 2006).

Water is a precious and scarce resource which needs to be conserved. The rapid urbanisation of major cities in South Africa and the population growth rate places our resources under severe pressure, making delivery of even the basic of services a considerable challenge (Mukheibir and Ziervogel, 2007). Domestic and industrial wastewaters treated by most municipalities are either pumped to an off-site location for unknown uses, or into released back into rivers, lakes or the ocean. Treated effluent can then be used for non-potable purposes, such as irrigation and groundwater recharge (CCT, 2006), but documented case studies of such direct uses in South Africa are scant.

Further, these wastewaters contain a nutrient load for removal while provision of similar nutrients for bioproduction of commodity products forms a considerable portion of their variable cost. This has resulted in the need to investigate more efficient solutions to treat wastewater as well as to evaluate the potential of value recovery from wastewater through use in bioenergy production or as a source of nutrients for bioprocesses (Burton *et al.*, 2009), whilst in turn recovering clean water at a reduced cost and ensuring increased resource productivity through the wastewater biorefinery concept (Zheng *et al*., 2008; Zhong *et al*., 2009). Suitable bioprocesses are those producing commodity products or bioenergy and able to function in a dilute environment.

Various *Bacillus* strains are found naturally occurring in wastewater; these are one of the genuses capable of producing poly-(γ-glutamic acid). γ-PGA has been identified as a possible product for production as a by-product of the proposed wastewater bio-refinery. This naturally occurring anionic polymer is biodegradable, edible and non-toxic towards humans and the environment. γ-PGA has a number of potential applications in food and skin-care products, fertilizers, water retention, wastewater treatment, bioplastics, tissue engineering and drug conjugates (Huang *et al.*, 2011), stimulating its development as production. Although γ-PGA has favourable properties resulting in renewed interest in the biopolymer, raw material costs cause considerable challenge for its economic production on a large industrial scale.

Thus, this project aims to evaluate the suitability of wastewater as a substrate for bacterial species to produce biomass and γ-PGA in a sustainable manner by developing an understanding of the key bioprocessing parameters which affect efficient bacterial growth and product formation. On the basis of such an understanding it is intended to make proposals of a design of a process which incorporates enriched bacterial cultures into existing WWTW which contain dilute nutrient sources.

1.2 Research objective and aims

The objective of this project was to investigate the potential for production of poly (γ-glutamic acid) (γ-PGA) using domestic wastewater and to assess whether suitable microbial species are naturally present in the WWTW. Furthermore, the evaluation of the kinetics of microbial growth would be used to develop sufficient knowledge of performance to inform a suitable process design for the WWTW.

The project aims were:

i. To determine the growth kinetics of *Bacillus licheniformis*

ii. To understand the basic growth nature of microorganisms isolated from the Mitchell's Plain wastewater treatment plant for the purpose of γ-PGA production

iii. To determine a suitable growth matrix through calculation and to test this experimentally

iv. To optimise the medium and environmental requirements for optimal biomass and γ-PGA production, and

v. To design a suitable experiment for a continuous bioreactor which uses wastewater as a source of nutrients using the growth kinetics of the chosen strain

1.3 Thesis outline

Chapter 2 presents the literature review, including background information on the properties of γ-PGA and its attraction as a polymeric bio-product. The microorganisms reported to be capable of producing γ-PGA, their general requirements and the various processes used are described. A special focus is placed on suggested optimisation strategies, and current knowledge gaps are identified. The prospect of utilising wastewater as a substrate, and associated requirements, are also discussed. All experimental protocols, including the materials, equipment and analytical techniques used are described. The experimental approach and rationale behind certain experimental approaches are also briefly explained in Chapter 3. The first section of the results and discussion is contained in Chapter 4. This chapter specifically focuses on the growth kinetics of *Bacillus licheniformis*. The growth rate, biomass, substrate utilisation and effect on pH over the cultivation period are reported. All experiments were conducted in shake flasks. Chapter 5 reports on the basis of the selection of the microorganisms isolated from the Mitchell's Plain WWTW and the screening results for the evaluation of various characteristics of the selected isolates. These results were used to provide insights on promising strains which could be further studied in more detail. In addition to growth screening in two chemically-defined media, which was similar to that of *B. licheniformis*, molecular biology analysis was used as a confirmatory tool for the selection of these promising strains. Six strains were then further screened and monitored in more detail. Additional growth kinetics such as the maximum specific growth rate (μ_{max}) and maximum biomass are presented and the two media and screening systems of

shake flasks and deep well plates are compared in Chapter 6. Chapter 7 contains the results and discussion of the media optimisation studies for the selected isolates using various carbon, nitrogen and phosphorus ratios. The effect of these on the bacterial growth in deep well plates and available substrates is analysed in detail. The results and discussion of the initial bioreactor studies which were performed in an effort to scale-up from the deep well plates micro bioreactors and strictly monitor the operation are presented in Chapter 8. The medium used was based on the optimisation studies. Kinetic parameters are presented for Isolate 1, which was selected for these experiments, and compared to the results obtained in deep well plates and previous studies found in the literature. A suitable bioreactor system design based on the findings of this study is proposed. Extracted and purified γ-PGA from *B. licheniformis,* Isolate 1 and Isolate 7 was quantified and characterised in terms of molecular weight and composition and this is reported in Chapter 9. The final results and discussion chapter, 10, reveals the identities of strains isolated in the initial stages of the project and selected for further investigations. Any phylogenetic relationships between the identified strains are also determined. Chapter 11 contains a summary of all the findings and linking these to the outlined objectives and aims of the study, the conclusions drawn and the general recommendations made from this study.

2. LITERATURE REVIEW

2.1 Introduction

Wastewater treatment works (WWTWs) in South Africa present a renewable resource for the recovery of energy and valuable products (Burton *et al.*, 2009). Traditionally, this harnessing of wastewater biomass or effluent has been focused on technologies for clean and renewable fuel production such as biogases, bioethanol and hydrocarbons (An *et al.,* 2003 ; Bain and Craig, 1988; Banerjee, 2007, Ito *et al.,* 2005).

The efficient operation of WWTWs has been subject to widespread scrutiny by various non-governmental stakeholders. The challenges encountered in this sector are especially highlighted in reports published by municipalities (CCT, 2006, 2009), which has led to the South African National Water Ministry to introduce interventions such as the Green Drop Programme (DWA, 2011). Applied research in this area may facilitate improved performance with the integration of wastewater treatment not only with energy recovery, but also for the recovery of valuable bio-products. Plants treating wastewater containing primarily high loads of sewage loads were identified by Burton *et al.* (2009) as an area with one of the greatest potential for research and innovation. The authors acknowledged that the exploitation of wastewater as a renewable resource has been poor, especially in South Africa. The successful use of this valuable renewable resource could not only contribute to the sustainable economic growth of the country derived from the trading of the secondary by-products produced, but also has the potential to improve the processing operations of WWTWs, resulting in effluent which can be reused, thus reducing the burden currently placed on potable water.

Microbial biopolymers produced from renewable sources are gaining increasing interest. These biomaterials could potentially serve as a replacement of petroleum-based commodity products in the future. Their potential applications range from high value drug delivery and tissue engineering to lower value agricultural and wastewater treatment applications, which require less stringent biopolymer properties such as purity and stereoisomer composition.

Poly amino acids are composed of repeating units of a single amino acid. Three poly-amino acids are reported, namely, poly (γ-glutamic acid) (γ-PGA), poly (ε-lysine) (ε-PL) and cyanophycin (Shi *et al.*, 2007). Poly (γ-glutamic acid) (γ-PGA) is anionic, biodegradable, water soluble, edible biopolymer which is naturally synthesized by various microorganisms.

Although γ-PGA has favourable properties which have led to renewed interest in the biopolymer, it is still a considerable challenge to produce in an economically feasible manner on a large industrial scale. Although concentrations may be variable, domestic wastewater streams typically contain high loads of COD, nitrogen and phosphates. The inefficient plant operations which result in a lack of compliant effluent, as well as the high cost associated with γ-PGA has resulted in the need to

investigate more efficient solutions to treat wastewater as well as to evaluate the suitability of wastewater as a substrate for bioprocesses.

To design and optimise a process for γ-PGA production, it is important to understand the growth kinetics of the various *Bacillus* strains, many of which are ubiquitous in the environment, including wastewater (Bramucci and Nagarajan, 2000; Buthelezi *et al.*, 2009). Additionally, an improved level of understanding of the challenges of using minimal media as a source of nutrients combined with understanding the key bioprocess parameters influencing γ-PGA production will allow for efficient optimisation of this system (Richard and Margaritis, 2004), providing the information required to design of a cost-effective and sustainable process with nutrient sources such as dilute wastewater streams.

2.2 The biopolymer γ-PGA

Poly (γ-glutamic acid) (γ-PGA) is a naturally occurring polyamide comprised of L- and D-glutamic acid isomers polymerized by α-amino and γ-carboxyl amide linkages (Jeong *et al.*, 2010). This unusual polyamide, shown in Figure 2.1, was first discovered by Ivánovics and colleagues in 1937 as part of a capsule of *Bacillus anthracis* (Ivánovics and Bruckner, 1937; Ivánovics and Erdös, 1937).

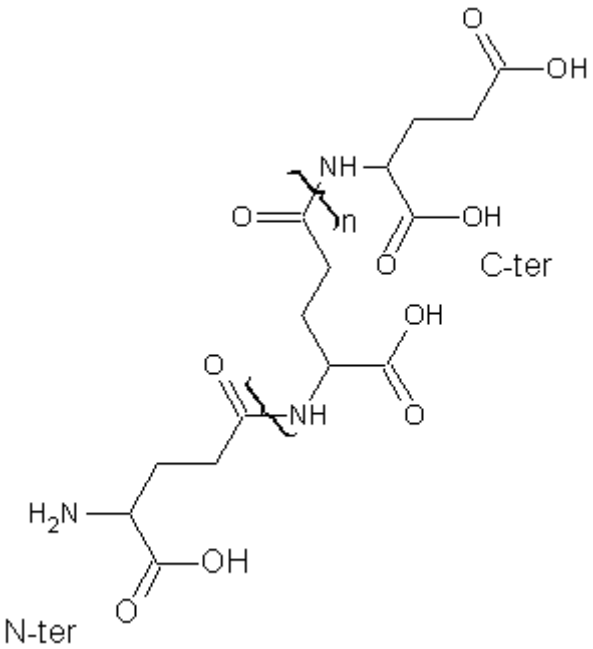

Figure 2.1: The structure of poly (γ-glutamic acid) (modified from Candela and Fouet, 2006).

γ-PGA is biodegradable, highly water soluble, edible and non-toxic towards humans and the environment. These favourable properties and versatility have led to renewed interest in γ-PGA for the use in the food, medical, plastic, cosmetic, textile, water and wastewater treatment industries. Other applications include its use in hydrogels, vaccines, biodegradable fibres, natto (a Japanese food), as

a thickener, bitterness relieving agent, humectant, cryoprotectant, bioflocculant, heavy metal absorber, sustained release material, drug carrier, biological adhesive and as part of animal feed additives (Shih and Van, 2001).

2.3 Applications of γ-PGA

2.3.1 Food

Two strains, *B. subtilis,* known to produce chungkookjang, and *B. subtilis,* known to produce natto, have been isolated from Asian food (Ashiuchi *et al.*, 1999; Sung *et al.*, 2005). These strains ferment soybeans and produce γ-PGA as an extracellular, viscous material as seen by the appearance of chungkookjang and natto in Figure 2.2. γ-PGA has also been applied to relieve bitter-tasting substances, stabilise ice cream and prevent aging in starches whilst improving the texture (Sakai *et al.*, 2000).

Figure 2.2: Chungkookjang, a traditional Korean food made from fermented soybeans (Sung *et al.*, 2005).

γ-PGA is considered as a nutritional supplement and has been shown to facilitate calcium absorption. Increased intestinal absorption was found after a single dose of γ-PGA, even in post-menopausal women. This increase in bioavailability is attributed to the inhibition of an insoluble calcium phosphate. γ-PGA also increases absorption of other minerals such as phosphorus when added to food or animal feeds (Tanimoto *et al.*, 2007).

2.3.2 Medicine

2.3.2.1 Drug carrier and vaccines

The use of γ-PGA for drug delivery is gaining much interest. The carboxyl groups on the side chains offer attachment points for the conjugation of chemotherapeutic agents, rendering the drug more soluble and easier to administer. For this application, the conjugated γ-PGA is able to enter the tumour sites and slowly release the drug (Bajaj and Singhal, 2011b). Glutamic acid, the product of the γ-PGA breakdown can then entre normal metabolism and not get excreted by the kidney (Singer, 2005). Paclitaxel (Taxol®, TXL) is a natural anti-tumour agent that has potent activity against human tumours (Rowinsky and Donehower, 1995; Holmes *et al.*, 1995). This drug, however, is insoluble in water. Polyglutamic acid paclitaxel conjugate (PG-TXL) is prepared by covalent bonding of native paclitaxel to glutamic acid polymer is a water soluble form of Taxol® .The conjugate was shown to possess enhanced efficacy in anti-tumour activity against murine and human tumor xenografts than TXL (Li *et al.*, 1998). Animal models showed complete tumour regression which was achievable by a single dose of intravenously administered PG-TXL. During the preclinical stages, data suggested the uptake of PG-TXL by tumour cells to be approximately 5-fold greater than that of TXL when equivalent doses were used. PG-TXL targets tumour cells and once inside the cell, the polymer is digested, delivering a more potent dose of TXL. It has fewer side effects, improved anti-tumour activity and allows for significantly increased maximum tolerated dosages. This conjugate is in human clinical trials. Other cancer drugs conjugated to polyglutamate show similar improvements (Avichezer *et al.*, 1998; De Vries *et al.*, 2000; Shih and Van, 2001; Singer, 2005). A number of biodegradable derivatives of poly-(L-glutamic acid) have been developed and show a great potential for drug delivery platforms and vectors for gene therapy (Dekie *et al.*, 2000; Hashida *et al.*, 1999; Markland *et al.*, 1999). Animal studies involving rabbits injected with γ-PGA showed the importance of γ-PGA in antibody production in high titres, which affects immune responses. Therefore, in addition to drug conjugates for treating existing illnesses, γ-PGA can also be successfully applied in vaccines (Chang *et al.*, 2008).

2.3.2.2 Biological adhesives

Surgical adhesives are commonly used ineffectively for tissue adhesion, hemostasis, aortic dissection repairs, wound closures and sealing of air and body fluid leaks in surgery. The currently used surgical adhesive, fibrin, has poor adhesion to the tissues and low mechanical strength. The use of fibrin renders patients to potential susceptibility to viral infection due to it being made from human blood. Synthetic and semi-synthetic adhesives are also available for use, but show cytotoxicity and low degradation rates, which results in chronic inflammations. Chemical crosslinking of gelatin and poly (L-glutamic acid), which are both biodegradable, has been shown to be promising as a biological adhesive and hemostatic agent. Animal studies have demonstrated such gelatine-L-PGA forms a gel

when crosslinked with water-soluble carbodiimide (WSC,) which has higher bonding strength and better hemostatic capability. Additionally, this hydrogel is slowly degraded in the body without inducing any problematic inflammatory response (Shih and Van, 2001; Otani *et al.,* 1996, 1998, 1999). Sekine *et al.* (2000) developed a biological adhesive made from porcine collagen and poly (L-glutamic acid), which is superior to fibrin in sealing lung air leakage.

2.3.3 Tissue engineering

Chitosan is considered the most appropriate biodegradable polymer for tissue engineering applications. The addition of polyglutamic acid improved the water absorption rate, swelling ratio of composite biomaterial, hydrophilicity and mechanical strength of the usually weak chitosan-based scaffolds. This modified matrix was also suitable for cell attachment and proliferation (Hsieh *et al.*, 2005).

2.3.4 Hydrogels

Highly water absorbable hydrogels prepared from natural polymers have received much attention. This is because hydrogels that can absorb several hundred times their dry weight of water or respond to external stimuli can be useful as materials for controlled release, enzyme immobilization or as moisture absorbents (Bae *et al.*, 1987; Park and Hoffman, 1992; Veronese *et al.*, 1991). Hydrogels from microbial γ-PGA show high water absorption ability and various swelling behaviours. There are various potential applications of hydrogels in the agricultural and biomedical industries. These are prepared by γ-irradiation and the effect of pH and ionic concentration on swelling equilibria and the rate of enzymatic have been studied (Choi and Kunioka, 1994; Kunioka, 1993, 2004).

2.3.5 Cosmetics

A natural composition containing aloe vera, γ-PGA and green tea has been shown to enhance the humectant effects on skin without disturbing the balance (Hasebe and Inagaki, 1999). Thus, γ-PGA can be used as a value-added component to enhance the properties of existing or novel personal care products as a moisturizer, exfoliant and wrinkle-remover. The chemistry of PGA allows it to be homogenously miscible as well as chemically stable in a variety of ingredients. γ-PGA also increases water retention and forms a protective barrier against chemicals making it applicable in hair products and facial masks (Ben-Zur and Goldman, 2007). Additional advantages of using γ-PGA include the ability to form a smooth, elastic, moisturizing and soft film on the skin (Bajaj and Singhal, 2011b).

2.3.1 Soil enhancer

γ-PGA is commercially available in environmentally-friendly fertilizer compositions for use in the agricultural industry. One such composition was developed by King *et al.* (2007). Further, its water retention or hydrogel properties (Section 2.3.4) offer good potential as a soil enhancer.

2.3.2 Wastewater remediation

2.3.2.1 Removal of heavy metals

γ-PGA is an anionic polymer, and is thus ideal for adsorbing metal ions in heavy metal removal in industrial wastewaters. Copper, mercury and lead ions have shown very good adsorption capacities with γ-PGA (Mark *et al.*, 2006; Inbaraj *et al.*, 2009; Bodnar *et al.*, 2008).

2.3.2.2 Production of bioflocculants

The most widely reported application of γ-PGA is in or as flocculants (Bajaj and Singhal, 2011a ; Buthelezi *et al.*, 2009; Shih *et al.*, 2001; Taniguchi *et al.*, 2005; Wu and Ye, 2007; Yokoi *et al.*, 1995, 1996; Zheng *et al.*, 2008; Zhong *et al.*, 2009). γ-PGA is considered as a superior bioflocculant because of its ability to flocculate a wide range of organic and inorganic compounds. Bioflocculants function by causing aggregation of particles and cells by bridging and charge neutralisation (Salehizadeh and Shojaosadati, 2001). A bioflocculant produced by *B. subtilis* DYU1 showed a flocculation ability of 97% in kaolin suspension as discovered by Wu and Ye (2007). Zhong *et al.* (2009) produced a novel bioflocculant ZS-7 from *B. licheniformis*. ZS-7 was applied to low temperature drinking water and showed a better permanganate index (COD_{Mn}) and turbidity removal efficiency when compared to conventional chemical flocculants. Conflicting reports exist about the influence of the addition of bivalent and trivalent cations such as Mg^{2+}, Ca^{2+} and Fe^{3+} on flocculation performance (Shih *et al.*, 2001; Zheng *et al.*, 2008). Industrially applied chemical flocculants such as polyaluminium chloride, ferric chloride and polyacrylamide are advantageous for use due to their high flocculation activity and low cost. However, these flocculants are at the helm of many health and environmental concerns. This includes evidence that they are neurotoxic, carcinogenic and are contributing factors to Alzheimer's disease. They are also not readily biodegradable. These harmful threats to the environment and humans have led to their use being restricted (Campbell, 2002; Rudén, 2004). Alternative flocculants to be applied on the industrial scale processes are urgently needed. Bioflocculants are expected to replace conventional chemical flocculants applied in the wastewater treatment, drinking water, fermentation and food downstream processing industries (Wu and Ye, 2007; Bajaj and Singhal, 2011a).

2.3.3 Other applications

An ester derivative containing crosslinked γ-PGA for use as biodegradable plastics was developed by Tsutomu and Makoto (2002). Wider application of this strong, elastic and most importantly biodegradable product could solve the huge problem of pollution caused by common plastics we are currently experiencing. Cryoprotectants have high acidic amino acid compositions and display high antifreeze abilities. Investigations showed γ-PGA to have this antifreeze ability and for this to increase with a decrease in molecular weight (Shih *et al.*, 2003). Enzymes immobilized on γ-PGA have shown improved performance. Therefore, γ-PGA can also be successfully applied in vaccines and in enzyme immobilization (Sung *et al.*, 2005).

The application of γ-PGA is safe, versatile and environmentally friendly. Much research over the past decade has been focused on the production of high value γ-PGA for biopharmaceutical applications (Li *et al*., 1998; Nair and Laurencin, 2007; Richard and Margaritis, 2001; Singer *et al*., 2005). Use of γ-PGA as a conjugate to anticancer drugs is a commercially viable alternative, but requires an experimental approach which uses expensive substrates and a process that specifically controls the molecular weight and chemical composition of the γ-PGA - an impractical and difficult feat (Shih and Wu, 2009). Conventional synthetic bioflocculants such as acrylamide, are carcinogenic and neurotoxic, and cause a myriad of health and environmental problems. The use of γ-PGA produced by *Bacillus* species has been studied by several researchers (Shih *et al*., 2001; Yokoi *et al*., 1995, 1996) as a promising replacement in wastewater treatment by use as a bioflocculant due to its high flocculating activity. This particular application of γ-PGA does not require exceptionally high purity or stringent control of molecular weight and stereochemistry.

2.4 Microbial potential for the production of γ-PGA

2.4.1 Microorganisms synthesizing γ-PGA

γ-PGA is synthesized by a variety of organisms, including bacteria, archaea and an eukaryote listed in Table 2.1. Most research and industrial γ-PGA production has been focused on *Bacillus* species, namely *B. subtilis* and *B. licheniformis*. Depending on the species producing γ-PGA and the surrounding environment, different functionalities exist. Bacteria use γ-PGA to sequester toxic metal ions as well as to store glutamate for use during late stationary phase, allowing for increased resistance to harsh conditions (Kimura *et al.*, 2004; McLean *et al.*, 1990). In archaea, γ-PGA is used to decrease high salt concentrations (Hezayen *et al.*, 2000, 2001). γ-PGA is also found in the stinging cells or nematocysts of *Cnidaria* and serves as a trigger when capturing prey or in defence (Weber, 1990). The two main types of γ-PGA have associated functions: released γ-PGA is a persistence factor increasing resistance to harsh environments and anchored γ-PGA serves as a virulence factor (Candela and Fouet, 2006). The two stereoisomers of γ-PGA can be used in all applications. Cao *et al.* (2011) reported γ-PGA with a relatively higher L-glutamic acid ratio to be more easily metabolized

and therefore is more favourable for use in drug delivery for medicinal applications. The synthetic peptide conjugate from *B. anthracis* capsular poly-(γ-D-glutamic acid) elicited higher levels of IgG antibodies than the D and L conformation mixture from *B. pumulis* (Schneerson *et al*., 2003).

Table 2.1: Microorganisms reported to produce γ-PGA (modified from Candela and Fouet, 2006).

Organism	Domain	Conformation	Reference
Bacillus anthracis	Bacteria	D	Ivánovics and Erlös (1937)
Bacillus mesentricus	Bacteria	D	Ivánovics and Bruckner (1937)
Bacillus licheniformis	Bacteria	D and L	Bovarnick (1942)
Bacillus megaterium	Bacteria	D and L	Torii *et al.* (1959)
Bacillus pumulis	Bacteria	D and L	Schneerson *et al.* (2003)
Bacillus subtilis	Bacteria	D and L	Bovarnick (1942)
Bacillus amyloliquefaciens	Bacteria	D and L	Meerak *et al.* (2007)
Staphylococcus epidermidis	Bacteria	D and L	Kocianova *et al.* (2005)
Fusobacterium nucleatum	Bacteria	D	Candela *et al.* (2009)
Planococcus halophilus	Bacteria	D	Kandler *et al.* (1983)
Sporosarcina halophila	Bacteria	D	Kandler *et al.* (1983)
Xanthobacter	Bacteria	L	Kandler *et al.* (1983)
Flexithrix	Bacteria	L	Kandler *et al.* (1983)
Bacillus halodurans	Bacteria	L	Aono (1987)
Natrialba aegyptiaca	Archaea	L	Hezayen *et al.* (2000)
Hydra	Eukaryote	Not determined	Weber (1990)

2.4.2 Biochemistry of γ-PGA production

Biosynthesis of γ-PGA in bacteria has been widely reported. Goto and Kunioka (1992) studied the pathway for γ-PGA synthesis in *B. subtilis* IFO 3336. They proposed that γ-PGA is produced mainly

from the citric acid and ammonium sulphate found in the medium via the tricarboxylic acid or TCA cycle. Citric acid is metabolized to isocitric acid and then α-ketoglutaric acid (or 2-oxoglutaric acid), a direct glutamate precursor. This α-ketoglutaric acid is further processed through one of two pathways: In the absence of glutamine, the glutamate dehydrogenase (GD) pathway, commonly found in microorganisms and first reported by Stadtman (1966) is used. In this pathway, L- glutamic acid is synthesized from α-ketoglutaric acid and ammonium sulphate, catalyzed by GD (Equation 2.1):

$$2-oxoglutaric\ acid + NH_3 + NAD(P)H + H^+ \overset{GD}{\rightarrow} L-glutamic\ acid + NAD(P) \quad (2.1)$$

In the presence of L-glutamine, the glutamine synthetase (GS) and glutamine-2-oxoglutarate aminotransferase (GOGAT) pathway is used, in which reaction of α-ketoglutaric acid and L-glutamine is catalyzed by GOGAT to form L-glutamic acid, and L-glutamine is regenerated from L-glutamic acid and ammonium sulphate is catalyzed by GS (Equations 2.2 and 2.3) (Holzer, 1969).

$$2-oxoglutaric\ acid + L-glutamine + (2\,H) \overset{GOGAT}{\rightarrow} 2L-glutamic\ acid \quad (2.2)$$

$$L-glutamic\ acid + NH_3 + ATP \overset{GS}{\rightarrow} L-glutamine + ADP + P_i \quad (2.3)$$

The proposed schematic for γ-PGA biosynthesis is illustrated in Figure 2.3.

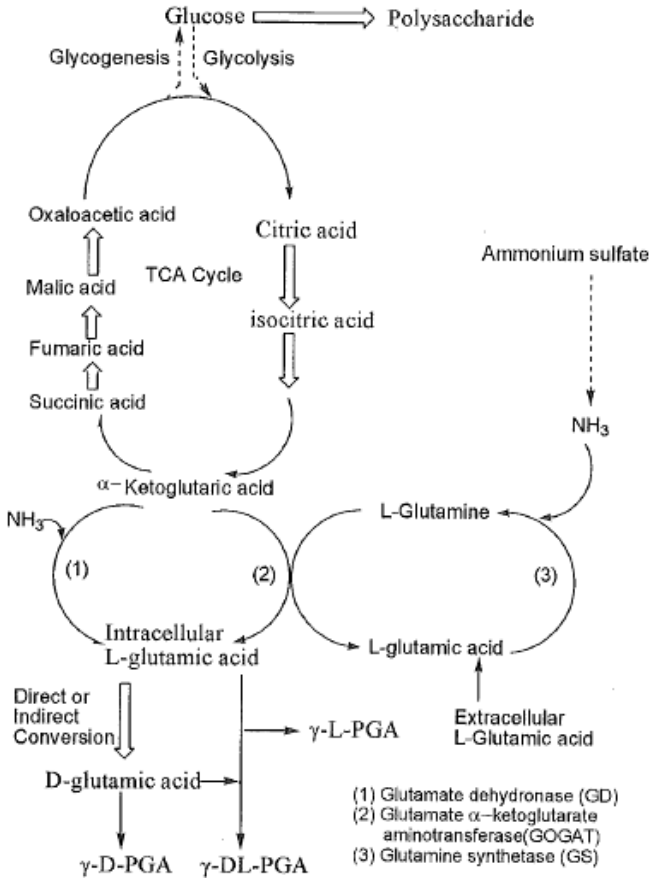

Figure 2.3: Biosynthesis mechanism for γ-PGA via the TCA cycle (modified from Shih and Wu, 2009).

The DL isomer content of γ-PGA is highly dependent on the presence and level of the enzymes which modulate D-glutamate (Shih and Van, 2001). Troy (1973) found that D-glutamic acid supplied in the medium was not incorporated into γ-PGA, regardless of the concentration added. The addition of D-glutamic acid, even in excess, did not inhibit the polymerization of L-glutamic acid. The D-glutamic acid stereoisomer is produced from direct or indirect conversion L-glutamic acid. The direct pathway involves the reversible catalysis of L-glutamic acid by the enzyme glutamate racemase (Glr) (Ashiuchi *et al*., 1998, 2001). The expression of the *glr* gene has been reported to be influenced by Mn^{2+}, which may affect the ratio of L- and D-glutamate residues in the racemization reaction (Ashiuchi *et al*., 2004). Thorne and colleagues (1955) first described the detection of alanine racemase and D- and L-glutamyl transaminase in cell extracts. These two enzymes are responsible for indirect conversion of L-glutamic acid in microorganisms such as *B.anthracis* and *B.licheniformis*, where glutamate racemase has not been detected (Kunioka, 1997). The indirect conversion mechanism involves a series of glutamyl transamidation and alanine racemization reactions in which γ-ketoglutarate is a key intermediate as shown in Figure 2.4.

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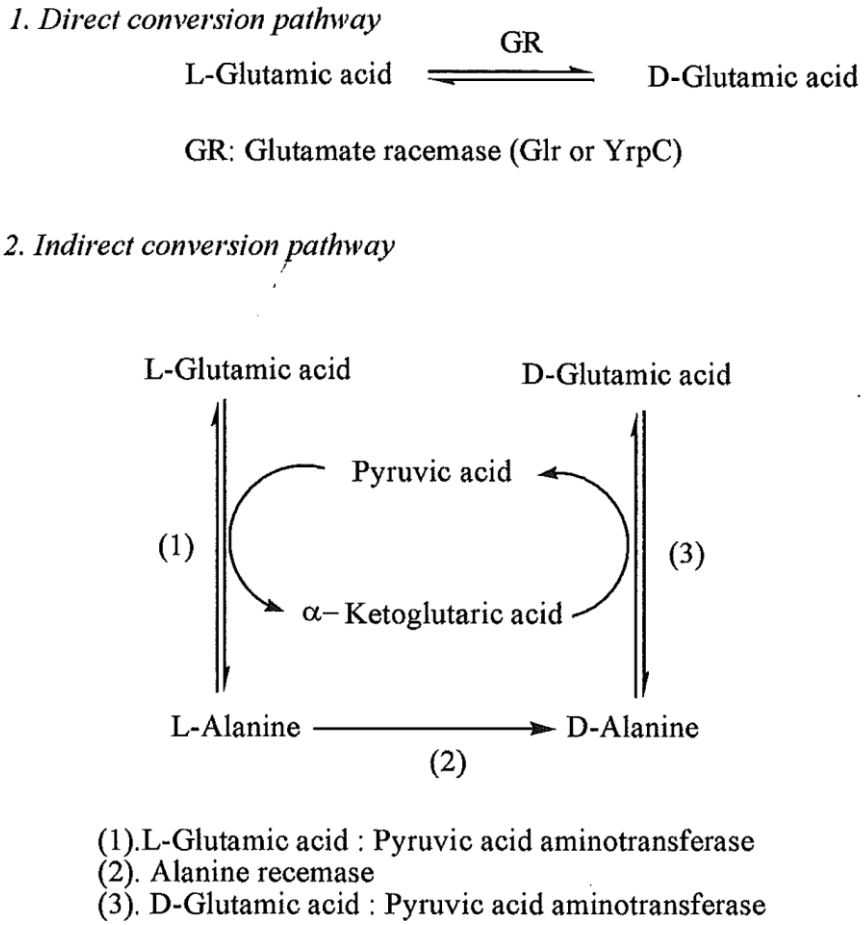


Figure 2.4: The direct and indirect pathways for the conversion of L-glutamic acid to D-glutamic acid (modified from Shih and Wu, 2009).

2.4.3 The genetic organisation of γ-PGA synthesis in bacteria

The genes required for the synthesis of γ-PGA in bacteria have been identified in several *Bacillus* species. In *B. anthracis*, the genes annotated as *capB, capC, capA* and *capE* encode a peptide which plays a role in capsulated γ-PGA synthesis. *CapD* encodes either a γ-glutamyltranspeptidase (GGT) or a γ-glutamylhydrolase enzyme which promote anchoring. Similarly, *B. subtilis* and *B. licheniformis* have been found to contain four polyglutamate synthase genes: *pgsB, pgsC, pgsA,* and more recently, *pgsE,* which are analogous to the *cap* genes. Similar genes *ywsC*, *ywsA*, *ywsB* and have also been found in *B.subtilis* Marburg 168, and correspond to *capBCA* (or *pgs*) (Kunst *et al*., 1997). The γ-PGA formed by this locus is secreted (facilitated by *pgsS)*. All these genes are organized in operons as illustrated in Figure 2.5 (Candela *et al.*, 2005; Urushibata *et al.*, 2002). There have been contradictions in the literature concerning the minimal genetic elements required to synthesize γ-PGA (Ashiuchi *et al.*, 2004; Urushibata *et al.*, 2002). Tarui and colleagues (2005) cloned *pgsB, pgsC* and *pgsAA* (collectively called *pgsBCA*) in tobacco plants and this appeared to be sufficient to produce γ-PGA, even though *PgsE* has been found to contain a small open reading frame which forms part of the *pgsBCA* operon (Ashiuchi *et al.*, 2001). Additionally, Candela *et al.* (2005) suggested that the PgsBCA proteins could form a complex at high concentration which was able to synthesize γ-PGA even in the absence of *PgsE*. Further studies revealed that a *pgs* null mutant could not produce γ-PGA. The contradictory report had an interrupted *pgsAA*, which needed to be deleted for the observed lack of functionality in the gene product to be conclusive. Thus, the *pgsBCA* system is the sole machinery of the PGA synthesis (Bajaj and Singhal, 2011b; Candela and Fouet, 2006).

Figure 2.5: The genetic apparatus required for γ- PGA synthesis in *B. subtilis* and *B. licheniformis* (modified from Bajaj and Singhal, 2011b).

2.5 The γ-PGA production process

2.5.1 Nutrient requirements for γ-PGA production

Glucose, sucrose, maltose, glycerol, ethanol and starch have been found to be suitable carbon sources for growth and γ-PGA production, depending on the production strain used (Dong *et al.*, 2010; Zhong *et al.*, 2009). Suitable nitrogen sources include tryptone, peptone, yeast extract, urea and ammonium chloride (Chen *et al.*, 2010; Cheng *et al.,* 1989; Das and Mukherjee, 2007). Citric acid, L-glutamine, α-ketoglutaric acid and L-glutamic acid serve as metabolic precursors to the TCA cycle of bacteria, thus addition of these may increase growth of the bacterium and offer a maximal γ-PGA yield (Bajaj and Singhal, 2009a). Certain bacteria are L-glutamic acid independent and do not require the addition of L-glutamic acid to the medium to stimulate *de novo* γ-PGA production and cell

growth. These strains include *B. subtilis* 5E (which produces γ-PGA from L-proline as the sole carbon and nitrogen source) (Murao *et al.,* 1969, 1971), *B. subtilis* TAM-4 (uses ammonium salt and glucose as the nitrogen and carbon source respectively) (Ito *et al.*, 1996), *B. licheniformis* A35 (which produced γ-PGA under denitrifying conditions using glucose and ammonium chloride) (Cheng *et al.*, 1989), *B. subtilis* C1 (produced a glycerol- γ-PGA bioconjugate when grown on glycerol and citric acid) (Shih *et al*., 2005), *B. lichenifomis* SAB-26 (which produces maximal γ-PGA when grown on casein hydrolysate and ammonium sulphate) and the recently isolated *B. amyloliquefaciens* LL3 (which grew on sucrose, $(NH_4)_2SO_4$ and $MgSO_4$) (Cao *et al.*, 2011) and *B. subtilis* C10 (sufficiently overproduces when grown in citric and oxalic acid medium) (Zhang *et al*., 2012b). *B. subtilis* TAM-4 and *B. licheniformis* A35 were both grown on Medium M, which contains relatively high concentrations of glucose and ammonium chloride of 75 $g.L^{-1}$ and 18 $g.L^{-1}$ respectively. Maximal γ-PGA (8 $g.L^{-1}$ *B. licheniformis* A35 and 22 $g.L^{-1}$ when glucose was replaced by the same concentration of fructose for *B. subtilis* TAM-4) were only reached after 5 and 4 days of cultivation respectively. A final concentration of 28 $g.L^{-1}$ γ-PGA was obtained when *B. subtilis* C10 was grown in a 10 L bioreactor with a medium which contained an initial concentration of 80 $g.L^{-1}$ glucose and 20 $g.L^{-1}$ citric acid as the major components after 33 hours of cultivation. Only 33% of the glucose was consumed. An increase in glucose utilisation to 46% resulted in a decrease in γ-PGA to almost half (17 $g.L^{-1}$). *B. amyloliquefaciens* LL3 required slightly less substrate (50 $g.L^{-1}$ sucrose and 2 $g.L^{-1}$ $(NH_4)_2SO_4$) and produced γ-PGA of 4 $g.L^{-1}$ after 48 hours. The only *de novo* γ-PGA synthesising strains which did not utilise a sugar carbon source were *B. subtilis* C1 and *B. lichenifomis* SAB-26. *B. subtilis* C1 was grown on 22 $g.L^{-1}$ citric acid and 170 $g.L^{-1}$ glycerol to produce 21 $g.L^{-1}$ γ-PGA after 6 days of cultivation, whilst *B. lichenifomis* SAB-26 perhaps presents the best example of a glutamic independent strain in the literature, growing on an optimised medium containing 12 $g.L^{-1}$ casein hydrolysate and 20 $g.L^{-1}$ $(NH_4)_2SO_4$, and producing 60 $g.L^{-1}$ γ-PGA after 72 hours.

Addition of trace elements such as $MgSO_4$, $MnSO_4$, K_2HPO_4, $CaCl_2$, NH_4NO_3 and $FeCl_3$ was beneficial in γ-PGA production (Cromwick and Gross, 1995; Xu *et al*., 2005b; Richard and Margaritis, 2003a). Medium E, detailed in Table 2.2, was formulated by Birrer and colleagues (1994) for the investigation of γ-PGA production in *B. licheniformis* 9945A. This medium has been widely used in other studies (Ashby *et al.*, 2005; Cromwick *et al.*, 1996; Cromwick and Gross, 1995; Du *et al.*, 2005; Ko and Gross, 1998; Perez-Camero *et al.,* 1999; Potter *et al.*, 2001; Shih *et al.*, 2002; Shih *et al.*, 2001; Yoon *et al.*, 2000).

Studies using complex solid substrates for γ-PGA production have also been reported. These include soybean cake powder, soybean meal, dairy manure, swine manure, wheat bran, potato peels (Bajaj *et al.*, 2008; Chen *et al.*, 2005a; Chen *et al.*, 2005b; Das and Mukherjee, 2007; Wang *et al.*, 2008; Xu *et al*., 2005b; Yong *et al.*, 2011). More details can be found in Section 2.5.2.

Table 2.2: The composition of modified Medium E

Component	**g.L^{-1}**	**Component**	**g.L^{-1}**
Glutamic acid	20	K_2HPO_4	1.5
Glycerol	80	$MgSO_4.7H_2O$	0.5
Citric acid	12	$MnSO_4.H_2O$	0.104
NH_4Cl	7	$FeCl_3.6H_2O$	0.04
		$CaCl_2.2H_2O$	0.15

2.5.2 Types of processes

The production of γ-PGA has been studied at various scales, and using submerged and solid state fermentation. During initial laboratory experimentation, the seed cultures were inoculated into shake flasks to determine the effect of medium composition on the bacterial growth and γ-PGA production. The next step involved the use of batch culture in laboratory scale cultivations using a bioreactor to examine the optimal γ-PGA production culture conditions and associated time profiles (Yoon *et al*., 2000). Chen *et al.* (2010) successfully scaled up this system from a 15 L bench top fermenter to a large scale 100 L fermenter, which yielded a γ-PGA concentration of 54 g.L^{-1}, compared to 46 g.L^{-1} obtained in the 15 L fermenter. The authors used yeast extract paste as a cost effective nitrogen source. More recently, Huang *et al.* (2011b) achieved 101 g.L^{-1} γ-PGA and a volumetric yield of 0.57 g.(g total substrate)$^{-1}$ from *B. subtilis* ZJU-7 using a fed-batch process in a 10 L fermenter after 50 hours. The glucose feeding rate was adjusted to maintain a concentration of 3 to 10 g.L^{-1} in during the cultivation, whilst the temperature was maintained at 37˚C, dissolved oxygen above 10% and pH at 6.5. This was also successfully scaled up to a 100 L fermenter, producing a lower average yield of 85 g.L^{-1} and 0.53 g.(g total substrate)$^{-1}$. Yoon *et al.* (2000) achieved 36 g.L^{-1} γ-PGA in a 2.5 L bioreactor by also using a fed-batch process. There has not been a report of using continuous culture to produce γ-PGA in the literature.

Submerged culture fermentation (SmF) in which cultures are grown in a liquid medium subjected to continuous, vigorous agitation are most commonly used for γ-PGA production. The advantages of using this method include the ability for control of the process and easier product downstream processing. However, γ-PGA is a highly viscous product, significantly increasing the viscosity of the production medium and reducing volumetric oxygen mass transfer rates. This limits cell growth and γ-PGA yield. A solution to this would be to increase aeration and agitation rates, but this could lead to γ-PGA degradation by high shear forces and uncontrolled foaming. Furthermore, the relatively high costs of the media constituents and energy requirements in SmF make it an expensive process (Bajaj and Singhal, 2011b; Cromwick *et al.*, 1996; Das and Mukherjee, 2007; Xu *et al.*, 2005b).

An alternative means called solid state fermentation (SSF) is slowly gaining increased interest and has been explored in recent studies for γ-PGA formation. This uses a solid substrate, such as those discussed in Section 2.5.1, for microbial growth in the absence or near absence of free water, resulting in a partially saturated system. The products are excreted into the medium. Advantages include reduced energy requirements, reduced water use, use of cheaper substrates (including domestic, agricultural and industrial waste), simpler cultivation equipment, no uncontrolled foaming, a stable product and a high product yield (Bajaj and Singhal, 2011b; Das and Mukherjee, 2007; Xu *et al.*, 2005b).

Comparative studies of SSF and SmF are lacking in the literature, probably due to the difficulties attributed by the major differences between these processes, culture conditions and *Bacillus* strains used. The yield of γ-PGA produced by *B.licheniformis* NCM 2324 growth in both SSF and SmF was investigated by Bajaj and colleagues (2008, 2009). A yield of 26 $g.L^{-1}$ γ-PGA was obtained during SmF on a screening medium similar to Medium E and 99 $mg.gds^{-1}$ in SSF on supplemented soybean meal. Although the same bacterial strain was used, the authors were unable to compare the efficiencies of these two processes due to the major differences in experimental methodology. In another study, Das and Mukherjee (2007) compared the efficiency of lipopeptide biosurfactant production by *B. subtilis* DM-03 and DM-04 in both SmF and SSF using dried potato peels as a cheap carbon source. The authors found that there was no statistically significant difference in biosurfactant yield and composition between SmF and SSF, showing efficiency in using either system. Wang and colleagues (2008) reported on co-producing lipopeptides and γ-PGA with a focus in using SSF. They found the γ-PGA yield to be 3.47% and 3.63% of the production medium using optimised SmF medium and a combination of sweet potato residues and soybean curd respectively. A summary of the reports of γ-PGA production in SSF in the literature is presented in Table 2.3.

Table 2.3: Production of γ-PGA in SSF

Strain	Key nutrients	Initial moisture (%)	Production time (hr)	Temperature (˚C)	γ-PGA yield (mg.g dry substrate^{-1})	References
B. subtilis CCTCC202048	Dairy manure, soybean cake, rapeseed cake, peanut cake, wheat bran, glutamic acid, citric acid	65	48	37	47	Chen *et al.* (2005b)
B. subtilis CCTCC202048	Swine manure, soybean cake, wheat bran, glutamic acid, citric acid	60	48	37	60	Chen *et al.* (2005a)
B. licheniformis NCIM 2324	Soybean meal, citric acid, L-glutamic acid, $(NH_4)_2SO_4$, mineral salts, glycerol, L-glutamine, α-ketoglutaric acid	60	72	37	99	Bajaj *et al.* (2008)
B. subtilis BLN-2	Soybean meal, glucose, fructose, $NaNO_3$, KNO_3	NA	48	37	89	Feng *et al.* (2008)
B. subtilis B6-1	Sweet potato, soybean curd, perlite, mineral salts	60	72	37	36	Wang *et al*. (2008)
B. subtilis ME714	Sodium glutamate, urea, trisodium citrate, starch	NA	NA	NA	75	Wu *et al.* (2007)
B. subtilis CCTCC202048	Soybean cake powder, wheat bran, glutamic acid, citric acid, NH_4NO_3	65	42	40	84	Xu *et al.* (2005b)
B. amyloliquefaciens C1	Dairy manure, soybean cake, corn flour, monosodium glutamate residues, rapeseed cake, wheat bran, citric acid, mineral salts	50	48	37	44	Yong *et al.* (2011)

NA: Not available

2.6 Downstream processing

2.6.1 Product location

γ-PGA is secreted through the cell wall. It either forms a capsule around the cell or is transferred directly into the culture medium. The cells capsulated with γ-PGA possess a negative surface charge near neutral pH due to the ionized carboxyl groups in the γ-PGA. These cells thus have high stability in culture broth due to their negative surface charges. This makes the sedimentation of cells difficult during separation processes. The high stability of the capsulated cells combined with the high viscosity of the culture supernatant present major problems in the separation of cells from the γ-PGA and recovering the γ-PGA from the culture medium. It is necessary to reduce the stability of the capsulated cells and the viscosity of broth for the efficient separation of γ-PGA (Do *et al.,* 2001).

2.6.2 Recovery after SmF

Recovery and downstream processing usually involves three steps:

1. Removal of the cells by centrifugation or filtration,
2. Alcohol precipitation of the polymeric product from the cell free extract, and
3. Dialysis to remove low molecular weight impurities.

This approach was developed by Goto and Kunioka (1992). It contributes substantially to operating costs as an alcohol concentration of 75–80% (v/v) is required for cell-free culture supernatant containing 1–2% (w/v) of γ-PGA (Ito *et al.*, 1996). It is important to note that the amount of alcohol required for γ-PGA precipitation decreases with the increase in γ-PGA concentration.

To reduce the alcohol requirement, Do *et al.* (2001) developed another method involving two steps:

1. Separation of the γ-PGA from the medium by manipulating the pH and hence the charge, and
2. Ultrafiltration for concentration.

Since ultrafiltration concentrates γ-PGA, this allows for the use of significantly lower amounts of alcohol for its subsequent precipitation. The authors showed the volume of ethanol was reduced by four fold after concentrating 20 $g.L^{-1}$ of γ-PGA solution to 60 $g.L^{-1}$ at pH 5 by ultrafiltration. Lowering the pH revealed a decrease in viscosity and the zeta (ζ) potential by a factor of six and three respectively. On removal of the negative surface charge, the cells aggregated together and sedimented out. This, in effect, lowered the energy requirements for the separation of the cells from the culture medium by centrifugation to 17%, compared to the culture without acidification.

2.6.3 Recovery after SSF

Chen *et al.* (2005b) reported the recovery of γ-PGA following small-scale SSF. At the end of the cultivation, ten volumes of distilled water (w/v, based on initial dry weight of substrate) were added to the fermented matter and mixed at room temperature (20 ± 2°C) on a rotary shaker at 150 rpm for 1

h. The contents were then filtered twice through muslin cloth and the total pooled volume of filtrate recorded. The filtrate was then centrifuged for 20 min at 12,000 rpm. The supernatant contains crude γ-PGA, which is poured into four volumes of ethanol to precipitate the γ-PGA. The precipitate was recovered by centrifugation at 12,000 rpm for 20 min to collect the crude γ-PGA and re-dissolved in de-ionized water. Any insoluble contaminants were similarly removed by centrifugation. Finally, salts and low molecular weight impurities from the γ-PGA solution were removed by dialysis.

2.7 Analytical methods for γ-PGA quantification

A number of analytical methods have been reported for γ-PGA characterisation. Gel permeation chromatography (GPC) is used to determine γ-PGA molecular weight and yield. High performance liquid chromatography (HPLC) is also used to quantify the γ-PGA concentration after hydrolysis to glutamic acid. Additionally, HPLC can be used to determine γ-PGA stereochemistry, glutamate racemase and D-amino acid aminotransferase enzyme assays for L-glutamic acid, citric acid and glucose utilisation rates. Potential exists to replace these methods by nuclear magnetic resonance (NMR) spectroscopy and electrode detectors (Chen *et al*., 2005a; Cromwick and Gross 1995: Shih *et al.*, 2002; Wu *et al.*, 2006), but no “gold standard” methods exist. Innovative technologies are arising, allowing for faster and more accurate analyses. Such an opportunity is the use of Fourier transform infrared or FTIR spectroscopy which records absorptions of IR light by chemical bonds in all molecules, using a wide spectral range. This can be used to provide a structural fingerprint of a compound. Advantages of this technique include its speed, high degree of automation and low cost (Bauer *et al.*, 2008).

2.8 Optimisation

2.8.1 Physicochemical conditions

The bacterial strain, medium composition, pH, temperature, aeration, agitation, type of process and bioreactor configuration all have an effect on the culture conditions for γ-PGA production. This is turn leads to specific environmental conditions.

Non-Newtonian behaviour is often observed for microbial polymers (Werner and Mersmann, 1998a, b). Parameters such as biomass concentration, cell morphology and substrate and product concentration influence the rheological properties of a culture (Doran, 2013). Because γ-PGA is an extracellular product, the production medium becomes highly viscous and exhibits non-Newtonian rheology in accordance with the high molecular weight molecule in solution. This behaviour makes mixing difficult, which in turn limits heat transfer and oxygen supply, affecting the product concentration, quality, recovery and manufacturing costs (Richard and Margaritis, 2003b). An understanding of the rheology can be used for bioreactor design, scale-up and downstream processing (Johnstone-Robertson, 2012; Richard and Margaritis, 2003b).

B. subtilis IFO 3335 was used by Richard and Margaritis (2003b) to investigate the oxygen mass transfer and broth rheology characteristics during γ-PGA production. The molecular weight of the γ-PGA was also measured by intrinsic viscosity using the laser light scattering method and compared to the broth viscosity measurements from a viscometer. The bacterium was able to reproduce previously observed (Richard and Margaritis, 2003a) growth and γ-PGA yield, with a biomass and γ-PGA concentration of 4 $g.L^{-1}$ biomass and 23 $g.L^{-1}$ respectively. An increase in γ-PGA concentration resulted in an increase in apparent broth viscosity, reaching a maximum of 275 cP during the exponential phase of growth. In this study, the increased viscosity did not limit the oxygen uptake rate, and only slightly decreased the volumetric mass transfer coefficient at the highest culture viscosity at the late exponential phase. This finding indicates that sufficient oxygen availability and transfer becomes critical during early and late exponential phase, when most γ-PGA accumulation occurs. The maximum γ-PGA molecular weight of $4x10^6$ Da was found during the stationary phase of growth when the γ-PGA was also at the maximal value. The flow behaviour index (n) decreased as the cultivation progressed, whilst the broth consistency index (K) was also reached at maximal γ-PGA concentration. A decrease in the flow index and increase in the flow index was found to closely follow an increase in biopolymer concentration, viscosity and molecular weight, thus the pseudoplastic nature of the culture was confirmed. Pseudoplastic fluids obey the Ostwald-de Waele or Power Law as described by Equation 2.4.

$$\tau = K\gamma^n \qquad (2.4)$$

Where τ is the shear stress, K is the consistency index, γ the shear rate and n the flow behaviour index (Doran, 2013).

2.8.2 The effect of the medium components

L-Glutamic acid is frequently added as a precursor in γ-PGA production. The addition of L-glutamic acid contributes significantly to production costs hence must be optimised for incorporation into the TCA cycle and polymerisation into γ-PGA. The utilisation rate can be used as a tool for calculating the optimal concentration required, and thus avoiding residual L-glutamic acid in the spent medium (Bajaj and Singhal, 2011b). The addition of citric acid, an intermediate of the TCA cycle must also be optimised. An optimisation strategy for this TCA cycle intermediate is discussed in Section 2.8.5.

The selection of the correct carbon source is the single most important item for γ-PGA across all bacterial strains. Glucose has been widely explored by many researchers; who support its suitability (Cheng *et al.*, 1989: Soliman *et al.*, 2005; Wu *et al.*, 2010; Xu *et al.*, 2005a; Zhong *et al.*, 2009). Ko and Gross (1998) suggested that γ-PGA synthesis by *B. licheniformis ATCC* 9945A can occur by the conversion of glucose to acetyl-CoA and the TCA cycle intermediates, which then form L-glutamic acid. When Yao *et al.* (2010) analyzed the conversion of carbon sources into γ-PGA using ^{13}C-labeled glucose in NMR, the results showed that the glucose was utilised mainly as the growth-limiting

substrate for cell growth and supplied the required energy during γ-PGA biosynthesis, while L-glutamic acid was preferred as the main substrate for incorporation into γ-PGA.

Glycerol is another, highly favoured carbon source. Troy (1973) suggested that glycerol may stimulate polyglutamyl synthetase, which catalyzes the polymerization of glutamic acid to γ-PGA. Wu *et al.* (2010) confirmed that glycerol was responsible for stimulating the production of γ-PGA. Further they showed that it regulated the molecular weight in *B. subtilis* NX-2 culture. This led to a decrease of supernatant viscosity during the cultivation, enhancing the uptake of substrates, and eventually improved cell growth and γ-PGA production.

Inorganic salts like $CaCl_2$ and $MnSO_4$ have a significant effect on yield as well as the stereochemical composition of γ-PGA (Cromwick and Gross, 1995; Shih and Van, 2001; Wu *et al.,* 2006*).* Huang *et al.* (2011a) observed that the addition of $CaCl_2$ to the medium also reduced viscosity of culture supernatant from *B. subtilis* CGMCC 2108 was effective in increasing the consumption of extracellular L-glutamic acid by 11.4%, leading to higher γ-PGA yield as compared to the control by increasing the activities of three enzymes of the γ-PGA biosynthesis pathway (isocitrate dehydrogenase (ICDH), glutamate dehydrogenase (GDH) and 2-oxoglutarate dehydrogenase complex (ODHC) as shown in Figure 2.6.

The biosynthetic pathway reported by Goto and Kunioka (1992) of *B. subtilis* IFO 3335 suggested the requirement of free ammonuim ions for γ-PGA production (Figure 2.3). Xu *et al*. (2005b) reported that the addition of NH_4NO_3 gave the highest yield of 59.7 g/kg and showed that inorganic nitrogen sources were more efficient than organic nitrogen sources in enhancing γ-PGA production.

Although highly dependent on bacterial species and strain, medium optimisation for improved growth and γ-PGA has moved towards to using modified versions of the extensively used Medium E. The addition of Ca^{2+} and Mn^{2+} ions is also highly favoured (Table 2.4)

2.8.3 Redistribution of metabolic flux

Wu *et al.* (2008) also investigated the flux redistribution with the presence of some additives. The authors constructed a metabolic reaction model for central carbon metabolism and γ-PGA synthetic pathways to analyze the metabolic flux redistribution. The results suggested the addition of Tween-80, DMSO and the presence of glycerol in the medium stimulated the conversion of glucose to glutamate by increasing the carbon flux from 2-oxoglutarate to glutamate. In similar studies by Shimizu *et al.* (2003), the flux distribution around 2-oxoglutarate was found to be influenced most by ODHC attenuation, resulting in an increase in monosodium glutamate production by *Corynebacterium glutamicum*. Zhang *et al*. (2012b) also observed the repressive effect of ODHC in γ-PGA synthesis in the *B. subtilis* C10 strain. These reports suggest that biosynthesis of γ-PGA could be regulated by the redistribution of the metabolic flux, which could be manipulated by alteration of culture conditions, without any genetic modification. This could provide a simple and efficient tool to realize metabolic engineering goals.

2.8.4 Improving the permeability of cell membrane

As discussed above, the efficient uptake of extracellular substrate is important for γ-PGA biosynthesis. The cell membrane regulates the transport of these substrates. If the cell membrane permeability is increased, the consumption rate of substrate increases, also increasing the secretion of γ-PGA and leading to more efficient production (Wu *et al.*, 2008). Bajaj and Singhal (2011b) state that additional γ-PGA secretion stimulation from the cell could decrease the stress associated with intracellular γ-PGA and intracellular substrate accumulation. This would enhance the formation of γ-PGA. Addition of glycerol, Tween-80 or DMSO facilitate the uptake of extracellular substrates and secretion of γ-PGA by improving the permeability of cell membrane and the yield of γ-PGA (Du *et al.*, 2005; Wu *et al.*, 2008).

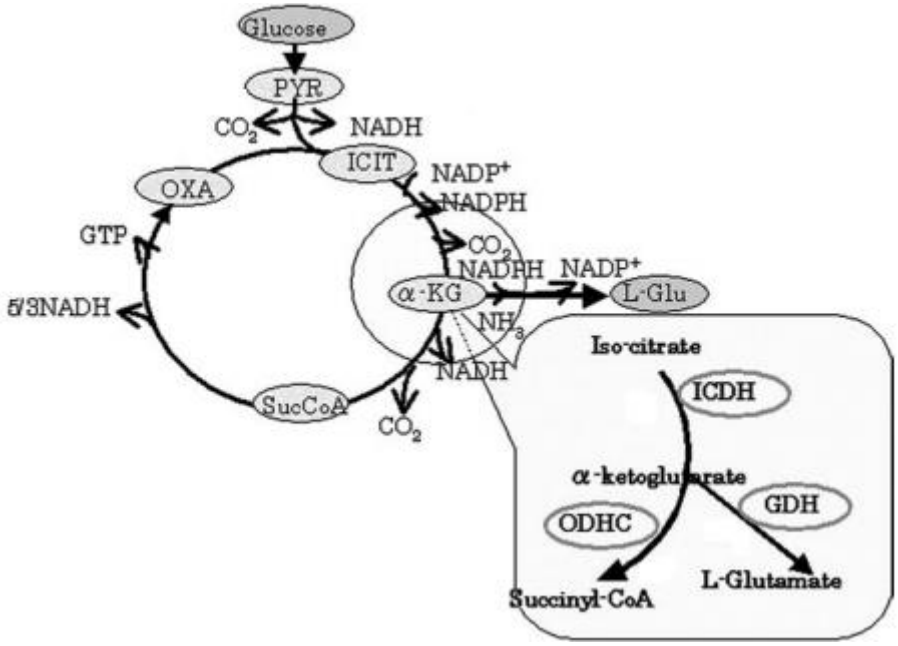


Figure 2.6: The metabolic pathway for central carbon metabolism and glutamate production with a more detailed view of the 2-oxoglutarate branch (modified from Shimizu *et al.*, 2003).

2.8.5 Metabolic precursors

The addition of metabolic precursors is another effective approach that has been used to increase γ-PGA production. Yoon *et al.* (2000) and Abdel-Fattah *et al.* (2007) pulse-fed citric acid, which is generally the best precursor for γ-PGA, when it was depleted from the medium in a fed-batch culture of *B. licheniformis.* They achieved a productivity of 35 $g.L^{-1}$ and 88 $g.L^{-1}$ respectively, compared to 23 $g.L^{-1}$ and 60 $g.L^{-1}$ in batch experiments without supplementation. *B. subtilis* NX-2 cannot assimilate citric acid, and produced no γ-PGA when this precursor was used as the sole carbon source. However, this strain was able to produce 30 $g.L^{-1}$ γ-PGA after 24 hours, when 30 $g.L^{-1}$ glucose and L-glutamic acid were used as carbon sources (Xu *et al*., 2005a). Bajaj and Singhal (2009a) studied the effect of the addition of different amino acids and TCA cycle intermediates as metabolic precursors on the production of γ-PGA by *B. licheniformis* NCIM 2324. Glutamic acid family (L-glutamine, L-arginine,

L-ornithine and L-proline) and PGA biosynthetic pathway amino acids (L-alanine and L-aspartic acid) were investigated. The authors found that these improved the yield, molecular weight of γ-PGA and the utilisation of L-glutamic acid. Of the TCA cycle intermediates tested, L-glutamine supported the highest yield, producing 31 $g.L^{-1}$ γ-PGA. A combination of L-glutamine and α-ketoglutaric acid further enhanced γ-PGA production by 39% to 36 $g.L^{-1}$ compared to 26 $g.L^{-1}$ in the absence of these precursors.

2.8.6 pH

The γ-PGA production depends strongly on the pH of the medium. Further utilisation of acid precursors affects solution pH. The pH is known to influence the physiology of a microorganism by affecting enzyme activity, cell membrane morphology, nutrient solubility and uptake, by-product formation and oxidative–reductive reactions (Bajaj *et al*., 2009). During shake flask experiments with uncontrolled pH, the product molecular weight, similarly to pH, decreases as a function of time (Birrer *et al.*, 1994; Cromwick and Gross, 1995). The control of pH (usually 7.0 for *B. subtilis* and 6.5 for *B. licheniformis*) has been shown by a large number of studies to aid in increased biomass and γ-PGA yields, with most studies opting to use pH 6.5 (Table 2.4) (Bajaj *et al*., 2009; Cromwick *et al*., 1996). Wu *et al.* (2010) used *B. subtilis* CGMCC 0833 for γ-PGA cultivation with an initial pH of 7.0. This pH enabled the bacterium produce maximum biomass. Thereafter, the pH was shifted to 6.5 to obtain high level of glutamate transport to form intracellular glutamate for utilisation and synthesis of γ-PGA. This increased yield of γ-PGA by 25% compared to cultivation without pH control, and proved to be an effective strategy for this strain. The authors also demonstrated the influence of efficient extracellular glutamate utilisation for improved γ-PGA production. Moreover, the optimal pH for cell growth was different to that for extracellular glutamate utilisation and γ-PGA production, as utilisation of N-compounds influenced pH. Citric acid utilisation has also been shown to be pH-dependent and highly favoured at pH 6.5 (Cromwick *et al.*, 1996).

2.9 The kinetics of γ-PGA production

Literature reports on γ-PGA production kinetics are scant (Bajaj and Singhal, 2011b; Hoppensack *et al.*, 2003; Potter *et al.*, 2001; Wu *et al.*, 2010). This is illustrated by Table 2.4. A fundamental understanding of key cultivation parameters such as medium composition, the microorganism used, bioreactor conditions and the effect of broth viscosity is necessary, but there is also a need to investigate kinetic models to further understand, control and optimise γ-PGA production (Richard and Margaritis, 2004). Richard and Margaritis (2004) proposed an empirical kinetic model for batch growth of γ-PGA from *B. subtilis* IFO 3335 that could be used to fit kinetic data from other bacterial strains to approximate not only γ-PGA production, but also other biopolymers. This model was based on the assumption that the biopolymer was a component of the biomass. The authors noted that this model needed refinement and testing for generalised applicability with respect to bacterial γ-PGA production. This model and other batch kinetic modelling for other processes (Rodrigues *et al.*, 2008; Dhanasekar and Viruthagiri, 2005) could provide a good framework to study other fermentation kinetics.

Table 2.4: Optimisation studies for submerged cultivations found in the literature for γ-PGA production arranged by date from 1989 to 2013. The taxa, major medium components, pH and available basic kinetic data are given.

Bacterium	Medium components	pH	μ_{max} (hr^{-1})	γ-PGA ($g.L^{-1}$)	Biomass ($g.L^{-1}$)	Reference
B. licheniformis A35	K_2HPO_4, $MgSO_4$, NH_4Cl, KNO_3, glucose, $CaCO_3$, $MnSO_4$, $FeCl_3$	7.2	NA	8	NA	Cheng *et al.* (1989)
B. subtilis IFO 3335	L-Glutamic acid, $(NH_4)_2SO_4$, citric acid, KH_2PO_4, Na_2HPO_4, $MgSO_4$, $FeCl_3$, $CaCl_2$, biotin	7.5	NA	10	6	Goto and Kunioka (1992)
B. subtilis F-2-01	L-Glutamic acid, veal infusion broth, glucose	NA	NA	48	NA	Kubota *et al.* (1993)
B. subtilis IFO 3335	L-Glutamic acid, $(NH_4)_2SO_4$, citric acid, KH_2PO_4, Na_2HPO_4, $MgSO_4$, $FeCl_3$, $CaCl_2$, biotin	7.5	NA	20	NA	Goto and Kunioka (1994)
B. licheniformis ATCC 9945A	Modified Medium E, $MnSO_4$	7.4	NA	17	NA	Cromwick and Gross (1995)
B. subtilis IFO 3335	L-Glutamine, $(NH_4)_2SO_4$, citric acid, KH_2PO_4, Na_2HPO4, $MgSO_4$, $FeCl_3$, $CaCl_2$, biotin	7.5	NA	5	NA	Kunioka (1995)
B. licheniformis ATCC 9945A	Medium E	6.5	0.11	23	4	Cromwick *et al.* (1996)
B. subtilis TAM-4	NH_4Cl, fructose, K_2HPO_4, $MgSO_4$, $MnSO_4$, $CaCO_3$	7.2	NA	22	NA	Ito *et al*. (1996)
B. subtilis (natto)	Maltose, soy sauce, L-glutamic acid, NaCl	NA	NA	35	NA	Ogawa *et al*. (1997)
B. licheniformis ATCC 9945A	Medium E , glucose, $MnSO_4$	6.5	NA	12	NA	Ko and Gross (1998)
B. licheniformis	Medium E, Mn^{2+}	7.4	NA	6	NA	Perez-Camero *et al.* (1999)
B. subtilis IFO 3336	$MgSO_4$, $MnSO_4$, NaCl, L-glutamic acid	7.5	NA	24	NA	Ashiuchi *et al.* (1999)
B. licheniformis ATCC 9945A	Medium E	6.5	NA	35	NA	Yoon *et al.* (2000)
B. subtilis (chungkookjang)	L-Glutamate, sucrose, $(NH_4)_2SO_4$, KH_2PO_4, Na_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, MS vitamin	7	NA	14	NA	Ashiuchi *et al.* (2001)
B. licheniformis ATCC 9945A & *B. subtilis*	Supplemented liquid manure, Medium E	7.5	NA	0.9 & 0.8	NA	Potter *et al.* (2001)
B. subtilis 1551	Supplemented liquid manure, Medium E	7.5	NA	5	NA	Potter *et al.* (2001)
B. subtilis CCRC 12826	Medium E	6.5	NA	14	NA	Shih *et al.* (2001)
B. subtilis CCRC 12826	Optimised Medium E	6.5	NA	20	NA	Shih *et al.* (2002)
B. licheniformis S2	Swine manure, soybean cake, wheat bran, L-glutamic acid, citric acid, $MnSO_4.H_2O$	NA	NA	0.2	8	Hoppensack *et al.* (2003)
B. subtilis IFO 3335	L- Glutamic acid, citric acid, $(NH4)_2SO4$, K_2HPO4, Na_2HPO_4, $MgSO_4$, $FeCl_3$, $CaCl_2$, $MnSO_4$	7	NA	23	4	Richard and Margaritis (2003a)
B. licheniformis ATCC 9945A	L-Glutamic acid, citric acid, glycerol, NH_4Cl, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $MnSO_4 \cdot H_2O$	6.5	NA	33	NA	Lu *et al.* (2004)

Table 2.4: Optimisation studies for submerged cultivations found in the literature for γ-PGA production arranged by date from 1989 to 2013 (continued).

Bacterium	Medium components	pH	μ_{max} (hr^{-1})	γ-PGA ($g.L^{-1}$)	Biomass ($g.L^{-1}$)	Reference
B. licheniformis WBL-3	Modified Medium E, $MnSO_4$, glycerol	6.5	NA	29	4	Du *et al.* (2005)
B. subtilis IFO 3336, *chungkookjang*	L-Glutamate, sucrose, KH_2PO_4, Na_2HPO_4, NaCl, $MgSO_4$, $(NH_4)_2SO_4$, vitamin solution	NA	NA	NA	NA	Park *et al.* (2005)
B. subtilis C1	Medium T (Medium E with higher citric acid, glycerol & no L-glutamic acid)	NA	NA	21	NA	Shih *et al.* (2005)
B. licheniformis SAB-26	Glucose, K_2HPO_4, KH_2PO_4, $MgSO_4$, $FeSO_4$, $CaCl_2$, $MnSO_4$	6.5	NA	34	18	Soliman *et al.* (2005)
B. subtilis NX-2	Glucose, yeast extract, KH_2PO_4, $MgSO_4$, L-glutamic acid	7	NA	41	NA	Xu *et al.* (2005a)
B. subtilis ZJU-7	Sucrose, tryptone, L-glutamic acid, NaCl	NA	NA	54	NA	Shi *et al.* (2006)
B. subtilis NX-2	Glucose, yeast extract, K_2HPO_4, L-glutamic acid, $MgSO_4$, $MnSO_4$	7.5	NA	31	NA	Wu *et al.* (2006)
B. licheniformis SAB-26	Casein hydrolysate, $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, citric acid, glucose, glycerol, $MgSO_4$, $MnSO_4$, $FeSO_4$, $CalCl_2$, $ZnCl_2$	6	NA	88	NA	Abdel-Fattah *et al.* (2007)
B. licheniformis CICC10099	Modified Medium E	7.5	NA	19	NA	Suo *et al.* (2007)
B. subtilis CGMCC 0833	Glucose, L-glutamate, glycerol, $(NH_4)_2SO_4$, K_2HPO_4, $MgSO_4$, $MnSO_4$, Tween-20	7.5	NA	34	4	Wu *et al.* (2008)
B. licheniformis NCIM 2324	Statistically optimised Du *et al.* (2005) medium	6.5	NA	26	2	Bajaj *et al.* (2009)
B. licheniformis NCIM 2324	Modified Medium E	6.5	NA	36	2	Bajaj and Singhal (2009a)
B. subtilis R 23	Modified Medium E, glucose, NaCl, KCl, $MgCl_2$, $NaHCO_3$	6.5	NA	25	3	Bajaj and Singhal (2009b)
B. licheniformis X14	Glucose, starch, urea, yeast extract, $(NH_4)_2SO_4$, NaCl, $MgSO_4$, K_2HPO_4, KH_2PO4, NH_4Cl	6.5 - 9	NA	NA	NA	Zhong *et al.* (2009)
B. subtilis ZJU-7	Glucose, tryptone, L-glutamic acid, NaCl, $CaCl_2$, $MgSO_4$	6.5	NA	46	NA	Chen *et al.* (2010)
B. subtilis RKY3	Glycerol, L-glutamic acid, yeast extract, K_2HPO_4	NA	NA	49	0.9	Jeong *et al.* (2010)
B. subtilis CGMCC 0833	Glucose, L-glutamate, $(NH_4)_2SO_4$, K_2HPO_4, $MgSO_4$, $MnSO_4$	7 & 6.5	0.15	28	5	Wu *et al.* (2010)
B. amyloliquefaciens LL3	Sucrose, $(NH_4)_2SO_4$, $MgSO_4$, KH_2PO_4, K_2HPO_4, $FeSO_4$, $CaCl_2$, $MnSO_4$, $ZnCl_2$	7	NA	4	NA	Cao *et al.* (2011)
B. subtilis CGMCC 2108	Glucose, L-glutamate, NH_4Cl, $K_2HPO_4.3H_2O$, $MgSO_4$, yeast extract, $CaCl_2$	7	NA	9	NA	Huang *et al.* (2011a)
B. subtilis ZJU-7	Glycerol, L-glutamic acid, yeast extract, NaCl, $CaCl_2$, $MgSO_4$, $MnSO_4$	6.5	NA	101	NA	Huang *et al.* (2011b)
B. subtilis NX-2	Cane molasses or monosodium glutamate waste liquor with L-glutamate, $(NH_4)_2SO_4$, $K_2HPO_4.3H_2O$, $MgSO_4$, $MnSO_4$	7	NA	50 & 52	7 & 8	Zhang *et al.* (2012a)
B. subtilis C10	Glucose, citric acid, oxalic acid, NH_4Cl, $MgSO_4{\cdot}7H_2O$, K_2HPO_4, $FeCl_3{\cdot}6H_2O$, $MgSO_4{\cdot}H_2O$, $CaCl_2$	7.2	NA	28	2	Zhang *et al.* (2012b)
Bacillus subtilis BL53	Medium E, Zn^{2+}	6.5	0.1	17	9	da Silva *et al.* (2013)

2.10 Co-cultivation

Many industrially applicable γ-PGA-producing strains are L-glutamic acid dependent. To counteract the problem of extracellular L-glutamic acid addition and its subsequent inefficient usage, Shi *et al.* (2007) developed a system in which *Corynebacterium glutamicum* S9114 (which produces L-glutamic acid) and *B. subtilis* ZJU-7 (which consumes the L-glutamic acid and produces γ-PGA) were used in simultaneous cultivations. Glucose was used as the sole carbon source, with urea serving as a nitrogen source. This system yielded 32.8 $g.L^{-1}$ γ-PGA after 24 hours.

2.11 Genetic engineering

Genetic engineering strategies, such as the expression of *pgsBCA* gene sequence in *Escherichia coli* or site-directed mutagenesis, might be used to modify the γ-PGA synthesizing enzymatic complex for controlled γ-PGA production (Ashiuchi *et al.*, 1999). Such efforts also could lead to the production of mono-isomeric γ-(L)-PGA, which seems to be more suitable for medical applications. Finally, employing PgsBCA as a bio- catalyst could open the door to new reactions that may be useful in the production of γ-PGA (Bajaj and Singhal, 2011b).

2.12 Wastewater as a potential source of nutrients for microbial product formation

Environmental biotechnology is concerned with the use of less radical tools (such as genetic engineering) to address challenges associated with the waste management and control of pollution in manufacturing processes, for more environmentally sound practices. The threat of rapid industrialization and urbanization on a clean environment have made the implementation of these approaches necessary, even though this is sometimes seen by corporations as an inconvenience (Evans and Furlong, 2003; Gavrilescu, 2010). In addition to the use of traditional molecular tools, effective for microbial ecosystems analysis and understanding requires essential engineering tools such as mass balances, kinetics and modelling to be applied. An improved understanding of the behaviour of these microorganisms can assist in providing us with information for more innovative solutions to address efficient service delivery to compliment the rising expectations of society (Rittmann, 2007).

For the γ-PGA process to be efficient and economically feasible, the optimised use of renewable, sustainable substrates as a source of nutrients is necessary. Wastewater is a potential source of nutrients as it is composed of 99.9% water, phosphorus, nitrogen, salts and organic matter (Shwartz, 2010). With the current worldwide challenge of freshwater scarcity, proper use and re-use of water is increasingly imperative. Utilising wastewater as the nutrient source for bioprocessing can decrease the operating cost of the bioprocess, partially treat the water and simultaneously produce valuable products (Polprasert, 2007). However, using wastewater for industrial applications does have considerable challenges. These include: wastewater being a dilute nutrient stream of variable

composition and a non-sterile ecosystem with various pathogens and competing microorganisms utilising the same carbon, nitrogen and phosphorus sources for growth and sustenance. The constituents typically found in wastewater streams are shown in Table 2.5.

An investigation of the use of *B. cereus* by Zhao *et al.* (2009) for the removal of organic matter from the Grandeur Garden Lake in Beijing, China resulted in a 69% removal of COD and an increase in the transparency of the water by 0.4 m. This reclaimed wastewater was then used in the park for irrigation purposes. In another study which was not focused on a wastewater treatment application, Drouin and colleagues (2008) were able to successfully demonstrate the use of municipal wastewater sludge as a substrate to produce proteases from *B. licheniformis* in the laboratory. Although these two studies focused of different aspects of wastewater use, they both highlighted the potential of wastewater as an alternative substrate for microbes.

Enhanced biological systems, which make use of existing organisms in their predominantly natural form, for faster and more efficient activities offers a promising alternative for the bioremediation of specific pollutants, whilst positively influencing the balance sheet (Evans and Furlong, 2003; Russel, 1998).

2.13 Future prospects

γ-PGA is a safe, versatile and environmentally-friendly polymer with favourable characteristics which has potential for numerous applications. Considerable research has been dedicated to its production since its discovery 74 years ago. However, γ-PGA still has high associated production cost with low productivity rates. There is a need for production optimisation and control that can be applied to industrial scale for this attractive and valuable product in order for it to be economically competitive with conventional synthetic chemicals.

Industrially, due to the popularity of natto in Japan, companies such as Meiji Seika Kaisha have been able to use *B. subtilis* F-2-01 to produce up to 50 $g.L^{-1}$ of γ-PGA (Tanaka *et al.*, 1997). Yigeda Bio-Technology, Shanghai Rundo Biotechnology Japan Co., Ltd. and Nanjing Saitai Si Biological Technology Ltd. possess patents and commercially produce γ-PGA for various applications.

A significant portion of the production cost of γ-PGA is associated with nutrient provision. Thus for production of commodity products, cost effective nutrient sources are required. Domestic wastewater has potential to provide these nutrients.

Table 2.5: Composition of typical residential untreated wastewater.

Constituent	Unit	Range	Typical
Total Solids	[mg.L^{-1}]	300-1200	**700**
Dissolved	[mg.L^{-1}]	250-850	**500**
Fixed	[mg.L^{-1}]	150-550	150
Volatile	[mg.L^{-1}]	100-300	150
Suspended	[mg.L^{-1}]	100-400	**220**
Fixed	[mg.L^{-1}]	30-100	70
Volatile	[mg.L^{-1}]	70-300	150
Setteable	[mg.L^{-1}]	50-200	100
BOD	[mg.L^{-1}]	100-400	250
TOC	[mg.L^{-1}]	100-400	250
COD	[mg.L^{-1}]	200-1,000	500
Total Nitrogen	[mg.L^{-1}]	15-90	**40**
Organic	[mg.L^{-1}]	5-40	25
Ammonia	[mg.L^{-1}]	10-50	25
Nitrite	[mg.L^{-1}]	0	0
Nitrate	[mg.L^{-1}]	0	0
Total Phosphorous	[mg.L^{-1}]	5-20	**12**
Organic	[mg.L^{-1}]	1-5	2
Inorganic	[mg.L^{-1}]	5-15	10
Chloride	[mg.L^{-1}]	30-85	50
Sulfate	[mg.L^{-1}]	20-60	15
Alkalinity	[mg.L^{-1}]	50-200	100
Grease	[mg.L^{-1}]	50-150	100
Total Coliform	colonies/100 mL	10^6-10^8	10^7
VOCs	[µg.L^{-1}]	100-400	250

Source: Burks and Minnis, 1994.

2.14 Conclusion

γ-PGA is a versatile and environmentally-friendly polymer which usually requires large amounts of carbon and nitrogen substrate. For every mole of γ-PGA produced, a range of 2 – 20 times the carbon and nitrogen input is generally used by bacteria (Kunioka, 1997). Many bacteria require L-glutamic acid as a precursor for γ-PGA polymerization and a carbon and nitrogen source. This compound is expensive (R 2800 per kg) and can thus render the γ-PGA production process economically unfeasible. This review has shown that γ-PGA can be produced from sustainable resources and by various wild-type bacterial strains which do not require L-glutamic acid.

The use of continuous culture cultivation is an effective tool for determining kinetic relationships and provides the best quality kinetic data for growth-associated products like γ-PGA. This system is efficient for industrial use and allows for low labour cost, high productivity and good space-time utilisation of plant. It favours propagation of the best adapted organism in the culture. *B. licheniformis* is a known γ-PGA producer and the basic nutrient requirements for the growth of this bacterium have been studied. The growth profile and derived biokinetics of *B.licheniformis* can be used as a benchmark for selecting for the microorganism of choice in wastewater. The study undertaken in this thesis, seeks to not only provide an alternative to more efficient is cost-effective bioremediation of wastewater sludge, but also leverage this renewable resource as a low-cost raw material to produce a value added bio-product. This research approach could assist in the development of a sustainable and economical process for industrial scale production of γ-PGA and other valuable products.

2.15 The focus of the current study

2.15.1 Scope of study

In this project, the suitability of using wastewater as a source of nutrients for microbial growth and bioproduct formation, using a biorefinery approach is explored. The aim is to reduce the nutrient load in wastewater while producing the biopolymer poly (γ-glutamic acid) as a by-product with minimal modification of existing WWTWs. As limited kinetic data exist for bacterial species used in γ-PGA production studies in the literature, this study focuses on the growth studies of *Bacillus licheniformis* JCM2505 as a model organism. This base case is then compared to various bacterial strains isolated from the WWTW. The growth studies aim to identify the factors influencing the production of high biomass concentrations and sustained growth through using a culture medium optimisation strategy. By using the associated kinetic data obtained, a suitable continuous culture system can be proposed for incorporation into a wastewater treatment plant to select positively for *Bacillus* species, allowing for maximal growth and sustainable γ-PGA production.

2.15.2 Key questions

The key research questions that posed in this project aims to answer are:

i. What are the maximum specific growth rates and biomass concentrations that can be achieved when *B. licheniformis* is grown on Medium E and modified Medium E?

ii. Are there other ubiquitous microorganisms, native to the WWTW, which have the ability to produce γ-PGA naturally?

iii. What are the maximum specific growth rates and biomass concentrations that can be achieved when selected isolates are grown on Medium E and modified Medium E? How do these compare to the benchmark organism *B.licheniformis*?

iv. What is the optimal ratio of C: N: P in modified Medium E which will result in maximum growth and biomass concentration?

v. Can a medium reflecting wastewater composition as closely as possible, allow for good microbial growth and product formation?

vi. What reactor type and configuration can be used to treat wastewater and produce γ-PGA?

3. MATERIALS AND METHODS

3.1 Introduction

Chapter 3 gives a detailed description of the experimental methodologies and materials used in this study. The experimental approach involves the initial screening, isolation and further screening of the various bacterial strains and the subsequent medium optimisation using the selected strains for sustained growth and γ-PGA production.

3.2 Bacterial strains

Bacillus licheniformis JCM 2505 was purchased from the Japanese Culture Collection and used as a reference strain. *B. licheniformis* was maintained on tryptone-soy agar plates at 37°C. Indigenous strains were isolated from the Mitchell's Plain wastewater treatment plant, as described in Section 3.4.

3.3 Medium preparation

Two types of media, namely a complex and chemically defined medium, were used to cultivate the *B. licheniformis* strain at the initial stage of this study. All media and glassware was autoclaved at 121°C for 20 min in order to render the equipment and materials free of undesired microbial contamination.

3.3.1 Complex medium

The tryptone-soy complex medium (Roychoudhury *et al.*, 2006) consisted of 17 $g.L^{-1}$ tryptone, 3 $g.L^{-1}$ soy peptone, 5 $g.L^{-1}$ NaCl, 2.5 $g.L^{-1}$ K_2HPO_4 and 2.5 $g.L^{-1}$ dextrose. For tryptone-soy agar plates, 15 $g.L^{-1}$ of bacto agar was added. The pH of the medium was adjusted to 6.5 with 5 M NaOH, prior to sterilization by autoclaving.

3.3.2 Defined medium

The defined medium, Medium E (ME), used in this study was adapted from Birrer *et al*. (1994). This consisted of the following: 20 $g.L^{-1}$ L-glutamic acid, 80 $g.L^{-1}$ glycerol, 12 $g.L^{-1}$ citric acid, 7 $g.L^{-1}$ NH_4Cl, 1.5 $g.L^{-1}$ K_2HPO_4 and mineral salts (0.5 $g.L^{-1}$ $MgSO_4.7H_2O$, 0.104 $g.L^{-1}$ $MnSO_4.H_2O$, 0.04 $g.L^{-1}$ $FeCl_3.6H_2O$ and 0.15 $g.L^{-1}$ $CaCl_2.2H_2O$). An additional Modified Medium E (MME) was derived for the purposes of this study, and used to screen for microorganisms which overproduced γ-PGA in the absence of L-glutamic acid in the medium (glutamic acid-independent). This medium contained 20 $g.L^{-1}$ glucose instead of L-glutamic acid. In order to prevent the Maillard reaction, glucose at pH 6.5 was filter-sterilized and added into the medium after autoclaving. Both media were adjusted to an initial pH 6.5 using 5 M NaOH. L-Glutamic acid was obtained from Sigma Aldrich (Missouri, USA),

glucose and NaOH were obtained from Merck Chemicals (Wadeville, RSA) and all other medium components were from Saarchem (Honeydew, RSA).

3.4 Isolation of isolates from the WWTW

An activated sludge sample was obtained from the Mitchell's Plain wastewater treatment plant, Cape Town. The wastewater sample was screened in a bacterial culture test for pathogens at the PathCare Reference Laboratory in Cape Town and found to be free of any of *Salmonella*, *Shigella* and *Campylobacter* species. The samples were suspended in sterile distilled water and diluted by a factor of 10^{-3} to 10^{-6}. Luria-Bertani (LB) medium plates were prepared using 10 $g.L^{-1}$ tryptone (Biolab Diagnostics, Krugersdorp, South Africa), 5 $g.L^{-1}$ yeast extract (Biolab Diagnostics, Krugersdorp, South Africa), 10 $g.L^{-1}$ NaCl (Merck Chemicals, Wadeville, South Africa) and 15 $g.L^{-1}$ bacto agar (Biolab Diagnostics, Krugersdorp, South Africa). These were spread with 100 µL of the dilution series of each isolate. The plates were cultivated at 37°C for 24 hours. Single colonies were selected, based on colony morphologies in terms of shape and colour and streak-plated and maintained on isolation medium containing (in $g.L^{-1}$): glucose, 10; L-glutamic acid, 10; NH_4Cl, 5; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.1 (Saarchem, Honeydew, RSA) and bacto agar, 15 (Bajaj & Singhal, 2009). The final pH was adjusted to pH 6.5 with the addition of a 5 M NaOH solution (Merck Chemicals, Wadeville, RSA). After incubation at 37°C for 48 hours, the appearance of highly mucoid colonies on the plates were characteristic of γ-PGA-producing microorganisms.

3.5 Microscopic analysis

To assist in assessing that the culture was pure, Gram staining was performed (Cowan and Steele, 1965). The Burke modification was used (Burke, 1922). Once pure cultures had been obtained, the morphology of each isolate was studied microscopically (model no. BX40, Olympus Optical Company Ltd, Japan). Cultures were also periodically withdrawn, and wet preparations checked for contamination microscopically. All microscope slides were viewed at 100x magnification under oil immersion.

3.6 Initial growth characterisation studies

3.6.1 Screening of *B. licheniformis* JCM 2505

Three different media were used for the initial screening studies, namely complex tryptone-soy medium, as well as the defined γ-PGA screening media ME and MME. A pre-inoculum was prepared in either tryptone-soy, ME or MME by transferring a loopful of the colony from the tryptone-soy agar streaked plates to 10 mL of medium in a 125 mL Erlenmeyer shake flask followed by incubation at 37°C and 200 rpm for 48 hours. The pre-inoculum was transferred to a 500 mL flask containing 100

mL of the same tryptone-soy, ME or MME medium. This inoculum was incubated at 37°C and 200 rpm for 16 hours and used to inoculate 250 mL sterile tryptone-soy, ME or MME medium contained in a 1 litre flask to obtain a culture with a starting optical density (OD) of 0.1 at $A_{600\ nm}$. The flasks were placed on a rotary shaker at 200 rpm and 37°C for 48 hours. Optical density at 600 nm, pH, cell dry weight and substrate concentration were monitored. The experiments were performed in duplicate.

3.6.2 Optimising the inoculation strategy for *B. licheniformis* JCM 2505

In order to improve the growth rate and biomass concentration of *B. licheniformis* and to evaluate the effect of different media on growth, the inoculum strategy was optimised in a systematic approach using five steps. To start, a pre-inoculum was prepared by transferring a loopful of a colony from the tryptone-soy agar plate to 10 mL sterile tryptone-soy medium in a 125 mL Erlenmeyer shake flask and incubating this at 37°C and 200 rpm for 48 hours. The pre-inoculum was transferred to a 500 mL flask containing 100 mL tryptone-soy broth. This inoculum was incubated at 37°C and 200 rpm for 16 hours and comprised of the first step in the strategy. In the second step, this inoculum was used to inoculate 250 mL sterile tryptone-soy medium contained in a 1 litre Erlenmeyer flask to obtain a culture with a starting OD of 0.1. The culture was incubated at 37°C and monitored for 48 hours at 200 rpm. Thereafter, this culture was used to inoculate 250 mL sterile ME or MME medium contained in a 1 litre flask to obtain a culture with a starting OD of 0.1 in the third step. The culture was incubated at 37°C and monitored for 48 hours at 200 rpm. In the fourth step, a second 250 mL aliquot of ME or MME medium contained in a 1 litre flask was used. The inoculum was obtained from the flask in the third step when the cultivation period had been completed. The same starting inoculum concentration, incubation conditions and duration were used. This was repeated in a similar manner in the fifth step, using an inoculum from the preceding step. The absorbance at 600 nm and growth rate over the cultivation period were monitored. The tryptone-soy complex medium provided the necessary nutrients to allow for maximal bacterial growth. The effects of nutrient carry-over and the adaptability of the *B. licheniformis* strain to a minimal medium were then studied across the consecutive steps. A summary of the protocol is given schematically in Figure 3.1.

Figure 3.1: Schematic diagram of the protocol followed to determine growth in a complex and chemically defined medium using the sequential inoculation strategy experiment for *B. licheniformis*.

3.6.3 Preliminary screening of the isolated strains

The purified isolates were inoculated from the isolation medium plates into 10 mL of ME and MME contained in a 125 mL Erlenmeyer shake flask and incubated at 37°C in a rotary shaker (Labotec, Midrand, RSA) at 200 rpm. The OD at a wavelength of 600 nm, biomass concentration and pH were measured after 48 hours. This was carried out in a single experiment as an initial investigation into the ability of the isolates to exhibit sustained growth in the relevant chemically defined media.

3.6.4 Isolate screening for production of γ-PGA

Medium E and Modified Medium E (Section 3.3.2) were used for the experiments to screen for γ-PGA production. A pre-inoculum was prepared in either ME or MME by transferring a loopful of each colony of interest from the streaked plates to 10 mL of medium in a 125 mL Erlenmeyer flask and incubated at 37°C and 200 rpm for 48 hours. The pre-inoculum was transferred to a 500 mL flask containing 100 mL ME or MME medium. This inoculum was incubated at 37°C and 200 rpm for 16 hours and used to inoculate 250 mL sterile ME or MME contained in a 1 litre flask or a deep well microtitre plate containing 2 mL ME or MME. The deep well plates were covered with airporous seals to prevent aerial and cross-contamination (Cat. no. 899410, Thomson Solutions, California, USA). All flasks and deep well plates were inoculated to obtain a culture with a starting OD of 0.1. The flasks and plates were placed on a rotary shaker at 200 rpm and 37°C for 48 hours. Samples were taken every 3 hours for the first 15 hours of incubation and every 8 hours thereafter for the 48 hour incubation period. Six isolates were selected for this more in-depth investigation to further understand their growth kinetics. Selection was based on morphological characteristics, the findings of the preliminary growth screening experiments and the *pgsBCA* PCR analysis as described in Section 3.9. All shake flask experiments were performed in duplicate and deep well plate experiments in triplicate.

3.7 Medium optimisation and further growth characterisation studies

3.7.1 Optimisation of growth matrix in terms of C:N:P ratio

The glucose modified medium E (MME) was optimised for optimal carbon, nitrogen and phosphorus ratios for high growth rate and biomass production. A Plackett-Burman factorial design was used to develop a 3 factor, 2 level design, allowing interactions to be explored while assuming a linear response to nutrient concentration over the range studied. The baseline MME concentrations were used to calculate the various nutrient input values in $g.L^{-1}$. Eight combinations of C:N:P ratios were tested (Table 3.2) (Plackett and Burman, 1946). The trace salt concentrations from MME (Section 3.3.2) were used. The optimisation studies were performed in deep well plates as outlined in Section 3.6.4. The samples were withdrawn from the deep well plates every 3 hours for the first 12 hours and at the end of the 48 hour cultivation period and analysed for the cell growth rate, biomass

concentration, pH, substrate utilisation and the effect of the carbon, nitrogen and carbon. Only five of the selected six isolates were studied. Each experiment was performed in triplicate.

3.7.1 Further optimisation of growth matrix in terms of carbon input

In an effort to further decrease the initial carbon source concentration, an optimisation study of five additional runs using various combinations of input concentrations for the three carbon sources used in MME were investigated. These are found in Table 3.1. The growth rate and biomass formation exhibited by the isolates in response to these lower concentrations were monitored. The study was also aimed to assist in determining the medium carbon source or sources which were most important for sufficient isolate growth. The baseline medium nitrogen source (NH_4Cl), phosphate source (K_2HPO_4) and trace salt concentrations were used (Section 3.3.2). The optimisation studies were performed in deep well plates as outlined in Section 3.6.4. The samples were withdrawn from the deep well plates every 3 hours for the first 12 hours and at the end of the 48 hour cultivation period and analysed for the cell growth rate and biomass concentration were determined. The two isolates that displayed good growth and a positive result for the *pgsBCA* gene complex were used for these triplicate experiments.

Table 3.1: The carbon sources and corresponding initial concentrations in $g.L^{-1}$ for the further optimisation study.

Carbon source	Run 9	Run 10	Run 11	Run 12	Run 13
Glycerol	1	5	1	1	1
Glucose	20	5	10	20	2
Citric acid	12	5	5	1	1

Table 3.2: C:N:P experimental matrix with relative nutrient inputs for the optimisation study.

Run #	**Factors**			**Ratio**			**Inputs ($g.L^{-1}$)**
	Carbon	Nitrogen	Phosphorus	C	N	P	
	High=4	High=0.26	High=0.0172				
	Low=2	Low=0.065	Low=0.0043				
1	low	low	low	2	0.065	0.0043	glucose = 10.82; glycerol = 43.27; citric = 6.49; NH_4Cl = 3.48; K_2HPO_4 = 0.87
2	low	low	high	2	0.065	0.0172	glucose = 10.82; glycerol = 43.27; citric = 6.49; NH_4Cl = 3.48; K_2HPO_4 = 2.99
3	low	high	low	2	0.26	0.0043	glucose = 10.82; glycerol = 43.27; citric = 6.49; NH_4Cl = 13.91; K_2HPO_4 = 0.87
4	low	high	high	2	0.26	0.0172	glucose = 10.82; glycerol = 43.27; citric = 6.49; NH_4Cl = 13.91; K_2HPO_4 = 2.99
5	high	low	low	4	0.065	0.0043	glucose = 21.63; glycerol = 86.53; citric = 12.98; NH_4Cl = 3.48; K_2HPO_4 = 0.87
6	high	low	high	4	0.065	0.0172	glucose = 21.63; glycerol = 86.53; citric = 12.98; NH_4Cl = 3.48; K_2HPO_4 = 2.99
7	high	high	low	4	0.26	0.0043	glucose = 21.63; glycerol = 86.53; citric = 12.98; NH_4Cl = 13.91; K_2HPO_4 = 0.87
8	high	high	high	4	0.26	0.0172	glucose = 21.63; glycerol = 86.53; citric = 12.98; NH_4Cl = 13.91; K_2HPO_4 = 2.99

3.8 Bioreactor studies

3.8.1 Batch growth culture conditions

Bioreactor growth studies were conducted in a series of 300 mL Sixfors® bioreactors with a 250 mL working volume using the optimised medium (Infors, Bottmingen, Switzerland). The optimised MME (Run 14) consisted of 20 $g.L^{-1}$ glucose, 1 $g.L^{-1}$ glycerol, 12 $g.L^{-1}$ citric acid, 3.48 $g.L^{-1}$ NH_4Cl, 2.99 $g.L^{-1}$ K_2HPO_4 and mineral salts (0.5 $g.L^{-1}$ $MgSO_4.7H_2O$, 0.104 $g.L^{-1}$ $MnSO_4.H_2O$, 0.04 $g.L^{-1}$ $FeCl_3.6H_2O$ and 0.15 $g.L^{-1}$ $CaCl_2.2H_2O$) as determined in Section 3.7. The growth medium was sterilized in the bioreactor at 121°C for 20 min. The inoculum strategy described in Section 3.6.4 was followed. The temperature of the medium was measured by a Pt100 temperature sensor and controlled by an aluminium heating block and cooling water. The pH was measured using a pH probe(405-DPAS-SC-K8S120, Mettler Toledo, Ohio, USA) and maintained at a constant value by automatic titration of 5 M NaOH and 5 M HCl. Dissolved oxygen was measured using an oxygen probe (InPro 6100/220/S/N, Mettler Toledo, Ohio, USA). Compressed air was filtered with a 0.2 µm Millipore Millex® membrane filter (Merck, Modderfontein, RSA) and sparged into the vessel to provide a source of oxygen. The stirrer system consisted of a regulated magnetic drive equipped with two six-blade Rushton impellers which allowed for the mixing of the contents in the bioreactor. Four baffles were also included around the bioreactor. Antifoam 204 was added to prevent excessive foaming (Sigma Aldrich, Missouri, USA). The IRIS version 5.1 software was used to monitor the temperature, dissolved oxygen, agitation rate and pH. The set values were as follows: temperature: 37°C, agitation: 200 revolutions per minute (rpm), initial aeration rate: 1.0 volume of air per volume of medium per minute (vvm) and pH: 6.5. Samples were taken every 3 hours for the first 12 hours of incubation and every 12 hours thereafter over the 48 hour incubation period. Cell growth rate, biomass concentration, and substrate utilisation were measured.

3.8.2 Continuous growth culture conditions

The Infors-HT Sixfors® stirred bioreactor was also used for the continuous culture experiments using the same medium and inoculation strategy and culture conditions described in Section 3.8.1. A working volume of 0.25 L was maintained. The strain was grown in batch culture until sufficient biomass was present to enable continuous culture to commence. On establishing the continuous feed, approximately one 2 mL sample was taken every three hours and analysed for biomass until constant values were obtained. This was used an indicator for steady state conditions. Steady state was assumed after three residence times and where the biomass concentration had stabilised. Triplicate samples were taken after steady state was reached for each of five dilution rates (0.11, 0.12, 0.13, 0.14 and 0.2 hr^{-1}) over at least three residence times and measured for cell dry weight.

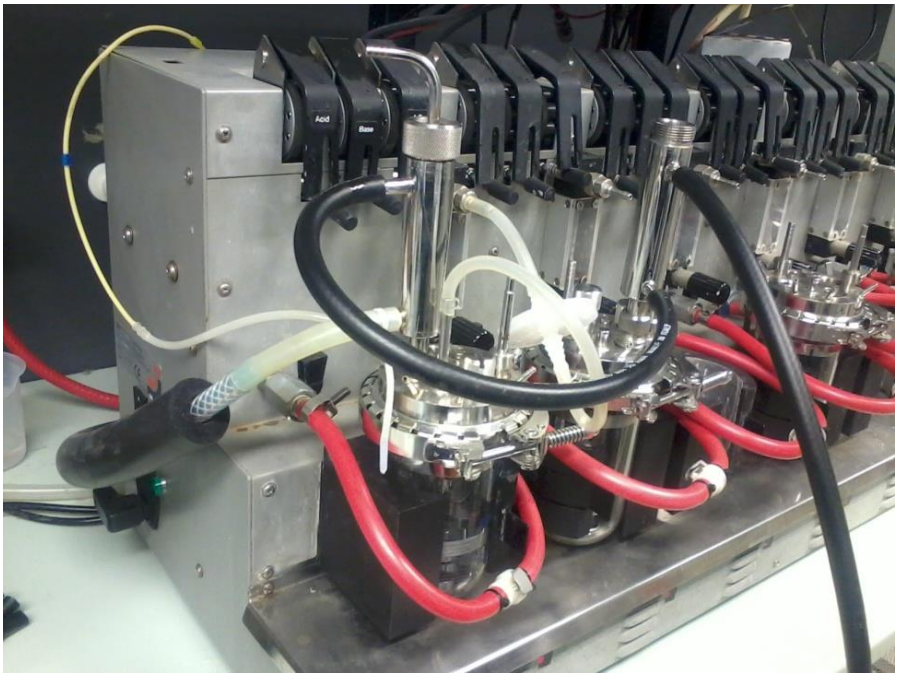


Figure 3.2: The Infors-HT Sixfors® bioreactor used in this study.

3.9 Isolate screening based on genetic capability to synthesize γ-PGA

3.9.1 Extraction of genetic material from isolated strains

A loopful of colony of all the purified isolates was selected from the individual LB agar plates and each inoculated separately into 5 mL of LB medium in a 50 mL Erlenmeyer flask and incubated at 37°C and 200 rpm for 16 hours. The cultures were then centrifuged at 10,000 x g for 10 min to pellet the cells (Heraeus Biofuge Pico, Kendro Laboratory Products, Hanau, Germany). The cells obtained were incubated in 500 µL lysis buffer (Roche Diagnostics, Mannheim, Germany) for 16 hours at -60°C. Genomic deoxyribonucleic acid (DNA) from the isolated strains was extracted using the High Pure PCR Template Preparation Kit® (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

3.9.2 Primer design

The nucleotide sequence (accession number AB016245) of the three genes which encode a poly-γ-glutamate synthetic system in *Bacillus* was obtained from the National Centre for Biotechnology Information (NCBI) database website (Ashiuchi *et al*., 1999). Poly (γ-glutamic acid) synthetase forward (PGSF) and poly (γ-glutamic acid) synthetase reverse (PGSR) primers were designed using DNAMAN version 4.13 software (Lynnon BioSoft) to amplify an internal fragment of this gene cluster (Figure 3.3).

Figure 3.3: The designed primers and their respective binding regions.

Additionally, a multiple sequence alignment was performed using all documented sequences for poly-γ-glutamate synthetic genes from the NCBI database (GenBank accession numbers JF343561.1, JF343562.1, JF343563.1, HM034756.1, HM034757.1, AL009126.3, CP002183.1, DQ086153.1, AB016245.1, EF066513.1, GQ249061.1, GQ249062.1, HQ599194.1, EF066513.1). The consensus sequence obtained was then used to design an additional set of primers, PGS2F and PGS2R (Table 3.3) which could bind to the same region. These primers also amplified an internal fragment but annealed to a different region. The two sets of primers were synthesised at the Department of Molecular and Cell Biology, University of Cape Town.

Table 3.3: The primers and corresponding sequences used for the detection of the presence of the poly (γ-glutamic acid) synthetase gene complex in the isolates.

Primer	Sequence (5' to 3')	Reference
PGSF	GACGTATTGCCTTATATTGAAGC	This study
PGSR	TCTGCCCCTTTTTGCTCCG	This study
PGS2F	GTGGTTACTCATTATAGCCTGTGC	This study
PGS2R	GCCGACGCCATATATGACACG	This study

3.9.3 Amplification of *pgsB*, *pgsC* and *pgsA* genes

An end-point polymerase chain reaction (PCR) was used to verify the ability of the selected isolates to produce γ-PGA by detecting the presence of the *pgsBCA* genes. The reaction mixture comprised 1 µL of each primer at a concentration of 0.25 µM, 200 µM deoxyribonucleotide triphosphate mixture, 2.5 mM $MgCl_2$, 2 units Taq polymerase, 2.5 µL reaction buffer (Kapa Biosystems, Cape Town, RSA) and 50 ng of genomic DNA in a 25 µL final reaction volume. Prior to addition into the reaction, the DNA was quantified using the NanoDrop 2000 (Thermo Fisher Scientific, Massachusetts, USA) and diluted accordingly using sterile nuclease-free water (Cat. no. 129114, Whitehead Scientific, Cape Town, RSA). After an initial denaturing step of 5 min at 95°C, amplification was performed for 32 cycles under the following conditions: 5 min at 95°C, 30 s at 95°C, 60 s at 58°C, 30 s at 72°C and a final

extension step of 72°C for 3 min. All PCR reactions were carried out in a PCR Sprint Thermal Cycler (Thermo Hyabaid, Middlesex, UK). The PCR product was analysed by loading 12 µL of each product on a 0.8% (w/v) agarose gel (prepared in TAE buffer (40 mM Tris-acetate and 1 mM EDTA) containing ethidium bromide (0.5 $\mu g.mL^{-1}$)) at 70 V for approximately 60 min and viewed under UV light using the G.Box SynGene system (SynGene, England, UK). The Fermentas GeneRuler™100 bp DNA Ladder (Cat. no. SM0243, Thermo Fisher Scientific, Massachusetts, USA) was used as a molecular weight marker. The presence of a 1.1 kb fragment confirmed the *pgsBCA* gene locus. The product of this locus is necessary to confer a cell the ability to produce γ-PGA.

The identification of the PCR fragment was confirmed by standard sequencing by Macrogen Inc. Europe (Amsterdam, The Netherlands) using the designed primers, following gel extraction and purification using the Qiagen QIAquick® Gel Extraction Kit (Cat. no. 28706, Whitehead Scientific, Cape Town, RSA), according to the protocol provided. The purified product was then quantified using the NanoDrop 2000 (Thermo Fisher Scientific, Massachusetts, USA).

3.10 Identification of unknown isolates by *16S rRNA* gene sequencing

The five strains which were used in the optimisation experiments were subjected to *16S rRNA* gene amplification and thereafter sequenced. *B. licheniformis* JCM 2505 was used as a positive control and Qiagen nuclease-free water as a negative control. Universal bacterial primers 27F and 1492R (Table 3.4), as previously described by Eden *et al.* (1991), were used for the PCR. The reaction mixture comprised of 1 µL of each primer at a concentration of 0.25 µM, 25 µL of Kapa SYBR Fast Mastermix (Cat. no. KK4601, Kapa Biosystems, Cape Town, RSA), and 50 ng of genomic DNA or water (control) in a 50 µL reaction volume. Amplifications included an initial denaturing step of 96°C for 5 min, further denaturing at 96°C for 30 s followed by annealing of the primers at 55°C for 30 s, with an extension for 90 s at 72°C performed for 32 cycles and a final extension step of 72°C for 3 min. After amplification, 12 µL of each reaction was loaded on a 0.8% (w/v) agarose gel, containing ethidium bromide, in TAE buffer by electrophoresis at 70 V for approximately 60 min. The KAPA™ Express Ladder (Cat. no. KK6304, Kapa Biosystems, Cape Town, RSA) was included in each gel. The gels were viewed and photographed under short wavelength UV light using GeneSnap software (SynGene, England, UK).

The 1.6 kb PCR product was extracted from the gel and purified using the Qiagen QIAquick® Gel Extraction Kit according to the manufacturer's instructions. The purified product was then quantified using the NanoDrop 2000 and sent for *16S rRNA* full sequencing by Macrogen Inc. in Europe (Amsterdam, The Netherlands).

Table 3.4: The primers and corresponding sequences used in experiments for the detection of the *16S rRNA* gene in the isolates.

Primer	Sequence (5' to 3')	Reference
27F	GAGAGTTTGATCCTGGCTCAG	Eden *et al.* (1991)
1492R	GTACGGTTACCTTGTTACGACTT	Eden *et al.* (1991)

3.11 Bioinformatics

The *pgs* and *16S rRNA* sequence results were edited using Chromas 2.01 (Technelysium, Queensland, Australia) and DNAMAN version 4.13 (Lynnon BioSoft). The identity of the gene and isolates was determined by comparing the query sequences to related DNA sequences found on the National Centre for Biotechnology Information (NCBI) database website. For this, a nucleotide Basic Local Alignment Search Tool (BLAST) was used (Altschul *et al.*, 1997).

3.12 Recovery and purification of γ-PGA

The γ-PGA isolation strategy, previously described by Ashiuchi *et al.* (1999), was employed. The culture medium was centrifuged at 10,000 x g for 1 hour to remove cells (JA-10 rotor, Avanté J-E centrifuge, Beckman Coulture, Johannesburg, RSA). The cells were washed twice with 8 mL 0.14 mM NaCl to collect the remaining polymer adhering to the cells. The wash solution was combined with the supernatant. The pH of the solution was adjusted to pH 3.0 with concentrated H_2SO_4 and incubated at 4°C for 12 hours to remove polysaccharides. The resulting solution was poured into three volumes of ethanol. The solution was centrifuged at 10,000 x g for 0.5 hour and the precipitate was collected and dissolved in 10 mL 0.2 M Tris-HCl buffer (pH 8.0) and dialyzed three times against 2 L 10 mM Tris-HCl buffer (pH 8.0) at 25°C overnight (Sigma Aldrich, Missouri, USA). The dialyzed solution was lyophilized, dissolved in 1 mL of 10 mM Tris-HCl buffer (pH 8.0) to form a concentrate, and then centrifuged at 10,000 x g for 1 hour. The supernatant was incubated at 37°C for 12 hours with 20 $\mu g.mL^{-1}$ Proteinase K to remove α-polypeptides (Roche Diagnostics, Mannheim, Germany). The resulting solution was dialyzed three times against 2 L distilled water at 25°C overnight, and centrifuged at 10,000 x g for 1 hour. The solution was lyophilized, and the dry matter was used to analyse the γ-PGA content and molecular weight as described in Section 3.13.6. Residual polysaccharide and lipid content were determined by the phenol-sulphuric acid (Dubois *et al.*, 1956) and direct transesterification (Griffiths, 2011) methods respectively, which are further described in Appendix B and C.

3.13 Analytical procedures

3.13.1 Measurement of optical density

Cell growth was monitored by measuring the OD or turbidity of the samples at a wavelength of 600 nm using a Helios Alpha spectrophotometer model no. UVA 161607 (Thermo Scientific, Kempton Park, RSA). Dilution of aliquots with distilled water was carried out to obtain absorbance values between 0.1 and 1.0 absorbance units, and these were corrected for the dilution factor after appropriate dilution with distilled water.

3.13.2 Determination of biomass concentration

An Adventurer Centrifuge (model no. AR2140, Ohaus Corp., New Jersey, USA) was used to centrifuge 1 mL cell suspension in pre-weighed Eppendorf tubes. The supernatant was filtered using a 0.22 µm membrane filter (Merck, Modderfontein, RSA) and used for substrate and product analyses as described in Sections 3.13.3 and 3.13.6.1 respectively. The harvested pellet was retained, washed with phosphate buffer saline (in $g.L^{-1}$: NaCl 8.00; KCl 0.20; Na_2HPO_4 1.44; KH_2PO_4 0.24; pH 7.4), dried at 80°C for 48 hours, left to cool in a desiccator cabinet overnight and thereafter measured to a constant weight to determine the dry cell weight. This was performed in triplicate.

3.13.3 Determination of substrate utilisation

The concentrations of glucose, glycerol and citric acid were determined by high performance liquid chromatography (HPLC) (Finnigan Surveyor Plus, Thermo Scientific, Kempton Park, RSA) equipped with a Refractive Index (RI) detector (Finnigan Surveyor RI Plus, Thermo Scientific, Kempton Park, RSA) using a Biorad Aminex® HPX-87H organic acid column (7.8 mm x 300 mm, Biorad, Cape Town, RSA) and 0.01M H_2SO_4 as the mobile phase. A flow rate of 0.5 $mL.min^{-1}$ was used with the column oven set at 60°C. The injection volume was 10 µL. A standard calibration curve of known concentrations was used to determine the corresponding substrate concentration in the samples. A more detailed description is given in Appendix A.

3.13.4 Crude protein assay

The purified γ-PGA was hydrolyzed with 6 N HCl and 15% phenol (Merck, Modderfontein, RSA) at 110°C for 24 h in vacua according to the procedure as described in the AOAC official method 994.12 (Horwitz, 2003). A homemade hydrolysis system which comprises of two pipes supplying gas (oxygen and nitrogen) that is connected to a “T” glass tube was used. The third open end connects the hydrolysis tube. The total crude protein was determined in the hydrolysate using the FP-528 Protein/Nitrogen Determinator instrument (LECO Corp., Michigan, USA). Alfafa (LECO Corp.,

Michigan, USA) was used as a calibration standard (King-Brink and Sebranek, 1993). This assay was performed in triplicate at the Department of Animal Sciences, University of Stellenbosch.

3.13.5 Amino acid analysis

A 10 µL aliquot of the undiluted hydrolysate obtained as described in Section 3.13.4 above was added to the Waters AccQ Tag Kit (Cat. No. 186003836, Waters, Massachusetts, USA) constituents and placed in a heating block at a temperature of 55°C for 10 min. The Waters AccQ Tag Ultra Reagent Powder (US Patent 5 296 599 and European Patent EP 0 533 200 B1) in the AccQ Tag Kit contains the derivatizing reagent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), which converts both primary and secondary amino acids into stable fluorescent derivatives that are amenable to UV-absorbance and mass spectrometric detection (Kaspar *et al.*, 2009; Pappa-Louisi *et al.*, 2007). Thereafter, it was analysed on the Waters Micromass Quattro *micro* API™ spectrometer after derivatization (Waters, Massachusetts, USA). The flow rate was set at 0.7 $mL.min^{-1}$ with 1 µL of sample injected and eluted with acetonitrile on an AccQ Tag C18 column (1.7 µm, 2.1x100 mm, Waters, Massachusetts, USA). This assay was performed by the Central Analytical Facility, University of Stellenbosch.

3.13.6 Characterisation of poly (γ-glutamic acid)

3.13.6.1 Quantification of γ-PGA

The γ-PGA analysis was carried out by HPLC (model no. AS3000, Thermo Scientific, Kempton Park, RSA) equipped with a RI detector using a TSKgel G5000 PW_{XL} gel permeation chromatography (GPC) column (7.8 mm x 300 mm, Tosoh, Tokyo, Japan). A 10 µL filtered sample was injected in each run. Samples were eluted with distilled water at a flow rate of 0.5 $mL.min^{-1}$ with the column oven set at 80°C. The purified γ-PGA was used as a standard to determine the concentration. Polyethylene oxide standards of known molecular weight were used to construct a calibration curve (Waters, Bellville, RSA) (Zhong *et al*., 2009).

3.13.6.2 Molecular size verification

The lyophilized γ-PGA was re-suspended in 0.2 M $NaNO_3$ to a final concentration of 1 $mg.ml^{-1}$. Particulate matter was removed by passing the samples through a 0.45 µm membrane filter (Merck, Modderfontein, RSA). A 100 µL sample was analysed on a size exclusion Ultrahydrogel Linear column (7.8 mm x 300 mm, Waters, Bellville, RSA) with 0.2 M $NaNO_3$ as an eluent after filtering and degassing. In addition to the samples, nine polyethylene oxide standards ranging from 20 000 Da to 895 500 Da were also analysed. The column temperature was set at 45°C and the flow rate at 0.6 $mL.min^{-1}$. The Waters R401 refractive index detector was used. Data was captured using the A/D data logger with REFLOG3 program. One point per second recorded and every 2 points averaged to give

one data point per 2 seconds. The obtained data were transferred to MS Excel and analysed on a template spreadsheet containing sample information, the linear regression equation from the calibration curve, and calculations of Mw, Mn and PD (Parolis and Harris, 2007).

The graph of the elution volume (ev) of the standards at the peak *vs.* log of their peak molecular weight (given by manufacturer) was plotted and used to obtain the equation of the linear regression line (a straight line with y = mx + c). To obtain the molecular weight of an unknown sample, the following formula was used:

$$\mathrm{Mw} = 10^{[(m \times ev) + c]} \qquad (3.1)$$

The average molecular weight (Mw) is calculated as follows:

$$\mathrm{Mw} = \frac{\Sigma\,(detector\ response_i \times Mw_i)}{\Sigma\,(detector\ response_i)} \qquad (3.2)$$

Number average molecular weight (Mn) is calculated as follows:

$$\mathrm{Mn} = \frac{\Sigma\,(detector\ response_i)}{\Sigma\,(detector\ response_i / Mw_i)} \qquad (3.3)$$

Polydispersity (PD) is defined as Mw/Mn. All measurements were performed in triplicate. The average was reported.

3.14 Kinetic parameters

In order to compare the growth curves for each isolate in the different media, various parameters were calculated based on the changes of the OD over time. From the growth curve for each bacterial strain in both growth media, the following parameters were calculated:

μ_{max} = Maximum specific growth [hr^{-1}]

X_{max} = Maximum biomass concentration [$g.L^{-1}$]

ΔX = Change in biomass concentration [$g.L^{-1}$]

Integration of the Malthus Equation (3.4) yields the straight line function, given in Equation 3.5. In the exponential phase where μ remains constant at μ_{max}, a straight line of slope μ_{max} is obtained on plotting the natural log of the cell number as a function of time:

$$\frac{\mathrm{dx}}{\mathrm{dt}} = \mu_{max} X \quad (3.4)$$

$$\ln \mathrm{X} = \mu_{max} t + ln X_i \quad (3.5)$$

where X is the cell biomass concentration, t the incubation time and X_i is the initial cell biomass concentration at time 0 hour.

The X_{max} was determined after 48 hours when cell growth was in its stationary phase, and the ΔX was determined by subtracting the biomass concentration at the beginning of the experiment from $X_{max.}$

3.15 Experimental approach

To investigate the growth requirements, substrate utilisation and the effect of these on product formation of typical γ-PGA producers, a *B. licheniformis* reference strain was obtained from a culture collection and grown in 48 hour shake flask cultures using a medium and specific growth conditions as recommended in the literature. The growth was assessed by measuring absorbance and cell dry weight over the cultivation period. Additionally, the suitability of a more cost-effective modified alternative for Medium E was studied using the same experimental methodology and the effect of the two media on the growth characteristics of this bacterium were compared.

In order to assess whether suitable microbial species were naturally present in the chosen Mitchell's Plain WWTW, an aerobic sludge was collected and inoculated on Luria-Bertani medium agar plates and 18 isolates were selected based on differing plate morphologies. These isolates were initially screened in terms of: morphological growth characteristics on isolation medium agar plates for γ-PGA producers as described in the literature, the presence or absence of the polyglutamate synthetase (*pgs*) gene complex which confers the ability to synthesise γ-PGA, and finally, growth in ME and MME. Based on these three screening tools, six isolates which showed the best potential for robust growth, biomass formation and γ-PGA production were selected for further screening in both shake flasks and deep well plates using the same methodology as the initial *B. licheniformis* screening and these results were compared to the *B. licheniformis* base case. A complete methodology using both shake flasks and deep well plates was presented and appropriate analytical protocols selected.

The literature reports of studies which have found certain medium components to be necessary for growth and control. However, different bacterial strains have different nutritional requirements. Medium optimisation was undertaken to identify optimal carbon, nitrogen and phosphorus molar ratios in the media and to assess the impact of medium composition on growth and biomass accumulation by the selected isolates in grown deep well plates. An optimised medium, which was comparable to the nutritional composition of domestic wastewater sludge, was used in batch and continuous

cultures. These cultivations were conducted by growing one isolate in a carefully monitored bioreactor and analysing the biomass and substrate concentrations throughout the cultivation period.

Finally, the extracted polymer from the *B. licheniformis* reference strain and two of the selected isolate cultures were characterised in terms of amino acid composition and molecular weight. The isolates selected for detailed screening and the medium optimisation studies were identified using *16S rRNA* gene sequencing and any existing phylogenetic relationships amongst the different isolates were studied.

The obtained growth kinetic data using this research approach were then used to inform the recommendations of the methodology to be employed when real wastewater experiments are performed to select for the optimised growth and polymer formation by the chosen bacterial strain amongst a non-sterile, mix-cultured and low nutrient environment.

4. INITIAL GROWTH STUDIES USING *BACILLUS LICHENIFORMIS*

4.1 Introduction

The *Bacillus licheniformis* strain JCM 2505 has been previously used in the literature as a reference strain when investigating the phylogeny of γ-PGA-producing strains (Meerak *et al*., 2007 and 2008).

In this chapter, the growth of *B. licheniformis* JCM 2505 is assessed in conical shake flask cultivations containing ME and MME. The results for the cell dry weight, substrate utilisation, growth rate, and change in pH over time are presented and discussed.

Furthermore, the effect of growth in a tryptone soy broth complex medium was compared to the growth displayed by the bacterium in the chemically defined media in a series of shake flask experiments. These results are also presented.

4.2 Initial screening results and discussion

Cultivations were conducted with *B. licheniformis* JCM 2505 in shake flasks containing ME and MME. The results are presented below.

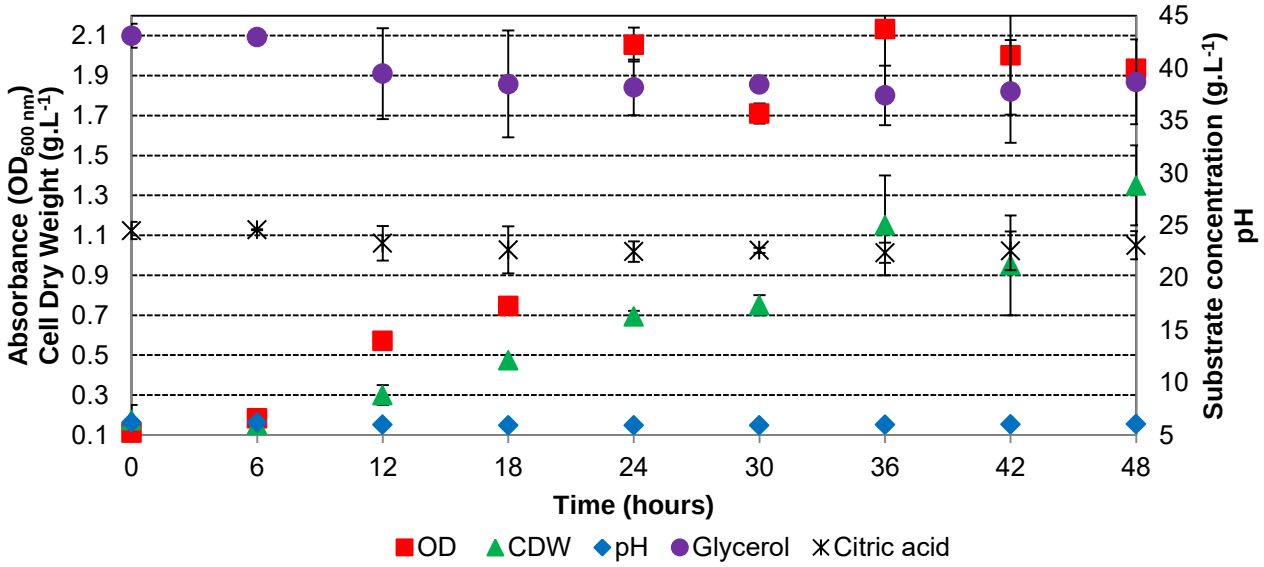


Figure 4.1: Growth profile for *B. licheniformis* grown in ME at 37°C. The averages of duplicate experiments are shown.

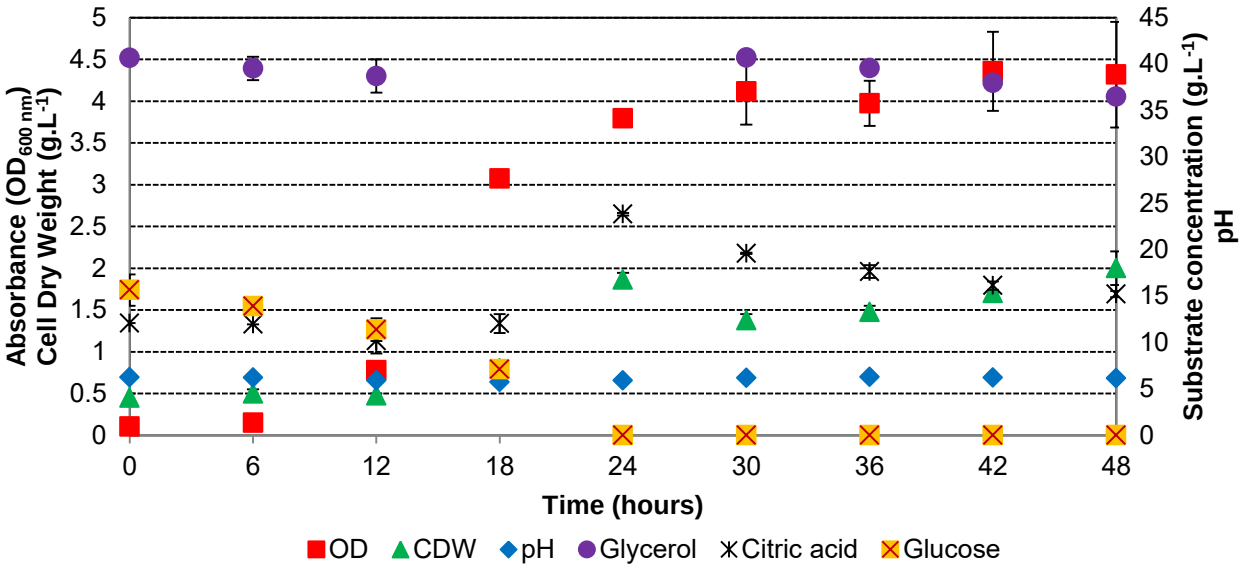


Figure 4.2: Growth profile for *B. licheniformis* grown in MME at 37°C. The averages of duplicate experiments are shown.

Figure 4.1 shows the growth profile, substrate consumption and pH values of *B. licheniformis* grown in the ME and Figure 4.2 that of MME. Cell dry weight values of 1.35 $g.L^{-1}$ and 2.0 $g.L^{-1}$ were obtained after the 48 hour cultivation in ME and MME, respectively. The pH values were similar in the two cultivations, and ranged between pH 5.73 and 6.27. The substrate analyses indicated minimal citric acid utilisation during the 48 hour cultivation. An increase in citric acid concentration was observed in Figure 4.2. This could be possibly due to an error in measurement. The sample could have also been compromised, as the chromatograms did not produce peaks for glycerol for the some samples obtained at the affected time points (18 and 24 hours post inoculation). The glycerol concentration decreased from 43.1 $g.L^{-1}$ to 38.7 $g.L^{-1}$, and 40.7 $g.L^{-1}$ to 36.5 $g.L^{-1}$, respectively, for ME and MME. For MME, glucose was fully consumed after 24 hours. The maximum specific growth rate in ME and MME were 0.082 and 0.119 hr^{-1}, respectively.

4.3 Optimising inoculum strategy results and discussion

Due to the low growth rates obtained, the effect of using a combination of nutrient-rich complex medium and minimal medium on *B. licheniformis* growth was investigated. Growth in a complex medium (tryptone soy broth) was compared to that in a chemically defined medium (ME and MME) in a series of shake flask experiments (Figure 4.3 to Figure 4.6). The inoculum was prepared in tryptone soy broth medium. A small sample was used to inoculate the first shake flask also containing tryptone soy broth medium to a final $A_{600\ nm}$ reading of 0.1 and incubated for 48 hours (step 2). Three subsequent inoculation and cultivation stages (similar to the first step) were conducted into flasks containing ME or MME, using a sample from the previous step as inoculum. The differences in the maximum absorbance and growth rates obtained after each step were monitored. No change in the

final maximum OD value was evident after three transfers to MME (Figure 4.4). In contrast, the maximum OD values achieved in ME decreased after two transfers (step 4, Figure 4.3). This could be due to the depletion of the additional growth factors supplied through the complex medium during the second transfer into a chemically defined medium. Interestingly, the maximum OD value during the third transfer to chemically defined medium (step 5) was again similar to the original maximum values achieved during step 2 and 3 in ME, indicating that the culture was slowly adapting better to the minimal medium and utilising the same amount of carbon in both media.

The maximum specific growth rates (μ_{max}) obtained during step 3 and 4 (first two transfers to the chemically defined medium) in both ME and MME was lower than the μ_{max} values measured during growth in the complex medium (step 2) (Figure 4.5 and Figure 4.6). The μ_{max} value decreased from 0.184 hr^{-1} to 0.05 hr^{-1} and 0.08 hr^{-1} for ME and MME, respectively, after two transfers (step 4). Maximum specific growth rates were higher in both media after the third transfer (step 5) with values of 0.11 hr^{-1} and 0.16 hr^{-1} for ME and MME, respectively, indicating that the cultures had adapted to the new medium. However, these maximal growth rates were not as high as in step 2 which meant that even though the maximum OD was similar, the cells were still growing more slowly in ME and MME. It is hypothesized that given more time, the cells could get back to the original growth rate after more adaptation.

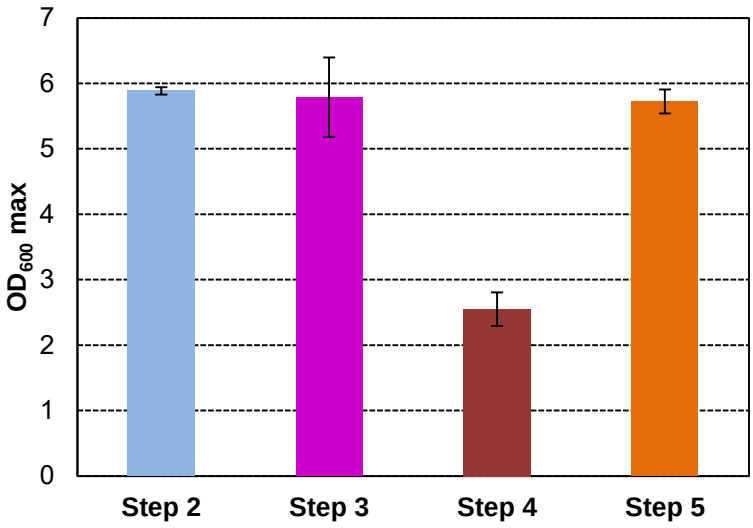


Figure 4.3: Maximum OD values obtained after growth of *B. licheniformis* at 37°C in shake flasks for 48 hours (average of duplicate experiments). The medium used for Step 2 was tryptone soy broth and ME for Step 3 to 5.

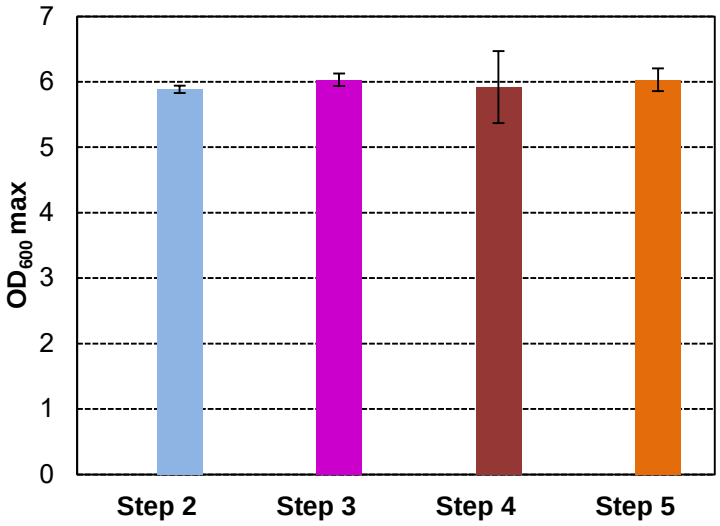


Figure 4.4: Maximum OD values obtained after growth of *B. licheniformis* at 37°C in shake flasks for 48 hours (average of duplicate experiments). The medium used for Step 2 was tryptone soy broth and MME for Step 3 to 5.

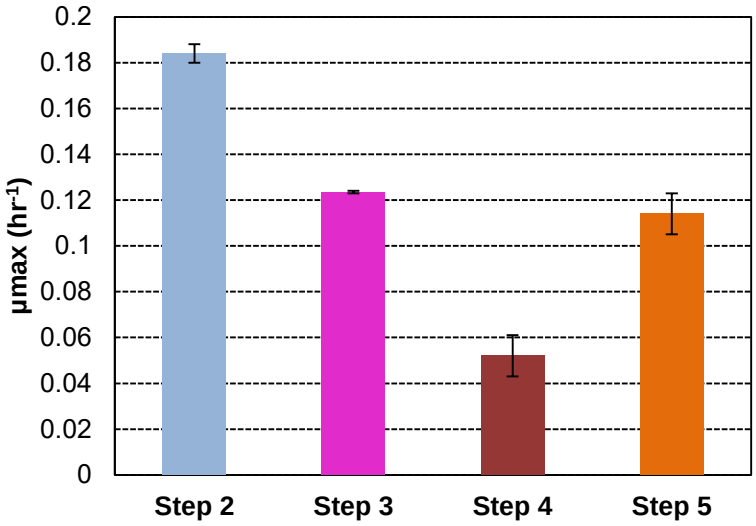


Figure 4.5: Maximum specific growth rate obtained after growth of *B. licheniformis* at 37°C in deep well plates for 48 hours (average of duplicate experiments). The medium used for Step 2 was tryptone soy broth and ME for Step 3 to 5.

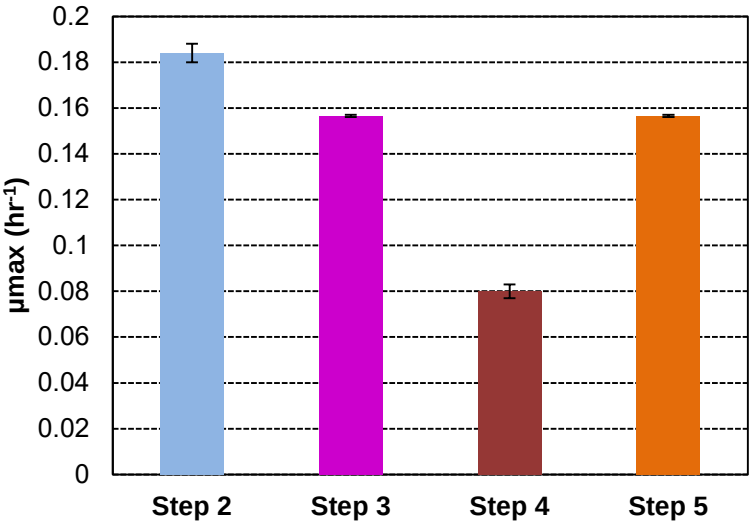


Figure 4.6: Maximum specific growth rate obtained after growth of *B. licheniformis* at 37°C in deep well plates for 48 hours (average of duplicate experiments). The medium used for Step 2 was tryptone soy broth and MME for Step 3 to 5.

4.4 General discussion

B. licheniformis JCM 2505 was screened in three media – from a highly nutritious complex medium to a more cost-effective modified chemically defined medium. The differences in growth kinetics between these media were elucidated. As expected, the bacterium displayed a prolonged adaptation phase to the chemically defined media, producing final biomass concentrations and maximum specific growth rates which averaged 1.7 $g.L^{-1}$ and 0.1 hr^{-1} respectively. Previously, the presence of L-glutamic acid in the medium has been shown to be important (Cromwick and Gross, 1995), albeit a glutamate independent strain has been isolated (Abdel-Fattah *et al*., 2007). The bacterium was able to use the medium containing glucose even effectively, producing higher measured values for biomass and μ_{max} than the medium containing conventional L-glutamic acid. It is important to note that glucose contains six carbon molecules, whereas L-glutamic acid contains five. The L-glutamic monosodium salt used in the medium does however; contribute to an additional nitrogen source. It is hypothesized that the improved growth displayed by the bacterium in the glucose could be either due to more carbon in the medium, or a more efficient metabolic assimilation of glucose in the TCA cycle.

The biomass produced by the *B. licheniformis* strain as well as the rate of growth were much lower than those previously reported in the literature. Cromwick *et al.* (1996) reported an increase of biomass to 4 $g.L^{-1}$ using another strain of *B. licheniformis*, ATCC 9945A, grown Medium E in controlled batch bioreactor cultivations at pH 6.5. Du *et al.* (2005) grew WBL-3, a mutant strain of ATCC 9945A under similar conditions and obtained 4.1 $g.L^{-1}$ biomass. An optimisation strategy which included an initial step in complex media, then carryover into the chemically defined media was implemented. Even though the OD, which can be used as an indicator for biomass, improved after three transfers into both media, matching growth in complex medium with readings of 6 and 5.7 for

MME and ME respectively, the μ_{max} improvement in this minimal medium required a longer period. The growth rate of 0.18 hr^{-1} obtained in complex medium could not be matched, even after three transfers. *B. licheniformis* grew at a maximal specific growth rate of 0.16 hr^{-1} in MME and 0.11 hr^{-1} in ME. Due to these findings, the focus of the project was diverted from *B. licheniformis* to obtaining a bacterial strain which could produce biomass more efficiently in dilute minimal medium, with a shorter adaptation period.

5. ISOLATION AND CHARACTERISATION OF POLY (Γ-GLUTAMIC ACID) PRODUCING STRAINS

5.1 Introduction

A sludge sample obtained from the Mitchell's Plain wastewater treatment plant in Cape Town was used to isolate several microorganisms. These microorganisms were selected based on their morphology. Strains which displayed characteristics that are reflective of the ability to produce γ-PGA are highlighted. The results are presented below.

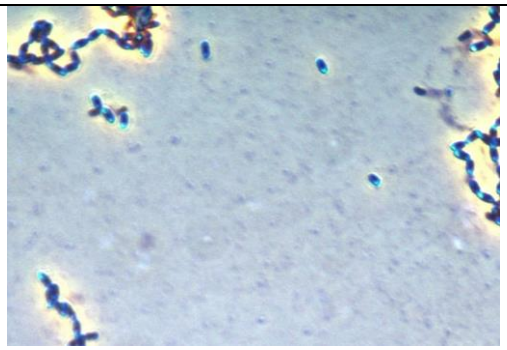
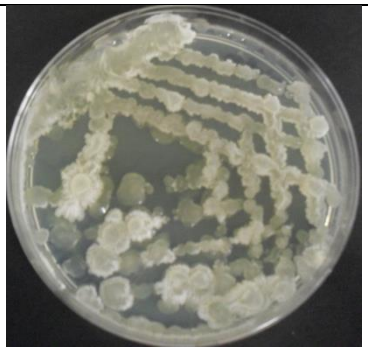
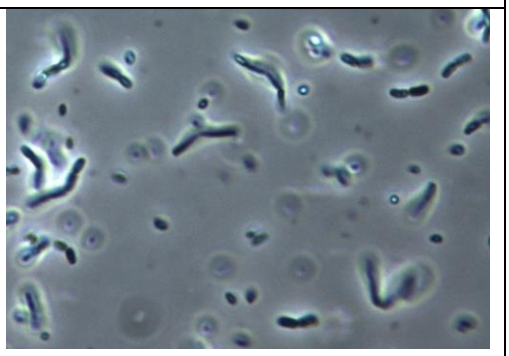
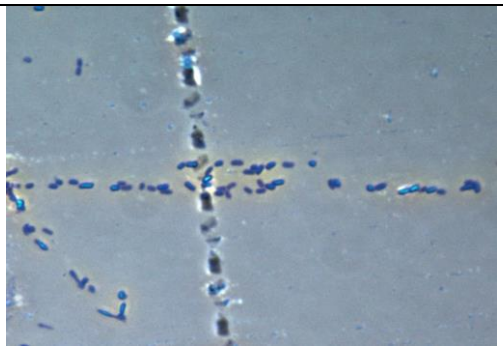
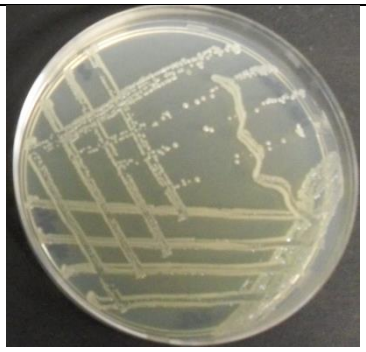
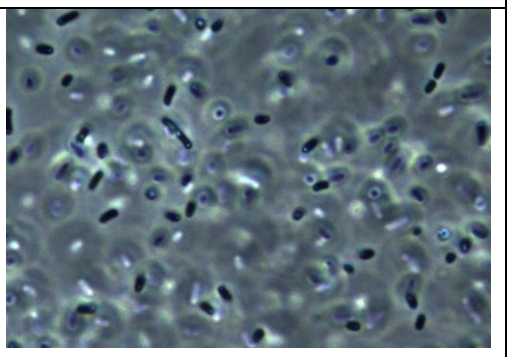
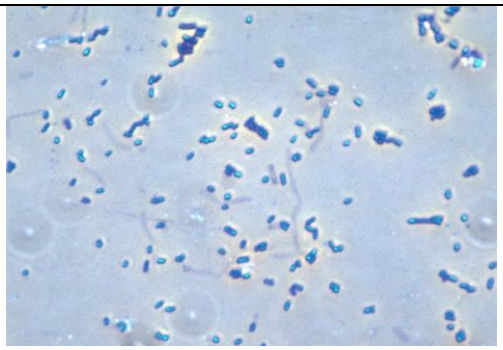
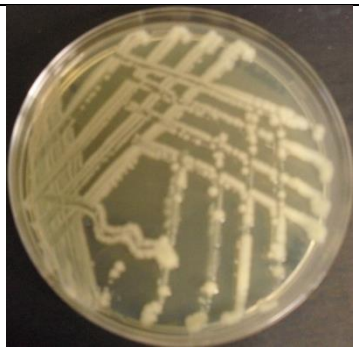
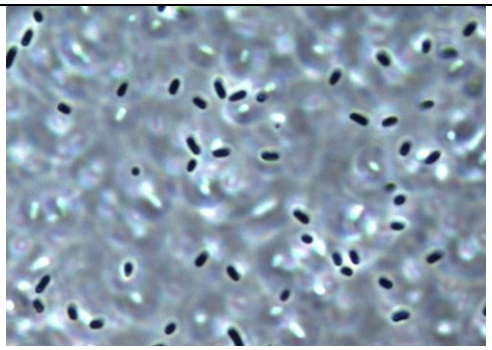
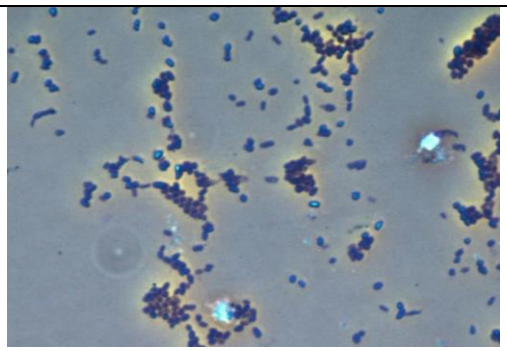
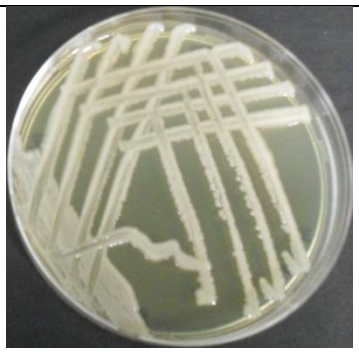
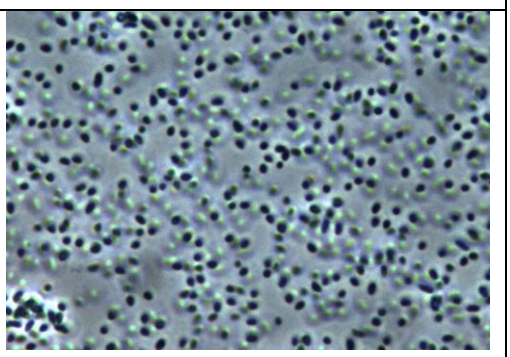
5.2 Isolation results and discussion

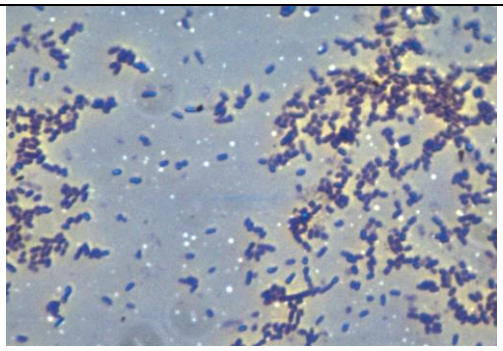
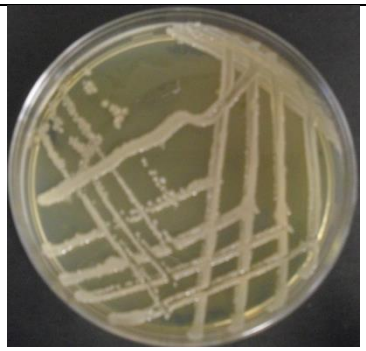
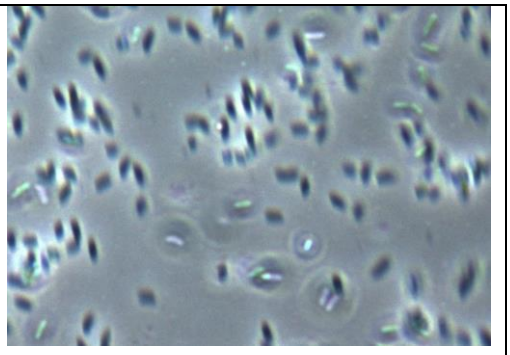
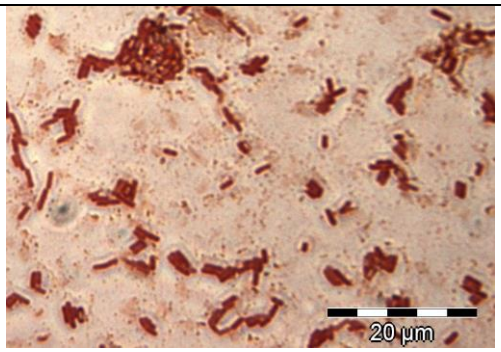
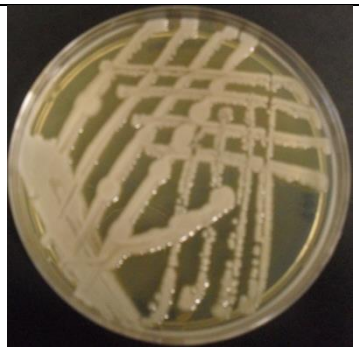
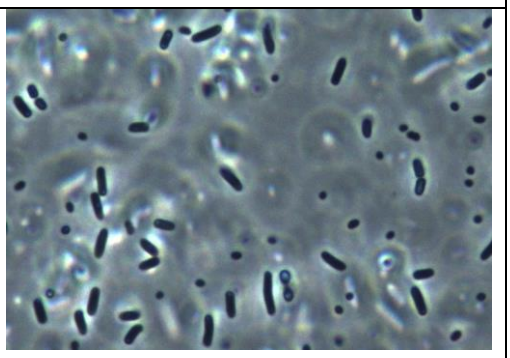
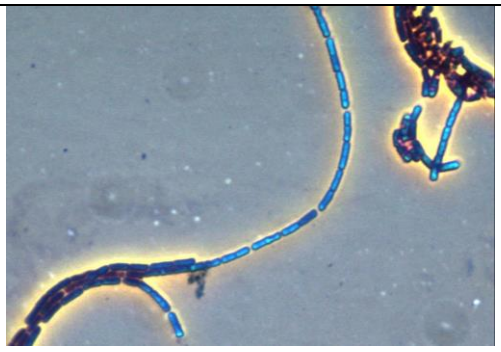
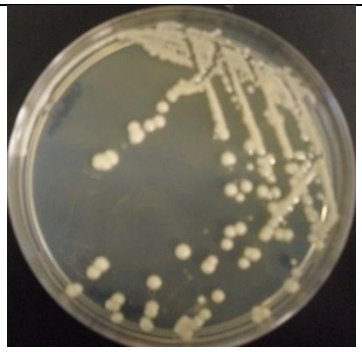
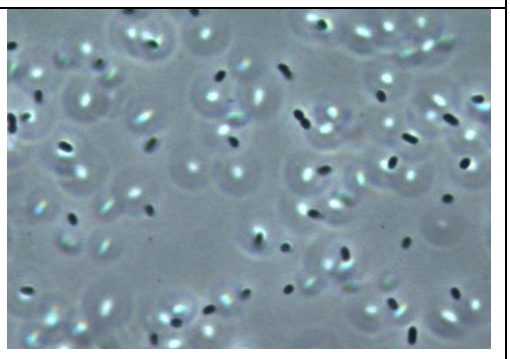
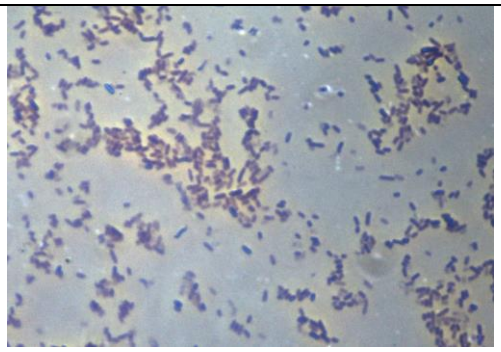
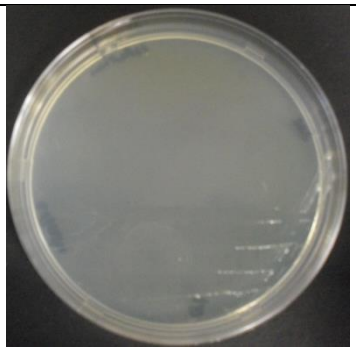
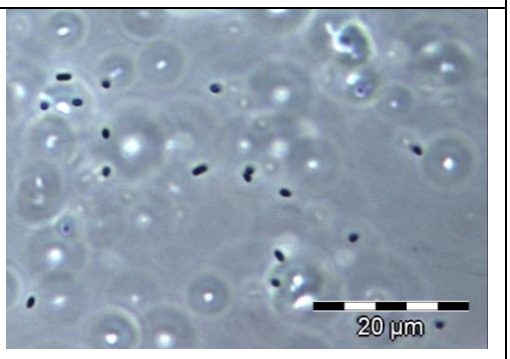
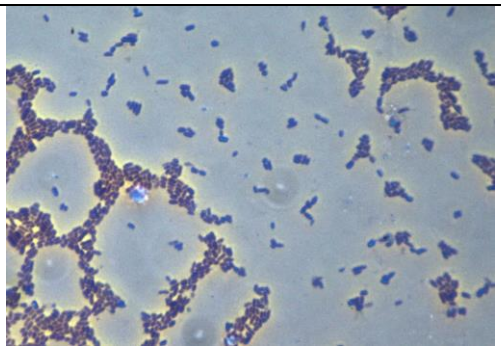
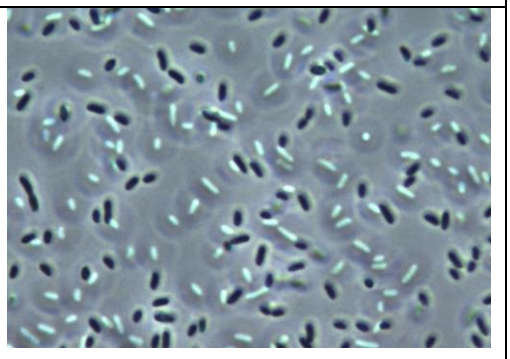
The isolation plates were inspected for various colony morphologies and 20 different colonies were selected. After numerous plate re-streaking, pure culture of Isolates 4 and 18 could not obtained. The remaining 18 isolates were further investigated. Table 5.1 shows the cell and colony morphologies of these 18 isolates. The Gram stains did not yield clear classification in all isolates; however 15 isolates appeared dark blue or purple i.e. Gram positive. These were mostly rod-shaped. Isolates 7, 12 and 14.2 showed a pink stain, which indicated that these strains were Gram negative. These could be enteric bacteria since they were isolated from a domestic wastewater treatment plant. The long, linked rods of Isolate 8 provided the clearest *Bacillus*-like morphology. The Gram stain is a good basic diagnostic tool, but has been shown to produce many inconclusive results (Pepper *et al.*, 2011). To counteract this, the isolates were also grown on MacConkey agar (20 $g.L^{-1}$ peptone, 10 $g.L^{-1}$ lactose, 1.5 $g.L^{-1}$ bile salts, 5 $g.L^{-1}$ NaCl, 0.03 $g.L^{-1}$ neutral red, 0.001 $g.L^{-1}$ crystal violet and 13.5 $g.L^{-1}$ agar), a Gram negative selective and differential media which can be used to detect and isolate microorganisms from wastewater (Eaton *et al.*, 1995). All isolates displayed varying degrees of growth, except Isolate 11 which did not grow at all on both the isolation and MacConkey agar plates. The Gram negative isolates aforementioned did show considerably greater growth compared to the Gram positive isolates on MacConkey agar.

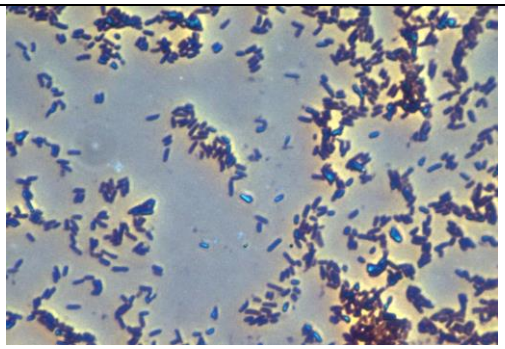
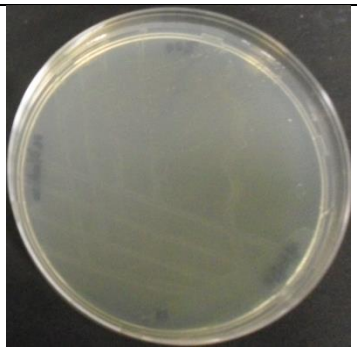
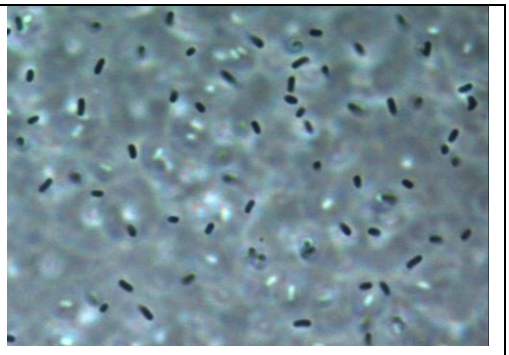
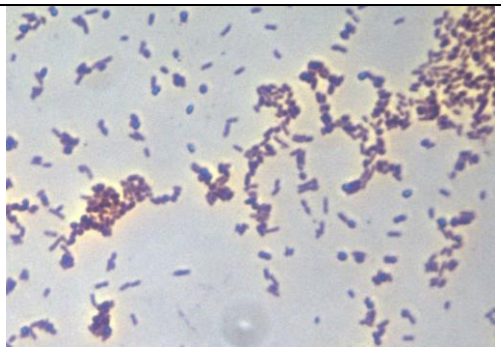
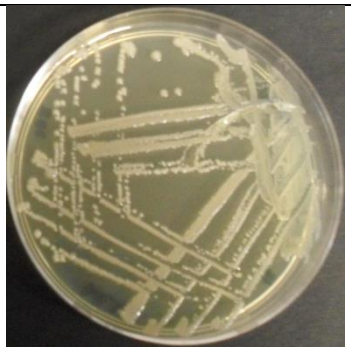
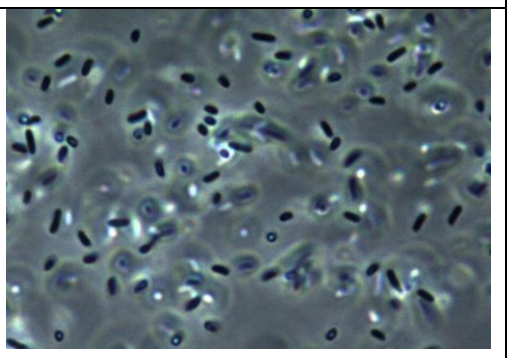
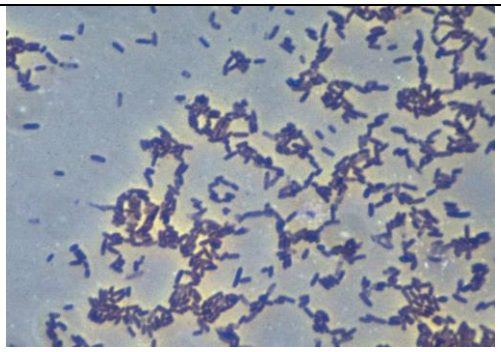
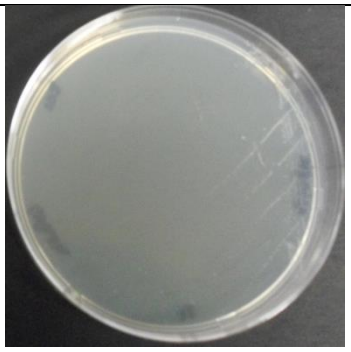
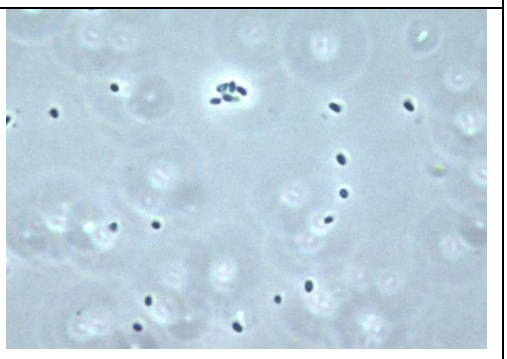
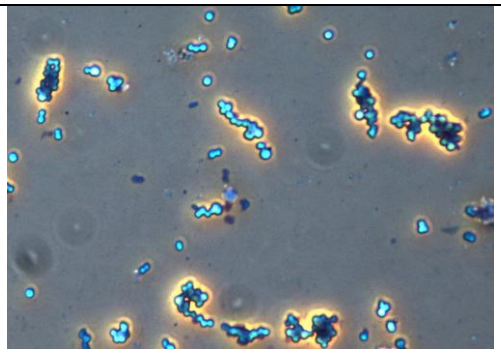
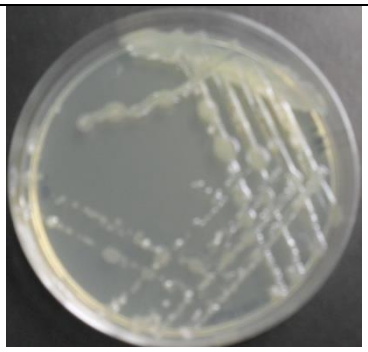
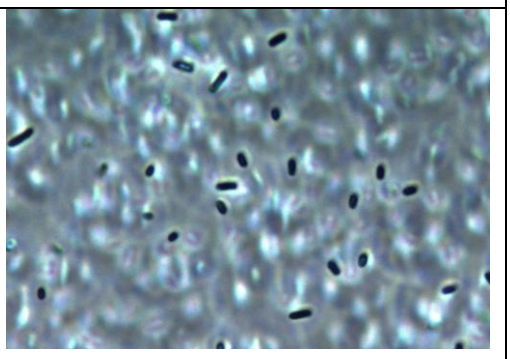
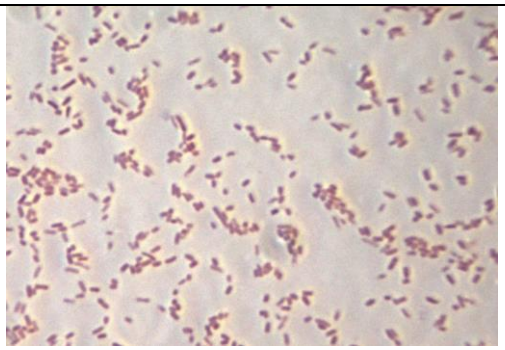
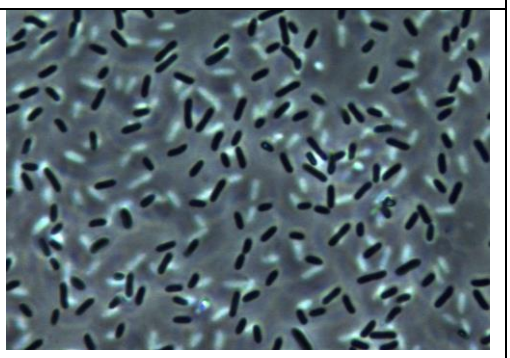
Growth on MME agar plates is also shown in Table 1. The cell morphologies can be seen more clearly. Isolates 1, 2, 7 and 12 were medium to long rods, some comma-shaped and some with spores. Sporulating rods are indicators consistent with the *Bacillus* bacterial species (Holt *et al.*, 1994). The rod shape was also observed for Isolates 3, 6, 8, 9, 10, 11, 13 and 17, but the rods were smaller in size. Medium rods were observed for Isolates 14.2, 15, 16 and 19 whilst 14.1 had a slightly longer cell size. Most of the cells were randomly and singly dispersed in the medium, except Isolate 1 which had cells in pairs, and 11 and 16 had regions where the cells aggregated. Isolate 5 had coccus-shaped cells. The isolates produced similar cell morphologies when grown in ME (results not shown).

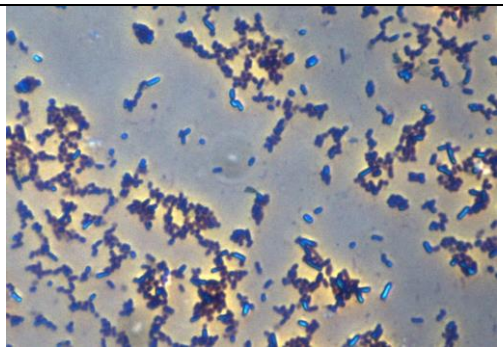
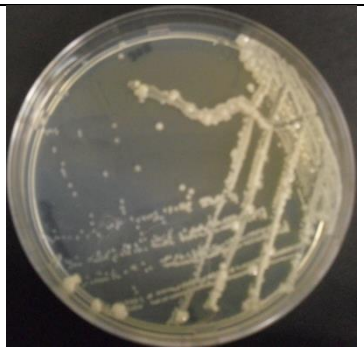
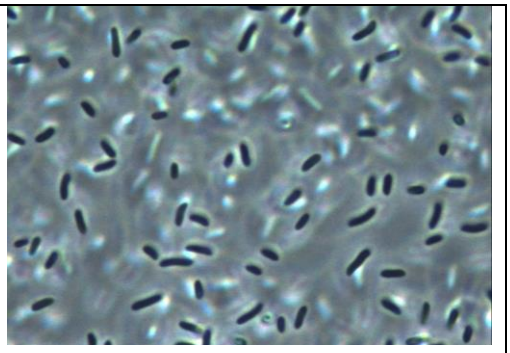
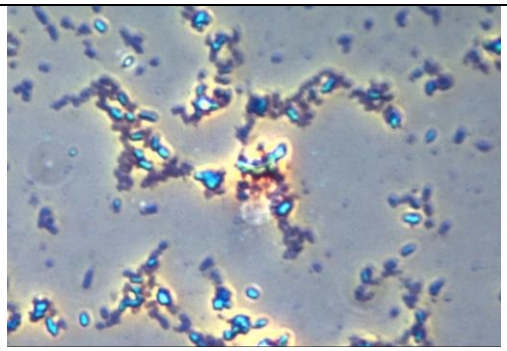
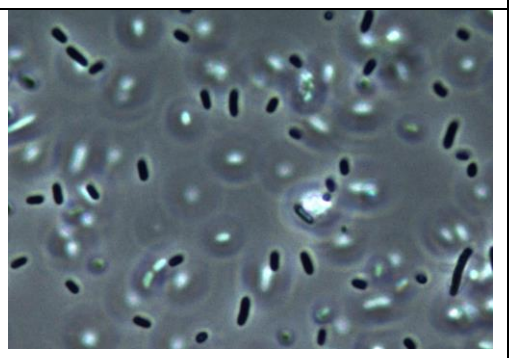
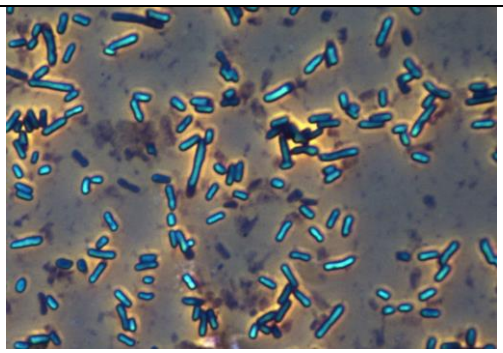
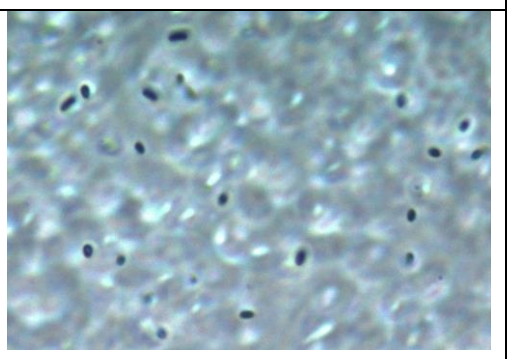
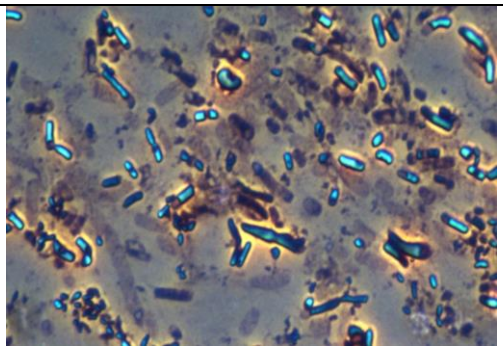
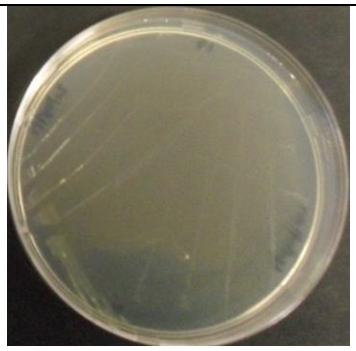
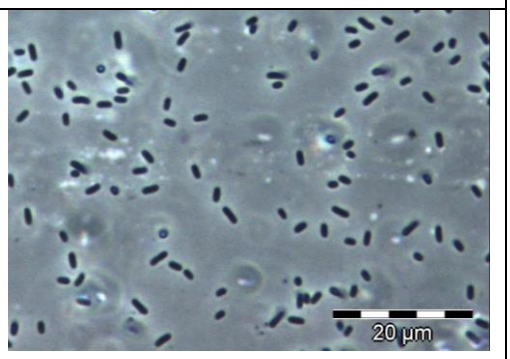
The most mucoid colonies on the isolation plates were displayed by Isolates 1 and 10. The medium in which Isolate 6 was grown produced a very viscous and elastic material after the cultivation which in addition to mucoid colonies, is also an indicator for the presence of γ-PGA (Yoon *et al.*, 2000).

Table 5.1: The morphological characteristics of the isolates obtained from the Mitchell's Plain wastewater treatment plant after growth at 37°C for 48 hours on isolation agar plates and MME.

Isolate	Gram stain	Isolation medium	MME
1			
2			
3			
5			

6			
7			
8			
9			
10			

11			
12			
13			
14.1			
14.2			

15			
16			
17			
19*			

*Due to non-growth in MME, the ME microscope picture is shown for Isolate 19

5.3 Initial growth studies: screening for γ-PGA producers

The 18 isolates obtained were subjected to further screening for good growth. Based on these findings, a sub-set of isolates would then be selected for further screening experiments.

In this chapter, the initial growth screening of all the isolates is presented and compared to *B. licheniformis*. Furthermore, the results of the molecular studies to characterise these isolates in terms of γ-PGA formation is presented and discussed. This was carried out in order to confirm the presence of the genetic elements required for γ-PGA synthesis.

5.3.1 Preliminary screening results and discussion

The results from the screening experiments with *B. licheniformis* and the 18 isolates in ME are shown in Table 5.2. Compared to *B. licheniformis*, all the isolates achieved higher biomass concentrations after 48 hours of cultivation. The strains with biomass production above 10 $g.L^{-1}$ included strains 1 (29.15 $g.L^{-1}$), 6 (20.35 $g.L^{-1}$), 8 (16.9 $g.L^{-1}$), 10 (14.05 $g.L^{-1}$), 11 (10.95 $g.L^{-1}$), 13 (10.4 $g.L^{-1}$), 15 (13.9 $g.L^{-1}$) and 17 (17.3 $g.L^{-1}$). Isolate 1 had a final pH value of 8.78. In the remaining cultures, the final pH ranged between 5.15 and 5.95. The final pH value for the *B. licheniformis* culture was 6.02.

The screening experiment was also repeated with MME and the results are shown in Table 5.3. The final pH values were slightly lower compared to the ME screening experiment, with values ranging between pH 4.54 and 5.77 for all isolated strains. The final pH for *B. licheniformis* was 6.14. Isolates 9 and 19 failed to grow in MME, and Isolates 2 and 16 achieved very similar final biomass values (2.4 $g.L^{-1}$ and 2.05 $g.L^{-1}$) when compared to *B. licheniformis* (2 $g.L^{-1}$). Isolates with biomass concentrations which were above 10 $g.L^{-1}$ included strains 1 (12.35 $g.L^{-1}$), 5, (11.7 $g.L^{-1}$), 6 (30.3 $g.L^{-1}$), 7 (19.6 $g.L^{-1}$), 8 (32.1 $g.L^{-1}$), 10 (15.05 $g.L^{-1}$) and 15 (32.45 $g.L^{-1}$).

Table 5.2: A summary of the OD values, biomass and pH values of the different isolates and *B. licheniformis* after growth in ME in shake flasks for 48 hours.

Isolate	**OD_{600}**	**Biomass ($g.L^{-1}$)**	**pH**
1	5.62	29.15	7.87
2	5.4	2.45	5.15
3	4.19	1.7	5.36
5	8.72	8.1	5.95
6	7.03	20.35	5.4
7	3.49	6.35	5.15
8	5.14	16.9	5.48
9	3.18	1.6	5.27
10	8.5	14.05	5.48
11	6.17	10.95	5.2
12	6.82	4.25	5.47
13	2.02	10.4	5.29
14.1	13.76	5.6	5.93
14.2	6.66	3.75	5.43
15	5	13.9	5.26
16	9.87	5.15	5.42
17	8.28	17.3	5.84
19	3.25	4.95	5.44
B. lich	1.94 ± 0.00	1.35 ± 0.20	6.02 ± 0.04

Table 5.3: A summary of the OD values, biomass and pH values of the different isolates and *B. licheniformis* after growth in MME in shake flasks for 48 hours.

Isolate	OD_{600}	Biomass ($g.L^{-1}$)	pH
1	21	12.35	4.6
2	5.57	2.4	4.57
3	5.92	3.2	4.61
5	15.06	11.7	5.77
6	15.7	30.3	4.78
7	5.38	19.6	4.75
8	7.92	32.1	5.1
9	0.81	0.3	5.01
10	20.4	15.05	4.64
11	8.58	9.3	5.02
12	9.39	4.35	4.89
13	1.06	3.85	5.12
14.1	7.49	3.55	4.73
14.2	14.34	6.9	4.49
15	17.1	32.45	4.81
16	4.3	2.05	4.54
17	6.11	8.95	4.77
19	0.12	0	6.37
B. lich	4.32 ± 0.63	2 ± 0.2	6.14 ± 0.15

5.3.2 Screening isolates for *pgsBCA* gene complex

5.3.2.1 pgsB, pgsC, and pgsA PCR results and discussion

PCR amplification using primers PGSF and PGSR was carried out as described in Section 3.9, to detect the presence of the *pgsBCA* genes as an indication of γ-PGA production potential. Based on the plate morphology and viscosity, Isolate 1 was selected as the isolate most likely to resemble a positive control, as no other control microorganisms or gene targets were available. Isolate 19 as the negative control. These primers failed to amplify the desired 1.1kb product, even after numerous temperature, time, and cycle optimisation steps, and therefore the presence of these *pgs* genes were not detected in any of the isolates tested (Figure 5.1).

Since some isolates had shown characteristics typical to γ-PGA producers such as the highly viscous broth (Section 5.2), a second attempt was made to design more optimal primers (see Section 3.9.2). PGS2F and PGS2R were obtained and tested. These primers produced multiple bands for most of the isolates. However, some of the isolates did produce the 1 kb desired fragment. The PCR

procedure was then further optimised by varying the PCR cycle parameters. Distilled water was used as a negative control.

Even after different PCR optimising strategies, the multiple bands could not be removed. This could be an indicator for either extremely unspecific binding or the presence of multiple copies of the gene complex. These multiple copies can be of different sizes and found on plasmids dispersed throughout the cells.

Isolates 1, 3, 6, 7, 8, 10, 11, 12, 13, 15, 17 and 19 produced the desired fragment size on the agarose gel, as shown in Figure 5.3. The thick bands in lanes 1 and 7 show that the *pgsBCA* gene fragment of Isolates 1 and 7 was strongly amplified. Isolates 10 and 12 also produced light bands at 1.1kb. Isolate 6 produced multiple bands, slightly smaller or larger than 1.1kb. *B. licheniformis* JCM 2505 also produced the desired fragment (Figure 5.2).

Although Isolates 3, 13, 17, 19 produced the desired fragment, these isolated did not grow well in MME (Table 5.3). Isolate 15 could not replicate the growth shown in Table 5.3. Isolate 11 and 19 did not produce the desired plate morphology in Table 5.1. The combined results for preliminary screening in the growth media, as well as the PCR were used to select isolates for further screening. These were Isolates 1, 6, 7, 8, 10 and 12 (Figure 5.4).

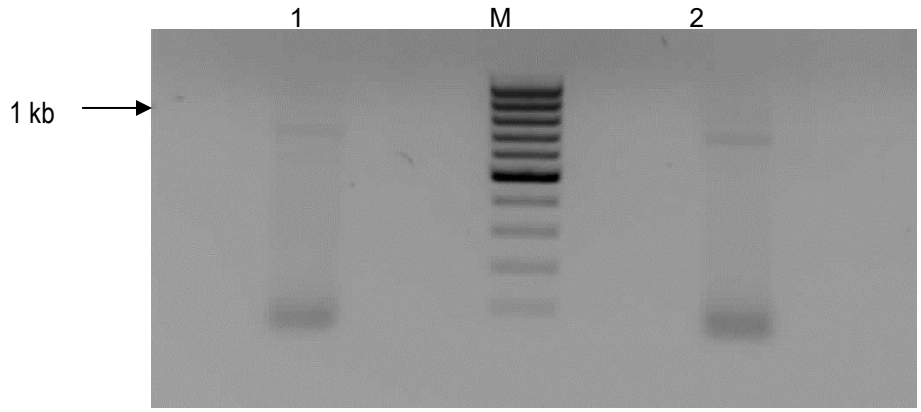


Figure 5.1: Detection of *pgs* genes by PCR using *pgsBCA* primers PGSF and PGSR. (1) Isolate 1; (M) 100bp molecular weight marker; (2) Isolate 19.

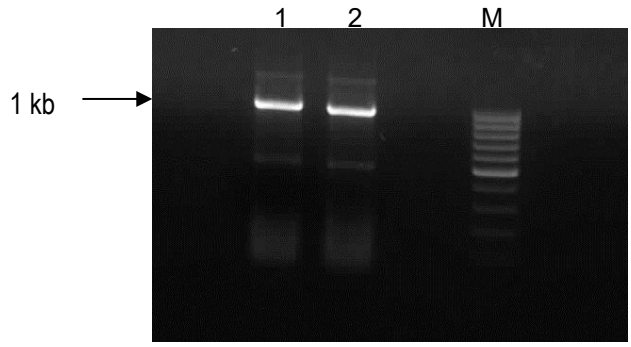


Figure 5.2: Detection of *pgs* genes by PCR using *pgsBCA* primers PGS2F and PGS2R. (1) and (2*) B. licheniformis*; (M) 100bp molecular weight marker.

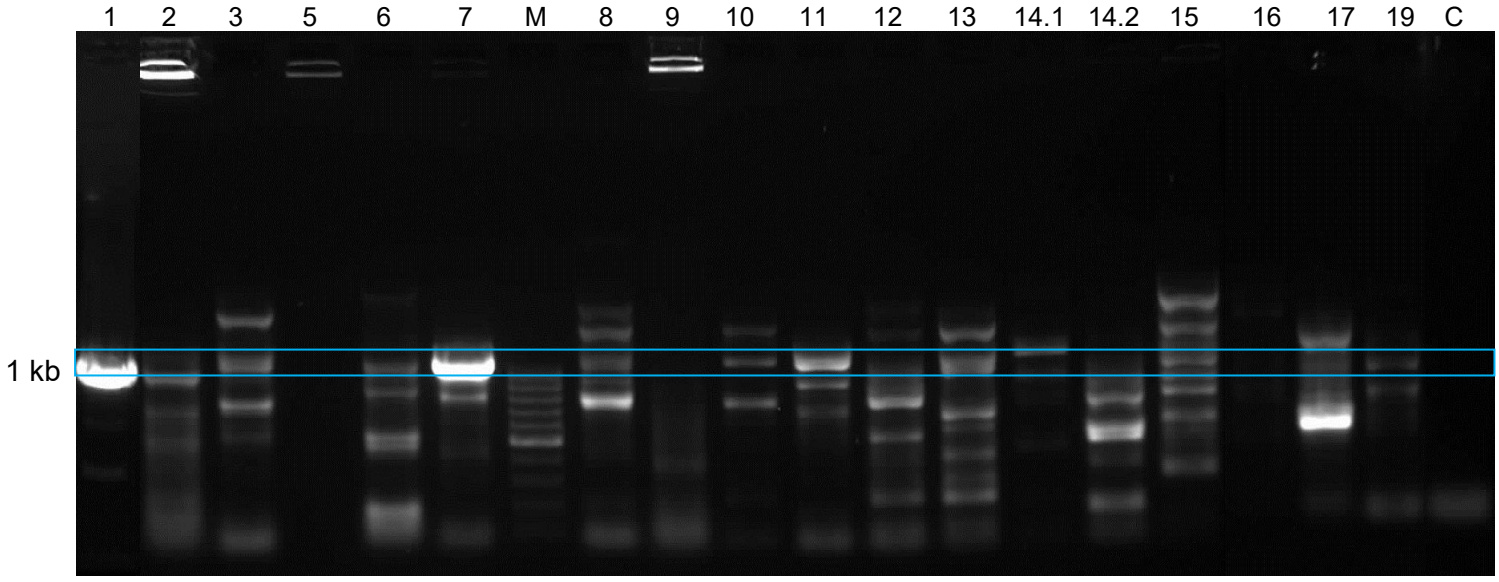


Figure 5.3: Detection of *pgs* genes by PCR using *pgsBCA* primers PGS2F and PGS2R in the isolates. The lane number correlates with the isolate number. (M) is the 100bp molecular weight marker and (C) the negative control.

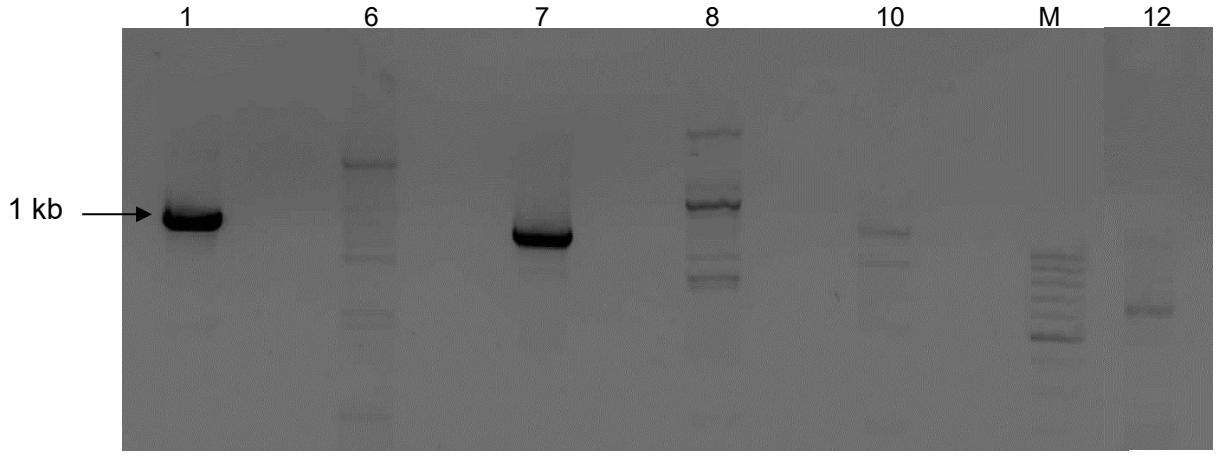


Figure 5.4: The optimised PCR of the selected six isolates using *pgsBCA* primers PGS2F and PGS2R. The lane number correlates with the isolate number. (M) is the 100bp molecular weight marker.

5.3.2.2 Sequencing of PCR product results and discussion

Sequencing was then conducted to ascertain that these fragments represent the desired gene fragment. *B. licheniformis* JCM 2505 genomic DNA was also extracted and used in the same PCR and sequenced with the purified fragments belonging to Isolates 1 and 7. The results are found in Table 5.4.

Table 5.4: The *pgs* gene sequencing BLAST results

Isolate	Identity	Accession number	Maximum identity %	Features in this part of subject sequence
Reference	*Bacillus licheniformis* ATCC 14580/ DSM13=14580	CP000002.3/ AE017333.1	99	DSM13=14580: glycerol-3-phosphate dehydrogenase GpsA GTPase EngA ATCC 14580: NAD(P)H-dependent glycerol-3-phosphate dehydrogenase GTP-binding protein
1	*Bacillus amyloliquefaciens*	CP003332.1/ HE774679.1	99	CP003332.1: ***gamma-polyglutamic acid synthetase*** HE774679.1: ***ywsC***
7	*Bacillus subtilis* BSn5	CP002468.1	99	GXT repeat-containing collagen-like protein ***capsular polyglutamate synthetase*** (ATP-dependent amide ligase)

The BLAST searches yielded scores with an E value equal to 0. The E value or Expect value describes the number of hits associated with a query sequence which one can expect by chance when searching a database. This is linked to background noise that exists for matches between sequences. Therefore, the lower the E value, the higher the significance. A match with an E value of 0 is of the highest significance.

The identity of the reference strain was confirmed to be *B. licheniformis* JCM 2505, which can also be annotated as ATCC 14580 or DSM13. The PCR fragment amplified was the correct size (Figure 5.2), but did not match any of the polyglutamate synthetase or related genes. Instead, it matched a glycerol-3-phosphate dehydrogenase gene. Glycerol-3-phosphate dehydrogenase, also known as G3P dehydrogenase is involved in the glycolytic pathway which is the stepwise degradation of glucose to pyruvate. This anaerobic process occurs in almost all living cells and the expression of this protein is essential for cell growth (Garret and Grisham, 2005).

The sequences obtained for Isolates 1 and 7 matched parts of the γ-polyglutamic acid synthetases belonging to *B.amyloliquefaciens* and *B. subtilis* BSn5 respectively. The *B.amyloliquefaciens* sequence deposited under accession number HE774679.1 was an equivalent match to CP003332.1, but *ywsC* was detected instead. This gene has been shown to be similar to *cap* and *pgs* genes and also involved in γ-PGA production as discussed in Section 2.4.3.

As the presence of these has been shown in the literature to be critical for γ-PGA synthesis, the ability of the reference strain to produce this biopolymer is questionable.

5.4 General discussion

In addition to the morphological characterisation presented in Section 5.2, the isolates were additionally characterised in terms of their growth nature and genetics in Sections 5.3.1 and 5.3.2. The isolates were screened in the same manner as *B.licheniformis* using ME and MME in shake flasks to compare their growth and biomass production in relation to *B.licheniformis*. Previous studies have shown a higher biomass concentration to be linked to more γ-PGA secretion in the medium (Du *et al*., 2005). For this reason, the biomass produced by the isolates was closely monitored. In general, most of these isolates grew better than *B.licheniformis* over a 48 hour cultivation period, producing higher biomass in both media. Isolates 1, 6, 8, 10 and 15 produced biomass concentrations greater than 10 $g.L^{-1}$, compared to a biomass concentration of 1.4 $g.L^{-1}$ and 2 $g.L^{-1}$ for *B. licheniformis* in ME and MME respectively. All isolates grew better in MME with higher biomass concentrations after a 48 hour cultivation, with the exception of Isolate 1 which produced 29.2 $g.L^{-1}$ in ME and 12.4 $g.L^{-1}$ in MME. The other isolates produced biomass values in the range of 1.6 to 20 $g.L^{-1}$ in ME and 0 to 32 $g.L^{-1}$ in MME. These results showed the potential of Isolates 1, 6, 8, 10 and 15 to be able to maintain good growth in minimal media which excluded L-glutamic acid

The PCR targeting the *pgsBCA* gene complex using designed primers required further optimisation. The final optimised PCR still produced multiple bands, so the gel image was carefully inspected for all

the isolates which produced a fragment of the desired molecular weight. This was the case for Isolates 1, 3, 6, 7, 8, 10, 11, 12, 13, 15, 17 and 19. Finally, the thick bands produced by Isolates 1 and 7 were sequenced to confirm the identity of the amplified gene. *B.licheniformis* was used as a reference. The identity of only the fragments from Isolates 1 and 7 was a positive match for the *pgs* gene.

The results from Section 5.2 (morphology) and Section 5.3 (growth characteristics in the minimal MME and PCR amplification of the desired fragment for *pgsBCA)* were analysed in parallel and used to select isolates for further screening experiments. Isolates 1, 6, 7, 8, 10, and 12 were selected on the basis of morphology, growth in MME and the PCR amplification of the desired fragment for *pgsBCA*. Although Isolate 7 and 12 did not produce biomass concentrations greater than 10 $g.L^{-1}$ in both media ME and MME, they were selected for further screening on the basis of morphological growth on the screening plates and the presence of the desired band in the PCR gels. Isolate 15 showed high final biomass concentrations (13.9 $g.L^{-1}$ in ME and 32.5 $g.L^{-1}$ in MME), but it was not included for further screening due to being unable to replicate this growth in both media in proceeding experiments.

6. FURTHER SCREENING OF ISOLATES

6.1 Introduction

Based on the results from the first set of screening experiments reported in Chapter 5, particularly in MME, the morphological appearance and viscosity of the cultures and PCR experiments, Isolates 1, 6, 7, 8, 10 and 12 were selected for further experimentation. The isolates were screened in both ME and MME. The growth results in shake flasks and deep well plates are presented and discussed in this chapter.

6.2 Results and discussion

6.2.1 Comparison of growth across isolates – studies in shake flasks

Figure 6.1 and Figure 6.2 show the growth curves for the different isolates that were cultivated in shake flask experiments in both ME and MME growth media, respectively.

The results were reproducible with repeated experiments. The growth curves for both media follow the expected trend for an active inoculum in a typical batch process. No adaptation phase was observed for Isolates 6, 7 and 12, and a very short adaptation phase of 3 hours was observed for Isolates 1, 8 and 10 before reaching μ_{max} as the cells were diluted from the inoculum into the same fresh growth medium for the experiment. Figure 6.3 and Figure 6.4 show how the pH of the growth medium changes over the cultivation period for ME and MME respectively. The pH decrease over the time is an indicator for the production of organic acids, including γ-PGA.

After the initial lag period, the cell numbers increased exponentially with time. In general, this exponential growth lasted for approximately 9 hours. After this, the growth started to slow down and a stationary phase was reached after 12 hours for most isolates, as system nutrients become limited, and conditions become unfavourable for optimal growth. The maximum biomass concentration was reported as the stationary phase concentration. The expected death phase was not evident in the cultivation period.

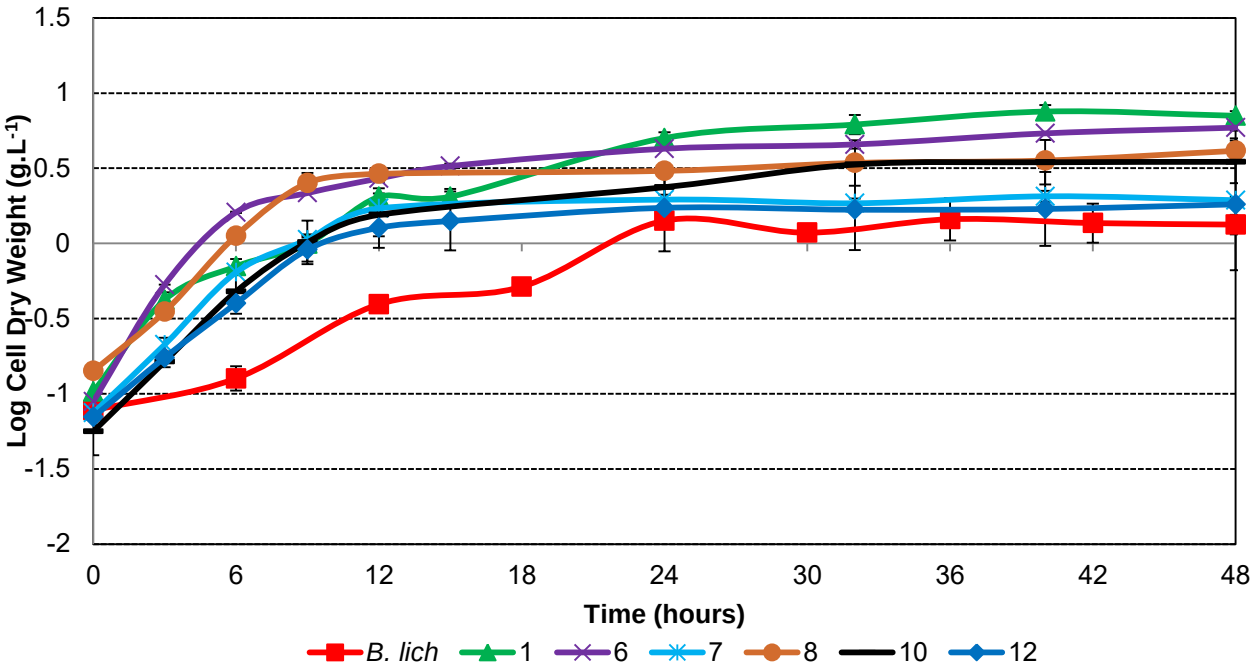


Figure 6.1: Growth profiles of *B. licheniformis* and Isolates 1, 6, 7, 8, 10 and 12 in shake flasks containing ME. The averages of duplicate experiments with standard deviation error bars are shown.

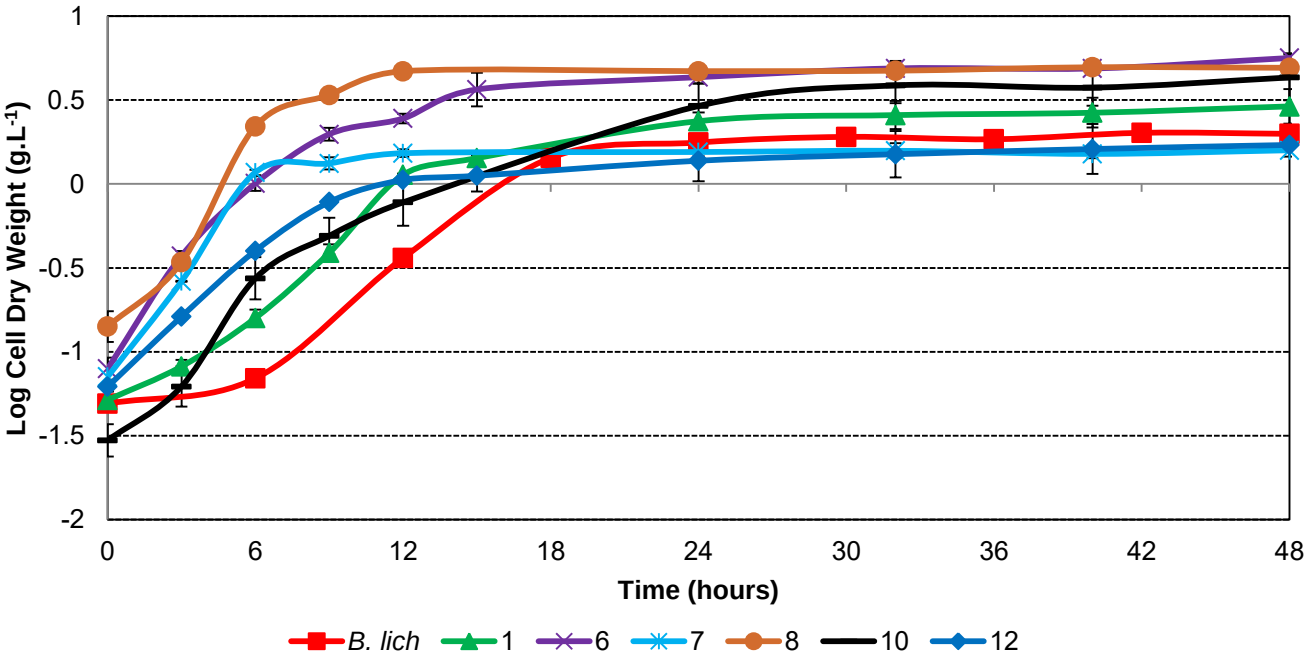


Figure 6.2: Growth profiles of *B. licheniformis* and Isolates 1, 6, 7, 8, 10 and 12 in shake flasks containing MME. The averages of duplicate experiments with standard deviation error bars are shown.

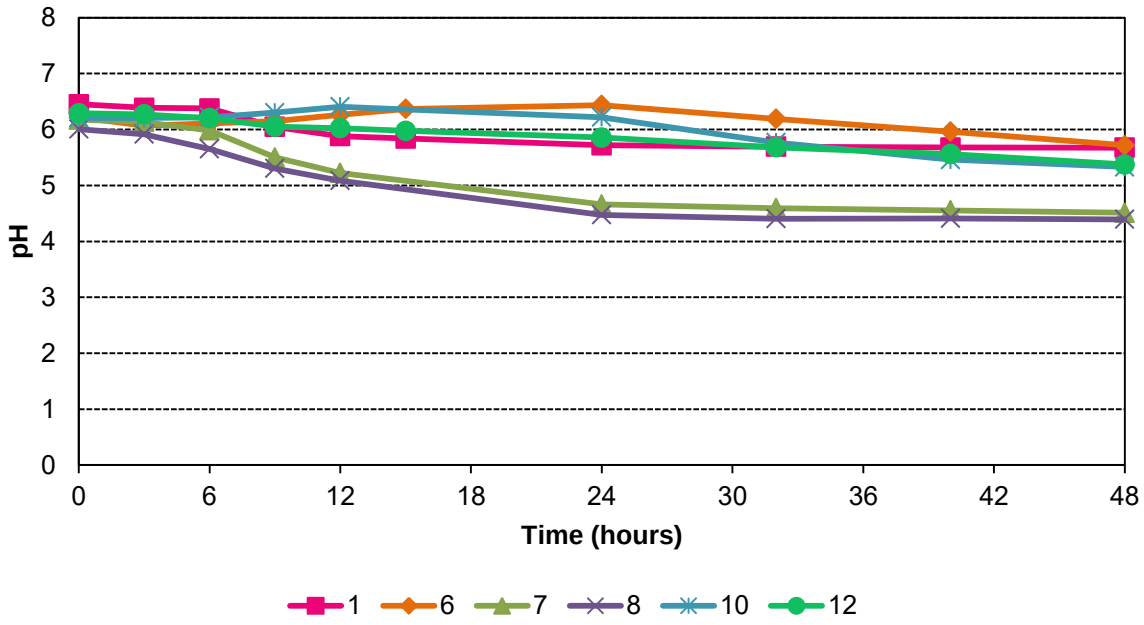


Figure 6.3: pH of Isolates 1, 6, 7, 8, 10 and 12 in shake flasks containing ME. The averages of duplicate experiments are shown.

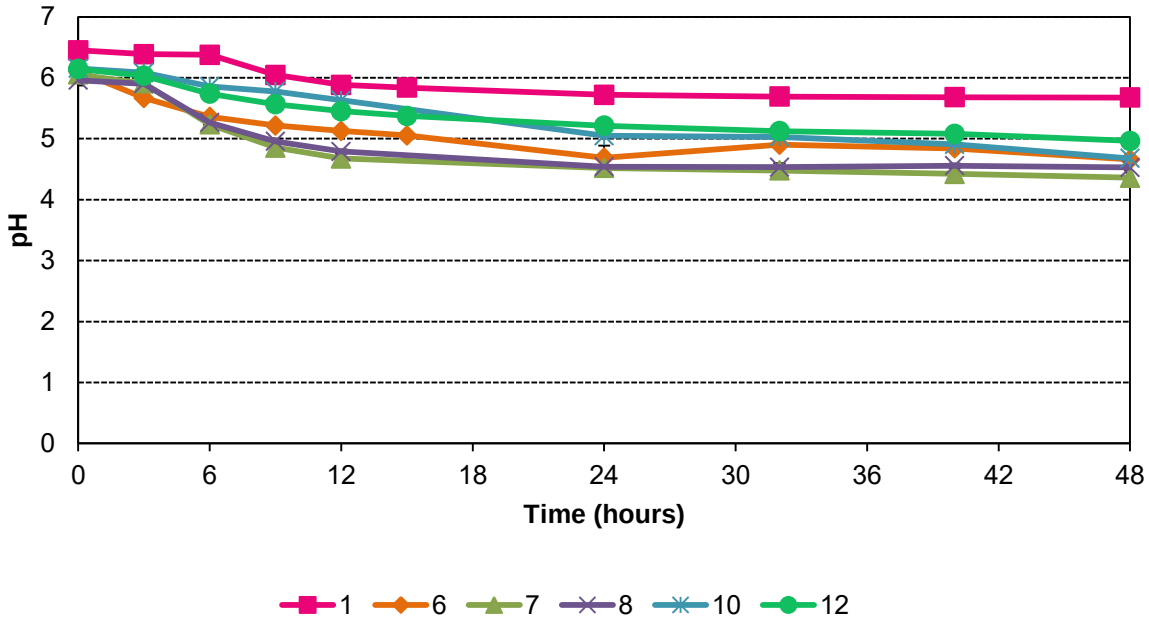


Figure 6.4: pH of Isolates 1, 6, 7, 8, 10 and 12 in shake flasks containing MME. The averages of duplicate experiments are shown.

The growth rate, final biomass concentration and change in biomass concentration determined for *B. licheniformis* and the six isolates in shake flasks containing growth media, ME and MME, are shown in Table 6.1. The maximum specific growth rate varied as a function of the isolate and the medium used, and ranged between 0.112 hr^{-1} and 0.269 hr^{-1}. Whilst Isolates 1 and 6 showed highest specific growth rates on ME (with μ_{max} of 0.203 hr^{-1} and 0.209 hr^{-1}, respectively), Isolate 7, 8 and 10 achieved higher growth rates in MME (μ_{max} of 0.202 hr^{-1}, 0.269 hr^{-1} and 0.214 hr^{-1}, respectively). The specific growth rates of Isolate 12 on ME and MME were similar; however, these growth rates and the maximum biomass concentrations were low compared to the other isolates, hence it was excluded from further experimentation.

The greatest biomass concentration of 7.08 $g.L^{-1}$ was achieved when Isolate 1 was grown in ME, with Isolate 6 showing the second highest biomass concentrations (5.64 $g.L^{-1}$ and 5.69 $g.L^{-1}$ in MME and ME, respectively). The biomass values for the other isolates ranged between 1.71 $g.L^{-1}$ and 4.96 $g.L^{-1}$.

6.2.2 Comparison of growth across isolates – studies in deep well plates

Figure 6.5 and Figure 6.6 show the summary for growth of the selected isolates in ME and MME in deep well plate experiments respectively. Similarly to the shake flask experiments, the growth curves for both growth media followed the expected trend for an active inoculum in a typical batch process. No adaptation phase was observed for Isolate 8 in both media, and a very short adaptation phase of approximately 3 hours was observed for Isolates 1, 6, 7 and 10. The exponential growth lasted for approximately 9 hours. The maximum biomass concentration was maintained in the stationary phase with the expected death phase not evident in the cultures.

The growth rate, final biomass concentration and change in biomass concentration calculated for the different isolates grown in deep well plates containing growth media, ME and MME are shown in Table 6.2. *B.licheniformis* was not grown in deep well microtitre plates.

Under the cultivation conditions of the deep well plates, the μ_{max} of the isolates ranged from 0.101 hr^{-1} to 0.196 hr^{-1} when grown in MME. When the isolates were grown in ME, the μ_{max} ranged from 0.073 hr^{-1} to 0.192 hr^{-1}. The maximum biomass ranged from 4.25 $g.L^{-1}$ to 5.98 $g.L^{-1}$ when the isolates were grown in MME and 5.88 $g.L^{-1}$ to 8.94 $g.L^{-1}$ when grown in ME. Isolate 1 yielded the greatest biomass of 8.94 $g.L^{-1}$ when grown in ME. Isolates 7 and 8 had the highest μ_{max} values (0.196 hr^{-1} in MME and 0.192 hr^{-1} in ME, respectively).

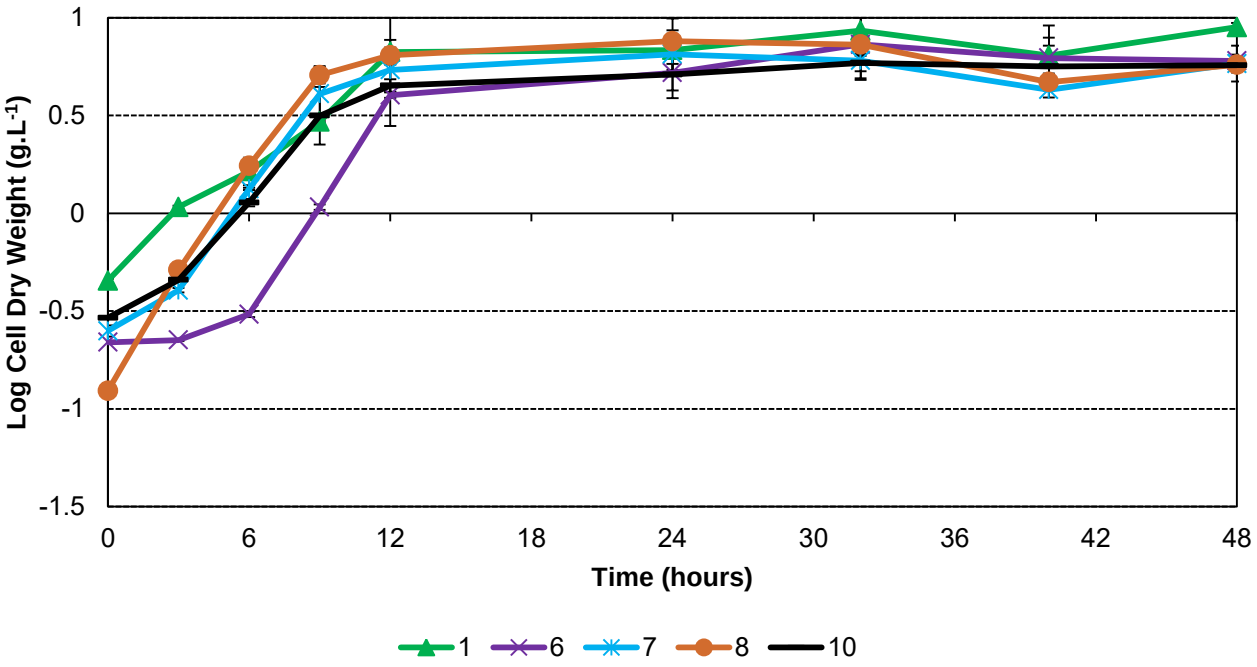


Figure 6.5: Growth profiles of Isolates 1, 6, 7, 8, 10 and 12 in microtitre deep well plates containing ME. The averages of triplicate experiments with standard deviation error bars are shown.

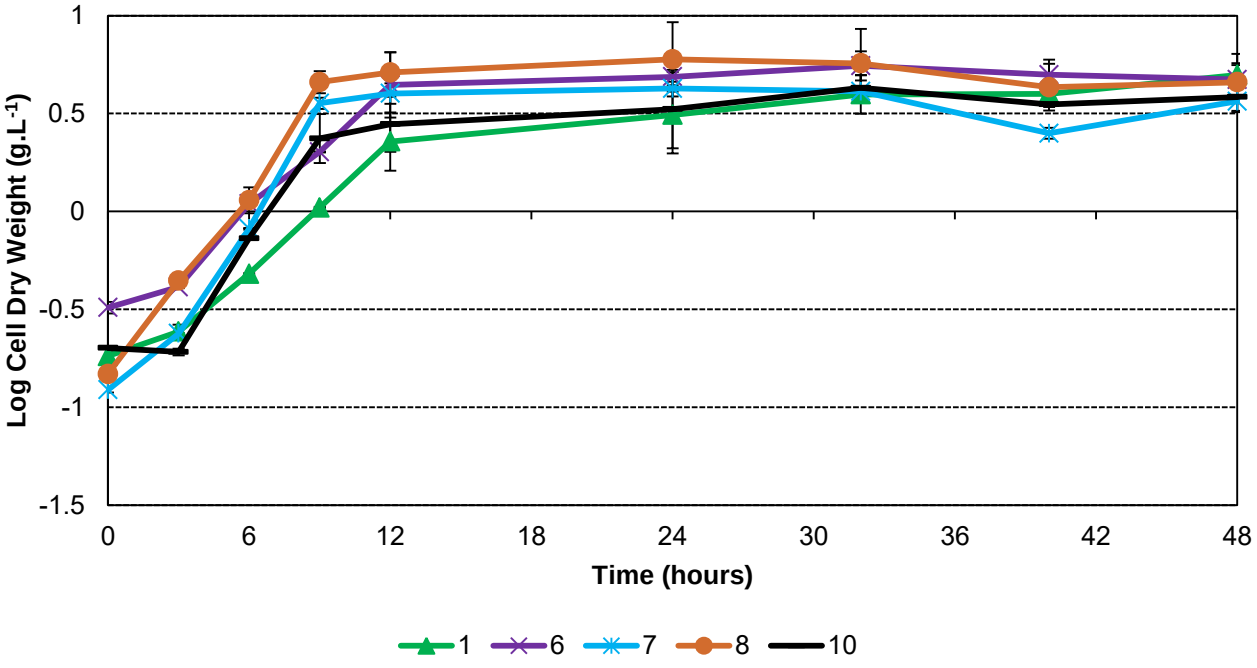


Figure 6.6: Growth profiles of Isolates 1, 6, 7, 8, 10 and 12 in microtitre deep well plates containing MME. The averages of triplicate experiments with standard deviation error bars are shown.

6.2.1 Substrate utilisation

Substrate utilisation of the isolates was calculated as a percentage difference of the initial measured concentration and the residual substrate after the 48 hour cultivation period. The results are shown in Figure 6.7 to Figure 6.10. These are compared to *B. licheniformis*. The glycerol utilisation in the experiments in shake flasks containing ME and MME was less than 14% (Figure 6.7 and Figure 6.8). A big variation in citric acid utilisation by the different isolates was noted in the shake flasks containing ME, and ranged between 0% and 84%. In general, the citric acid utilisation was also poor in MME, with less than 11% utilisation for most isolates, except Isolate 12 where 41% citric acid was used (Figure 6.8). The glucose utilisation in shake flasks with MME was the highest for Isolates 1, 8, 10 and *B. licheniformis* with values above 93% utilisation. Isolates 6, 7 and 12 achieved glucose utilisation less than 57%.

The cultivations in deep well plates using ME (Figure 6.9) indicated low citric acid utilisation (less than 7%) for all isolates except Isolate 8 with 83% citric acid utilisation. Isolates 1 and 8 achieved high glycerol utilisation in deep well plates using ME (above 79%), whereas the other isolates achieved glycerol utilisation of less than 11%. In the deep well plates using MME (Figure 6.10), a similar trend was noted for the citric acid and glycerol utilisation, when compared to the shake flask cultivations using the same medium. Low utilisation was reported, with utilisation values less than 17% and 12% for glycerol and citric acid, respectively. All isolates utilised glucose well (utilisation rates above 79%) in the deep well plates containing MME, except Isolate 1 which appeared to not primarily utilise any of the carbon sources in the medium.

Table 6.1: Summary of the kinetic parameters for the different isolates after growth in shake flasks in ME and MME. The average of triplicate experiments are shown,

Isolate	**1**		**6**		**7**		**8**		**10**		**12**	
Media	**MME**	**ME**	**MME**	**ME**	**MME**	**ME**	**MME**	**ME**	**MME**	**ME**	**MME**	**ME**
μ_{max} (hr^{-1})	0.112 ± 0.01	0.203 ± 0.01	0.184 ± 0.0	0.21 ± 0.01	0.203 ± 0.00	0.156 ± 0.01	0.269 ± 0.00	0.169 ± 0.00	0.215 ± 0.00	0.155± 0.00	0.125 ± 0.00	0.122 ± 0.00
Max Biomass ($g.L^{-1}$)	2.98 ± 0.70	7.57 ± 0.73	5.64 ± 0.36	5.89 ± 0.28	1.58 ± 0.07	2.06 ± 0.17	4.96 ± 0.20	4.13 ± 0.00	4.52 ± 1.38	3.67 ± 1.16	1.74 ± 0.03	2.04 ± 0.22
Δx ($g.L^{-1}$)	2.93 ± 0.69	6.98 ± 0.48	5.56 ± 0.34	5.8 0.27	1.51 ± 0.01	1.86 ± 0.09	4.77 ± 0.16	3.98 ± 0.00	4.49 ± 1.37	3.61 ± 1.14	1.68 ± 0.04	1.96 ± 0.22

Table 6.2: Summary of the kinetic parameters for the different isolates after growth in microtitre deep well plates in ME and MME. The averages of triplicate experiments are shown,

Isolate	**1**		**6**		**7**		**8**		**10**	
Media	**MME**	**ME**	**MME**	**ME**	**MME**	**ME**	**MME**	**ME**	**MME**	**ME**
μ_{max} (hr^{-1})	0.106 ± 0.02	0.073 ± 0.01	0.101 ± 0.00	0.187 ± 0.03	0.196 ± 0.00	0.167 ± 0.00	0.148 ± 0.01	0.192 ± 0.02	0.182 ± 0.00	0.140 ± 0.01
Max Biomass ($g.L^{-1}$)	4.98 ± 0.08	8.94 ± 0.08	5.54 ± 0.17	7.30 ± 0.37	4.25 ± 0.02	6.51 ± 0.17	5.98 ± 0.21	7.57 ± 0.26	4.27 ± 0.06	5.88 ± 0.10
Δx ($g.L^{-1}$)	4.80 ± 0.05	8.49 ± 0.12	4.40 ± 0.23	5.79 ± 0.09	3.53 ± 0.02	5.57 ± 0.20	4.42 ± 0.17	5.62 ± 0.09	3.64 ± 0.08	5.42 ± 0.01

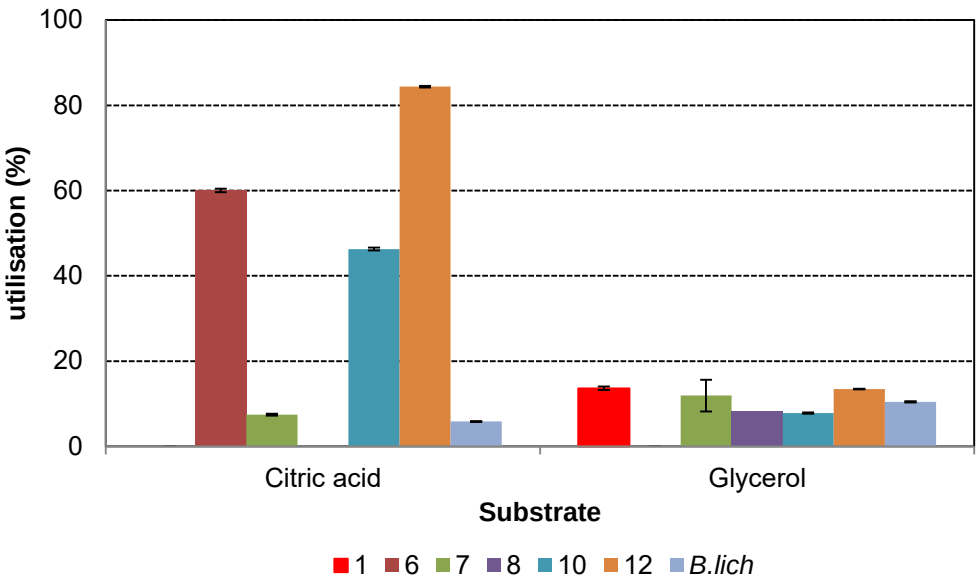


Figure 6.7: Extent of substrate utilisation of *B. licheniformis* and Isolates 1, 6, 7, 8, 10 and 12 in shake flasks containing ME. The averages of duplicate experiments with standard deviation error bars are shown.

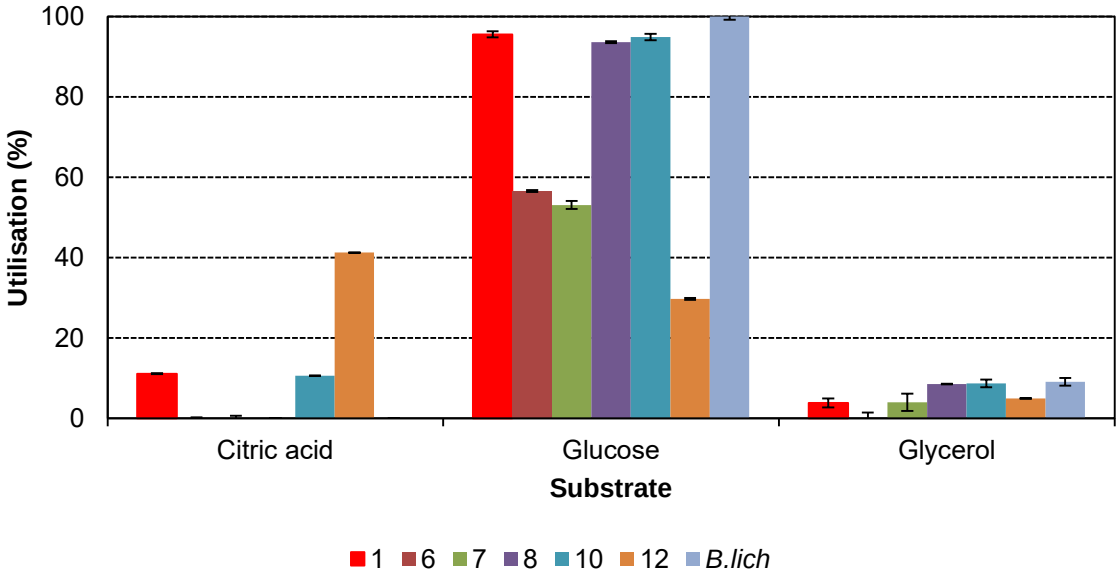


Figure 6.8: Extent of substrate utilisation of *B. licheniformis* and Isolates 1, 6, 7, 8, 10 and 12 in shake flasks containing MME. The averages of duplicate experiments with standard deviation error bars are shown.

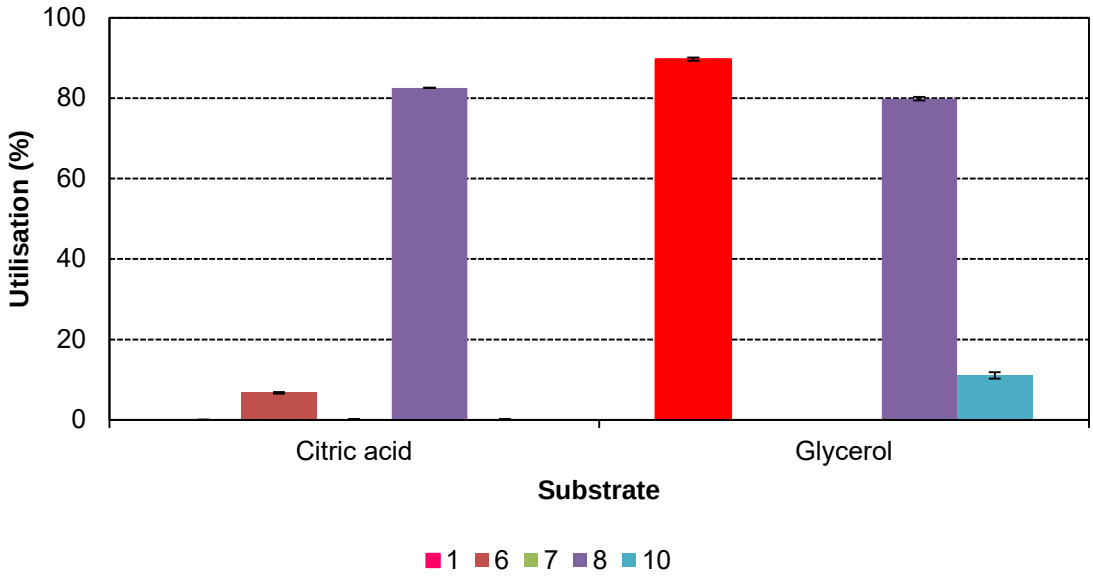


Figure 6.9: Extent of substrate utilisation of Isolates 1, 6, 7, 8 and 10 in microtitre deep well plates containing ME. The averages of triplicate experiments with standard deviation error bars are shown.

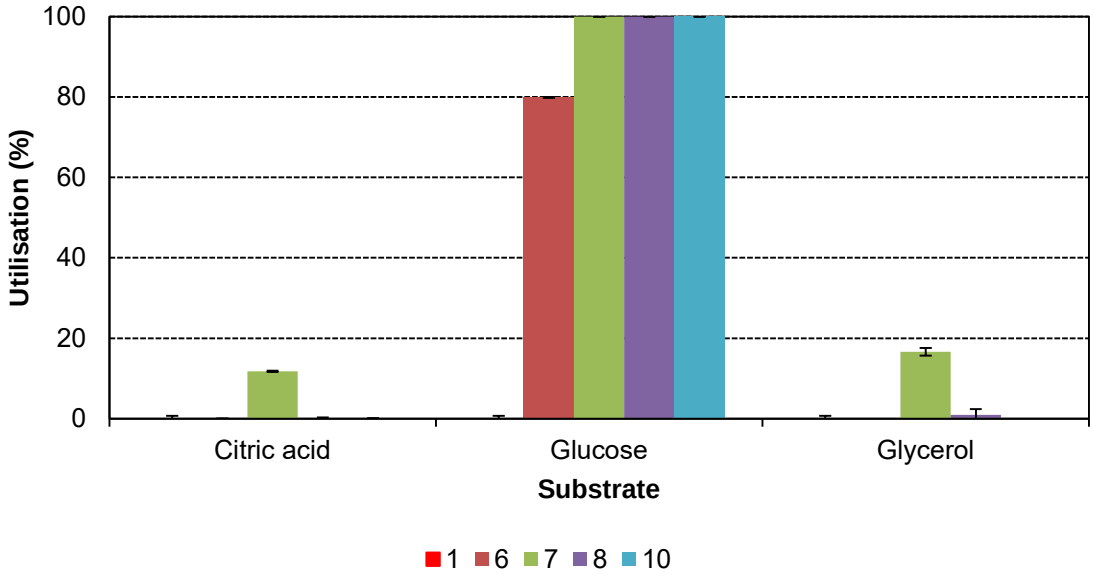


Figure 6.10: Extent of substrate utilisation of Isolates 1, 6, 7, 8 and 10 in microtitre deep well plates containing MME. The averages of triplicate experiments with standard deviation error bars are shown.

6.3 General discussion

When comparing the growth kinetics of the isolates obtained from shake flasks and deep well plates (Table 6.1 and Table 6.2 respectively), the deep well plates generally produced higher biomass concentrations. There was a noticeable increase in culture viscosity in the shake flasks during cultivation. Thus, the higher biomass concentrations in the deep well plates are hypothesized to be due to an improved gas liquid mass transfer in this system. ME favoured improved biomass, with Isolate 1 producing a maximum biomass of 8.94 $g.L^{-1}$ compared to 7.08 $g.L^{-1}$ in the shake flask. Isolates 6, 7 and 8 also produced high biomass amounts of 7.30 $g.L^{-1}$, 6.51 $g.L^{-1}$ and 7.57 $g.L^{-1}$ in the deep wells, whilst the shake flasks produced much lower values, in some cases, of 5.89 $g.L^{-1}$, 2.06 $g.L^{-1}$ and 4.13 $g.L^{-1}$ respectively.

The biomass yield for Isolates 1, 7 and 8 grown in MME also showed the same trend as ME, producing lower biomass in the shake flask experiments. Isolates 6 and 10 produced slightly lower biomass in deep well plates (MME) of 5.54 $g.L^{-1}$ and 4.27 $g.L^{-1}$ respectively. This corresponded well the results in the shake flasks (5.64 $g.L^{-1}$ and 4.52 $g.L^{-1}$, respectively for Isolates 6 and 10 in MME).

Substrate utilisation in the shake flasks was poorest for glycerol and citric acid. Only Isolate 12 was able to use more than 11% of citric acid in MME. Isolates 1 and 10 utilised 11%, and the rest of the isolates 0%. In ME, citric acid utilisation was better, with Isolates 6, 7, 10 and 12 using between 7.5% and 84%. The isolates which utilised more of the substrates, also displayed higher maximum growth rates and biomass. This was especially the case for Isolates 7, 8, 10, which all had μ_{max} values greater than 0.2 hr^{-1} in MME. The same correlation was only observed for citric acid and Isolate 6 in ME. The findings in the deep well plates followed a similar trend. Whilst all isolates, except Isolate 6, utilised low levels of the glycerol in the shake flasks, only Isolates 7 and 8 utilised this carbon source in MME. Isolates 1, 8 and 10 were able to utilise more of the glycerol in ME. The same was observed for citric acid – only Isolate 7 used 12% of citric acid in MME and Isolates 6 (7%) and 8 (83%) in ME. Isolate 7 was also the only strain which used all the carbon sources, including all the glucose. This isolate showed the best maximal growth rate in MME. Isolates 8 and 10 also used 100% of the glucose in this medium and did not grow as well as Isolate 7, but were the two isolates which followed most closely. Isolate 8 was able to utilise both the citric acid and glycerol in ME, and was the isolate with the highest μ_{max} in this particular medium. Glucose was better utilised in deep well plates. This is consistent with the formation of higher biomass concentrations.

A focus was placed on the isolates which grew better in MME, as this medium was a more cost-effective option owing to the replacement of L-glutamic acid with glucose. After the conclusion of the shake flask experiments, Isolate 12 was excluded from any further experimentation due to poor growth, especially in MME. Isolate 6 displayed preferentially better growth in ME, and produced the lowest growth rate in MME. Isolates 1, 7, 8 and 10 were selected for further optimisation based on a combination of the growth rates, biomass and, more especially, efficient substrate utilisation in MME.

7. OPTIMISATION OF GROWTH MATRIX

7.1 Introduction

The glucose modified medium E (MME), which was the cheaper medium alternative, was optimised for optimal carbon, nitrogen and phosphorus ratios for high growth rate and biomass production. The isolates which displayed the best growth and substrate utilisation in MME were selected for this study. These were Isolates 1, 7, 8 and 10.

Three factor and two-level factorial design was used. The three factors were carbon, nitrogen and phosphorus. The low and high level selected for carbon were 2 and 4 respectively, 0.065 and 0.26 were used for nitrogen, and 0.0043 and 0.0172 for phosphorus, all derived from the baseline medium. These were used to determine the various combinations of input concentrations for the three factors.

Following optimisation of the C:N:P ratio, combinations of lower initial carbon source concentrations were also investigated using Isolates 1 and 7. The results are included in this chapter.

7.2 Medium optimisation in terms of the C:N:P ratio using factorial design

7.2.1 Experimental approach

To determine the best combination of C, N and P, a factorial design was conducted, assuming linear response and using two levels. The experimental details and design are given in Section 3.7.1. An abridged version of the experimental matrix is given in Table 7.1 below.

Table 7.1: The C:N:P experimental matrix used for the medium optimisation study.

Run	**Factors**			**Ratio**		
	Carbon	Nitrogen	Phosphorus	C	N	P
	High=4	High=0.26	High=0.0172			
	Low=2	Low=0.065	Low=0.0043			
1	low	low	low	2	0.065	0.0043
2	low	low	high	2	0.065	0.0172
3	low	high	low	2	0.26	0.0043
4	low	high	high	2	0.26	0.0172
5	high	low	low	4	0.065	0.0043
6	high	low	high	4	0.065	0.0172
7	high	high	low	4	0.26	0.0043
8	high	high	high	4	0.26	0.0172

The growth of Isolates 1, 7, 8 and 10 was monitored by removing samples and measuring the absorbance, biomass and substrate concentrations. The maximum specific growth rates were also

determined. Following an analysis of the growth kinetics exhibited by the isolates, statistical analysis was performed to determine the influences and possible correlations of carbon, nitrogen and phosphorus to absorbance, biomass accumulation and maximum specific growth rate. An understanding of the relationship between various C:N:P ratios, their interactions and the effect of these medium components on microorganism growth can be used for the evaluation of suitable further process optimisation targets.

The growth kinetics are presented in Section 7.2.2.1, extent of substrate utilisation in Section 7.2.2.2, statistical analysis in Section 7.2.2.3. Further, the initial carbon source optimisation is presented in Section 7.3 and finally conclusions are drawn in Section 7.4.

7.2.2 Results and discussion

7.2.2.1 Growth kinetics

An example of an optimisation run is shown in Figure 7.1 using Run 1 and Isolate 1. The value of -1 for log (X) implies that the inoculum concentration resulted in a starting cell concentration of 0.1 g dry weight per mL. The growth curves were conducted in triplicate and average values used for the assessment. Following correction for evaporation by calculating the difference between the initial and measured remaining volume in the microtitre plate upon sampling, the effect of the media composition on growth was assessed in terms of maximum specific growth rate and biomass concentration.

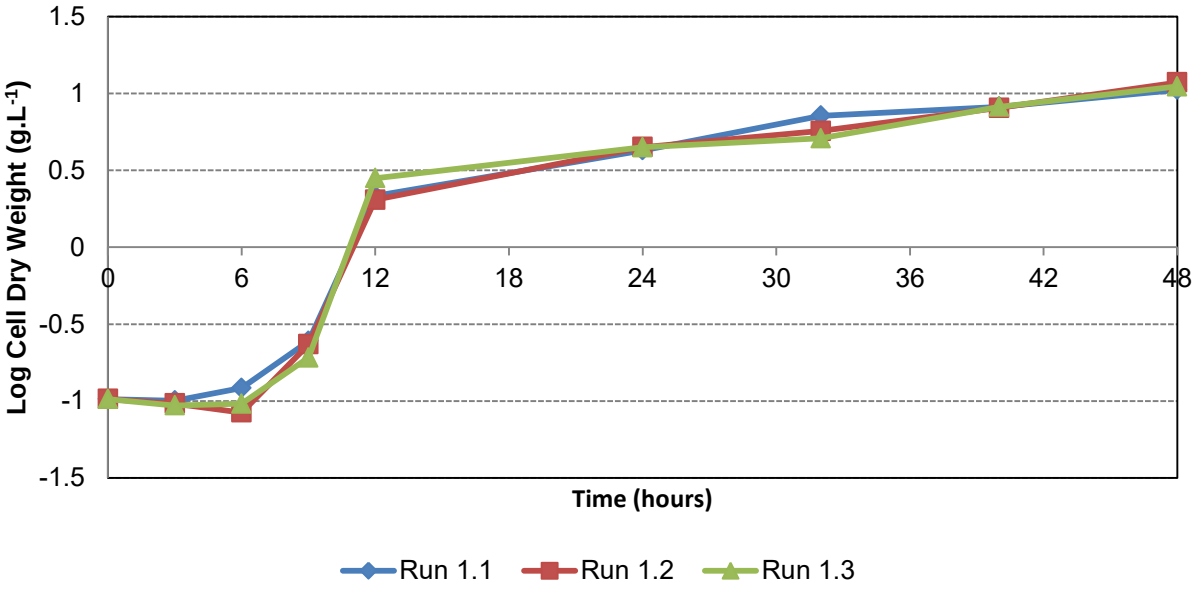


Figure 7.1: The triplicate growth curves for Run 1 of Isolate 1 in the medium optimisation experiment. The average was corrected using the volume and evaporation rate over the cultivation period to obtain the final values.

Summaries of the results for the optimisation experiments for Isolates 1, 7, 8 and 10 are shown in Table 7.2, Table 7.3 and Table 7.4 and respectively.

For Isolate 1, the highest average OD_{600} (10.56) and maximum cell dry weight (14.44 $g.L^{-1}$) at 48 hours were achieved using a C:N:P ratio of 2:0.0.065:0.0172 and 2:0.0.065:0.0043 respectively. The highest μ_{max} (0.274 hr^{-1}) over the 48 hour cultivation period was also achieved during Run 2 (2:0.0.065:0.0172). All ratios were expressed relative to nitrogen.

Isolate 7 had the highest average OD_{600} (11.70) and maximum cell dry weight (15.72 $g.L^{-1}$) were achieved using a C:N:P ratio of 4:026:0.0043 (Table 7.3).

In Table 7.4, Isolate 8 the highest average OD_{600} value for Isolate 8 was noted for a C:N:P ratio of 4:0.065:0.0172 at a reading of 9.31. The highest maximum cell dry weight (15.27 $g.L^{-1}$) was achieved with a C:N:P ratio of 4:0.065:0.0043.

For Isolate 10, the highest average OD_{600} (10.88) and maximum cell dry weight (12.50 $g.L^{-1}$) were achieved using a C:N:P ratio of 2:0.065:0.0172 and 4:0.065:0.0172 respectively, as shown in Table 7.5.

These data gathered suggest that optimal growth may occur at lower N and P levels; however this needs to be considered in terms of substrate utilisation and γ-PGA analysis too. The effect of the N and P is analysed in terms of substrate utilisation in Section 7.2.2.2 and discussed in terms of statistical significance in Section7.2.2.3. Chapter 9 discusses γ-PGA analysis in detail.

Table 7.2: Optimisation study results for Isolate 1 after growth for 48 hours in the various media[1] in microtitre deep well plates. The averages of triplicate experiments are shown.

Run	Average OD_{600}	Biomass ($g.L^{-1}$)	μ_{max} (hr^{-1})
1	8.74 ± 1.25	14.44 ± 1.08	0.116 ± 0.01
2	10.56 ± 2.47	13.65 ± 1.22	0.274 ± 0.01
3	7.90 ± 1.13	12.71 ± 0.22	0.121 ± 0.01
4	9.21 ± 1.64	13.41 ± 0.87	0.054 ± 0.01
5	7.68 ± 1.11	10.11 ± 0.95	0.244 ± 0.01
6	8.99 ± 1.79	9.74 ± 0.35	0.158 ± 0.02
7	7.80 ± 1.54	10.48 ± 0.86	0.139 ± 0.00
8	8.24 ± 1.30	12.11 ± 1.41	0.144 ± 0.01
*MME	3.21 ± 0.05	4.98 ± 0.08	0.106 ± 0.02

*MME - values for isolate in standard MME medium prior to medium optimisation.

[1]The concentrations of the media are described in Section 3.7.1.

Table 7.3: Optimisation study results for Isolate 7 after growth for 48 hours in the various media in microtitre deep well plates. The averages of triplicate experiments are shown.

Run	Average OD_{600}	Biomass ($g.L^{-1}$)	μ_{max} (hr^{-1})
1	2.65 ± 0.95	4.39 ± 1.15	0.256 ± 0.07
2	9.95 ± 0.79	11.86 ± 0.14	0.239 ± 0.02
3	7.16 ± 0.23	12.42 ± 0.07	0.204 ± 0.02
4	10.85 ± 0.75	14.51 ± 0.22	0.213 ± 0.02
5	10.65 ± 0.12	14.03 ± 0.88	0.211 ± 0.03
6	10.21 ± 1.36	8.96 ± 1.14	0.327 ± 0.01
7	11.70 ± 0.75	15.72 ± 0.98	0.321 ± 0.05
8	0.87 ± 0.30	1.28 ± 0.93	0.224 ± 0.03
MME	2.99 ± 0.01	4.25 ± 0.02	0.196 ± 0.00

Table 7.4: Optimisation study results for Isolate 8 after growth for 48 hours in the various media in microtitre deep well plates. The averages of triplicate experiments are shown.

Run	Average OD_{600}	Biomass ($g.L^{-1}$)	μ_{max} (hr^{-1})
1	3.35 ± 0.03	7.73 ± 0.00	0.273 ± 0.01
2	5.26 ± 0.25	10.92 ± 1.22	0.246 ± 0.01
3	4.53 ± 0.10	11.9 ± 0.3	0.235 ± 0.01
4	5.78 ± 0.17	10.82 ± 0.28	0.243 ± 0.02
5	7.43 ± 0.12	15.27 ± 1.41	0.236 ± 0.01
6	9.31 ± 0.55	13.35 ± 1.48	0.252 ± 0.01
7	9.00 ± 0.60	11.39 ± 0.12	0.078 ± 0.02
8	8.52 ± 0.15	11.81 ± 0.44	0.187 ± 0.01
MME	4.11 ± 0.15	5.98 ± 0.21	0.148 ± 0.01

Table 7.5: Optimisation study results for Isolate 10 after growth for 48 hours in the various media in microtitre deep well plates. The averages of triplicate experiments are shown.

Run	Average OD_{600}	Biomass ($g.L^{-1}$)	μ_{max} (hr^{-1})
1	9.52 ± 0.55	10.27 ± 0.15	0.264 ± 0.02
2	10.88 ± 0.31	11.23 ± 0.37	0.265 ± 0.01
3	9.66 ± 0.13	10.48 ± 0.20	0.200 ± 0.01
4	10.93 ± 0.84	10.68 ± 0.57	0.222 ± 0.02
5	7.27 ± 0.25	11.35 ± 0.43	0.230 ± 0.03
6	7.62 ± 0.68	12.50 ± 0.31	0.243 ± 0.01
7	4.67 ± 0.38	11.20 ± 1.15	0.241 ± 0.00
8	4.58 ± 0.64	12.00 ± 0.55	0.166 ± 0.01
MME	4.23 ± 0.08	4.27 ± 0.06	0.182 ± 0.00

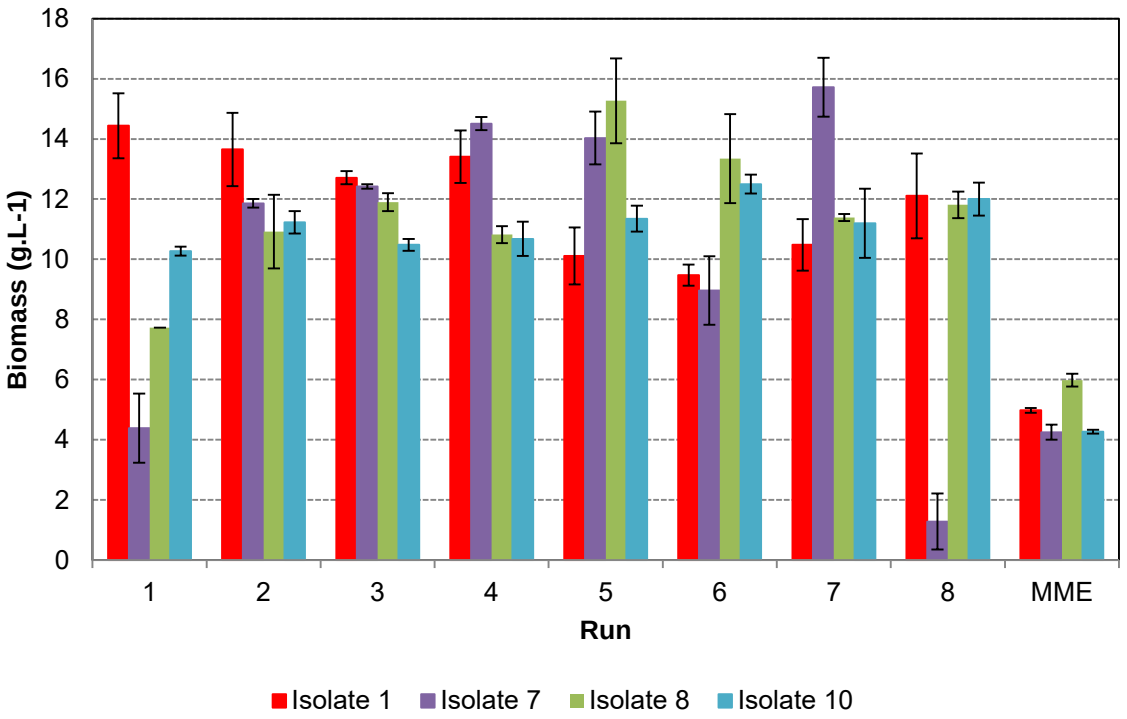


Figure 7.2: The biomass concentrations obtained for Isolates 1, 7, 8 and 10 in MME and the eight media used in this study. The experiments were conducted over a 48 hour period. The average of triplicate experiments is shown.

The graphical comparisons of the cell dry weights and various μ_{max} values for the isolates are displayed in Figure 7.2 and Figure 7.3 respectively.

Isolate 7 produced a high cell dry weight of 15.72 $g.L^{-1}$ during Run 7. This medium contained a higher proportion of carbon and nitrogen in the ratio of 4:0.26:0.0043. Isolate 8 grew similarly well in a high

carbon and low nitrogen and phosphorus, producing 15.27 $g.L^{-1}$ biomass. Although the measured biomass obtained for these two isolates can be used as an indicator of good growth in these media, when the error associated is taken into account, these biomass concentrations are not conclusively the highest overall obtained amongst the isolates and across all the media. Runs 1 and 8 produced the least biomass for Isolate 7, which showed intolerance for extremities.

Isolate 1 was the only isolate which grew better, on average, in the media which contained low carbon (Runs 1 to 4). Isolate 10 showed less discrimination, producing moderately high amounts of biomass across all the media. Overall, the isolates produced more biomass compared to the un-optimised MME, with the exception of Isolate 7, Run 8.

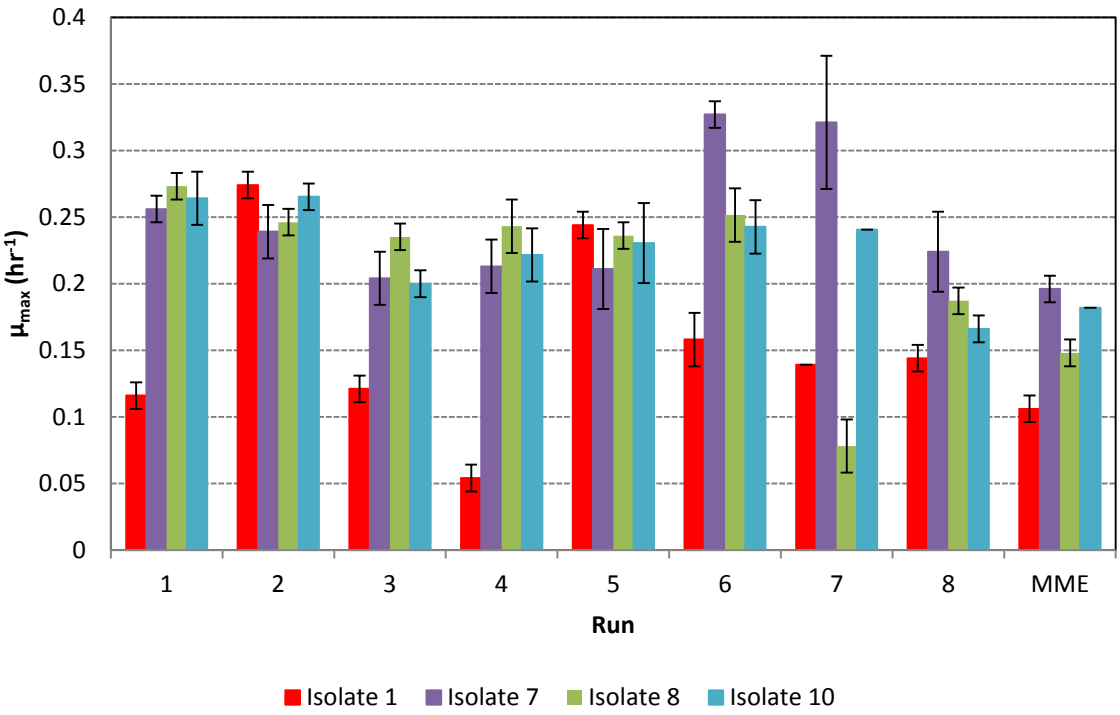


Figure 7.3: The maximum specific growth rates obtained for Isolates 1, 7, 8 and 10 in MME and the eight media used in this study. The experiments were conducted over a 48 hour period. The average of triplicate experiments is shown.

For Isolate 7, Run 6 (4:0.065:0.0172) produced the third least biomass amongst the eight runs. However, this run produced a maximum specific growth rate which was amongst the highest obtained. The lower final biomass produced by Run 6 could potentially be due to an unfavourable interaction between the medium components which resulted in the isolate suffering from a death phase towards the conclusion of the cultivation. The substrate consumption will be discussed in the Section that follows. The statistical analysis of the effects of carbon, nitrogen and phosphorus will also be discussed, in Section 7.2.2.3, in order to elucidate this finding.

The opposite was observed for Runs 1, 3 and 4 of Isolate 1. These runs produced high yields of biomass, but at the lowest growth rates. Whilst the growth rates for Runs 1 and 3 were comparable to

un-optimsed MME, the medium with the lowest growth rate (Run 4), did not exceed the growth measured in un-optimsed medium. The isolate also generally grew faster in the high carbon media, but produced lower biomass.

Isolate 8 and 10 were unable to grow efficiently during the first 12 hours in the medium for Run 7. Although these isolates experienced a prolonged lag phase, adaptation to the medium resulted in steady growth and considerably high final OD and biomass concentrations. During the exponential phase, Isolate 10 grew at a μ_{max} of 0.241 hr^{-1}. Both biomass values were around 11 $g.L^{-1}$.

These results illustrate that there are possibly different factors which depend on the medium composition that are responsible for biomass and growth rates.

7.2.2.2 Substrate utilisation

Citric acid, glucose and glycerol were analysed in detail for only Isolates 1 and 7 using HPLC. The utilisation of these carbon substrates after 48 hours for Runs 1 to 8 is found in Figure 7.4 and Figure 7.5 for Isolates 1 and 7 respectively. Isolate 1 was more efficient than Isolate 7 in glucose utilisation, completely consuming all glucose found in the media after 12 hours (data not shown). Glycerol was least efficiently utilised by both isolates. For Isolate 1, Runs 1 (87%), 2 (72%) and 7 (21%) successfully consumed some of this substrate. Some glycerol (34%) was only used by Run 4 for Isolate 7.

For Isolate 1, only Run 7 was able to utilise 21% of the citric acid found in this medium. Isolate 7 was able to consume more citric acid than Isolate 1. Runs 3, 4 and 5 were able to consume all the citric acid. Run 7 utilised 13%.

The use of glycerol in the media for Runs 1 and 2 may have been a contributing factor for the good biomass yields obtained for Isolate 1, but not exclusively so, as similar biomass concentrations were obtained for Runs 3, 4 and 8. Less biomass was produced for Run 7 compared to these two runs, even though an additional carbon source was utilised (21 % of citric acid). There was also 21% glycerol used in this medium, which was not as much as Runs 1 (87%) and 2 (72%). Runs 1 and 2 contained half the combined initial carbon concentration of Run 7.

Run 4 for Isolate 7 produced a biomass concentration which was amongst the highest, and utilised all the citric acid, glucose and 34% glycerol. For this isolate, Run 7 produced slightly higher biomass than Run 4 and displayed a faster growth rate, even though the organism consumed only 13% of the citric acid and no glycerol. The lower biomass concentration produced from Run 6 could be possibly due to inefficient use of citric acid and glycerol.

For both isolates, efficient use of some or all carbon sources did not automatically translate to an increased quantity of biomass or maximum specific growth rates.

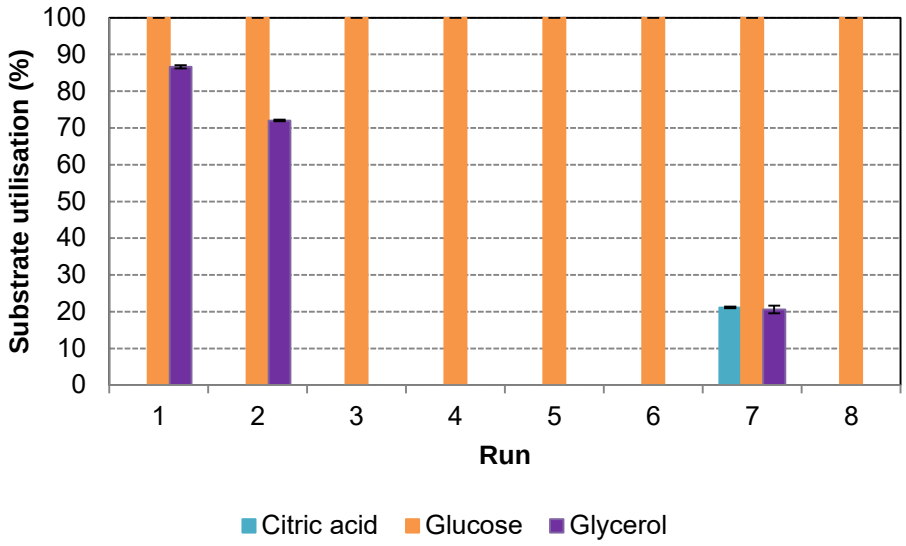


Figure 7.4: The carbon source substrate utilisation for Isolate 1 after a 48 hour cultivation period in the various media. The averages of duplicate experiments are shown.

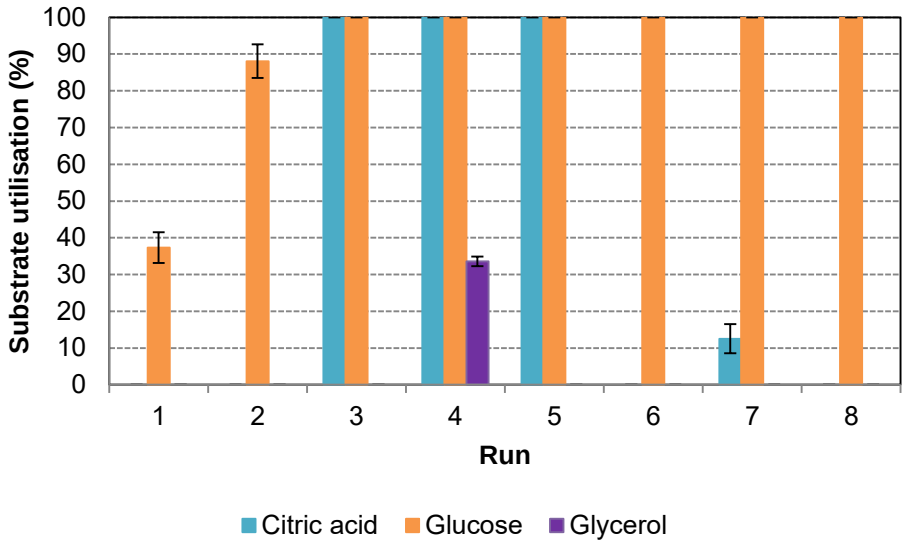


Figure 7.5: The carbon source substrate utilisation for Isolate 7 after a 48 hour cultivation period in the various media. The averages of duplicate experiments are shown.

7.2.2.3 Statistical analysis

In order to better understand the effects of carbon, nitrogen and phosphorus on absorbance, biomass accumulation and maximum specific growth rate were analysed in detail for each isolate using analysis of variance (ANOVA) on the Design Expert 8.0 software (Stat-Ease Inc., Minnesota, USA).

This software has the ability to identify the vital factors and interactions that affect a process, aiding in further process optimisation. A number of different factors can be screened simultaneously. In this work, each factor was tested at the two levels and combinations as described in Table 7.1. The

factors were annotated as A, B and C for carbon, nitrogen and phosphorus respectively. The responses were absorbance, biomass and maximum specific growth rate. The recorded data from Tables 7.2 to 7.4 was entered separately for all isolates into the software, corresponding to the same run C:N:P ratio. The software automatically lists the runs in randomized order, which protects against any lurking factors such as time, temperature or humidity. The user chooses the effects (or factors) to model, by selecting these (plotted as squares on a half-normal probability plot) starting from the extreme right hand side of the plot to the left. Design-Expert adjusts a line to exclude the chosen effects. This line should ideally match up with the majority of the remaining effects near zero as one keeps selecting squares from right to left.

An ANOVA analysis table is produced which includes all the selected effects and their coefficients.

7.2.2.3.1 Absorbance

The results of the ANOVA analysis for Isolate 1 are presented in Table 7.6. The model for Isolate 1 was found to be significant (p value less than 0.05) when interactions between the three effects (AB, AC, BC and ABC) were excluded. An interaction represents a non-linear response of second order.

The sum of squares (SS) is a mathematical approach to determining the dispersion of data points. This can be calculated using the following equation:

$$SS = \sum X^2 - \frac{(\sum X)^2}{N} \qquad (7.1)$$

where, X is the data point and N the number of data points available.

The mean square (MS) was calculated by dividing the sum of squares by the appropriate number of degrees of freedom.

The F-value further supports how well the regression line approximates the real data points.

The R^2 coefficient of determination is a statistical measure of how well the regression line approximates the real data points. An R^2 of 1.0 indicates that the regression line perfectly fits the data. R^2 = 1- (total sum of squares / residual sum of squares).

When the regression line is linear (y = mx + c) the regression coefficient is the constant (m) that represents the rate of change of one variable (y) as a function of changes in the other (x). This is the slope of the regression line. This trend line can show, graphically and numerically, relationships between the dependent and independent variables. The mean standard deviation from these points is the related standard errors.

Design Expert has additional regression coefficients, including the predicted R^2. This value determines the confidence level of the model predictability and how it fits the data in the design space. When insignificant terms are removed from the selected effects, both the R^2 and predicted R^2 values decrease. Therefore, insignificant terms (p value less than 0.05) which did not result in rendering the overall model insignificant were included in the models for better prediction of the particular responses.

The model F value of 9.51 implies the model is significant. There is only a 2.72% chance that a model F value this large could occur due to noise. Values of "Prob > F" falling below a p-value of 0.05 indicate model terms that are significant. In this case, A and C are significant model terms.

The sum of squares for phosphorus was the highest, indicating that this component had the greatest effect on the model, and hence aborbance.

The final model equation in terms of the actual factors was described by:

$$Absorbance = 9.59833 - 0.46250 \times carbon - 3.61538 \times nitrogen + 97.57364 \times phosphorus \quad (7.2)$$

The prediction equation is provided in actual units. The coefficients in the actual equation compensate for the differences in the ranges of the factors as well as the differences in the effects.

Table 7.6: Results of ANOVA analysis of the 2^3 factorial design experiments for absorbance values obtained from Isolate 1.

Source	Sum of Squares	DF	Mean Square	F Value	p value Prob > F	
Model	5.68	3	1.89	9.51	0.0272	Significant
A-Carbon	1.71	1	1.71	8.59	0.0427	
B-Nitrogen	0.99	1	0.99	4.99	0.0892	
C-Phosphorus	2.98	1	2.98	14.95	0.0180	
Residual	0.80	4	0.199			
Cor Total	6.45	7				

The model for Isolate 7 was not significant. Although the p value of phosporus was also not significant, this factor had the greatest effect . The interaction between carbon and nitrogen showed a similar, but slightly lower effect. Therefore, the larger influence of the phosphorus component on absorbance was also found in this isolate. The results are shown in Table 7.7. The residual, in this case, reflects the sum of squares of the remaining ABC interaction.

Table 7.8 shows the ANOVA analysis for Isolate 8. The model was significant when AB and ABC were excluded. Carbon, with a p-value of 0.0078, displayed the greatest effect on the model and was the only significant factor. The measured OD for this bacterium were found to be higher in the media

which contained higher carbon (Runs 5 to 8). Nitrogen displayed the least effect on absorbance, but when phosphorus was present, the interaction between these two factors was greater than carbon and phosphorus (AC). This indicates that this particular isolate may require phosphorus to effectively assimilate nitrogen.

Table 7.7: Results of ANOVA analysis of the 2^3 factorial design experiments for absorbance values obtained from Isolate 7.

Source	Sum of Squares	DF	Mean Square	F Value	p value Prob > F	
Model	49.20	6	8.20	3.25	0.4006	Not significant
A-Carbon	4.95	1	4.95	1.96	0.3947	
B-Nitrogen	0.044	1	0.044	0.017	0.9168	
C-Phosphorus	15.10	1	15.10	5.99	0.2469	
AB	13.08	1	13.08	5.19	0.2633	
AC	15.10	1	15.10	5.99	0.2469	
BC	0.93	1	0.93	0.37	0.6522	
Residual	2.52	1	2.52			
Cor Total	51.72	7				

Table 7.8: Results of ANOVA analysis of the 2^3 factorial design experiments for absorbance values obtained from Isolate 8.

Source	Sum of Squares	DF	Mean Square	F Value	p value Prob > F	
Model	34.31	5	6.86	29.38	0.0332	Significant
A-Carbon	29.41	1	29.41	125.96	0.0078	
B-Nitrogen	0.77	1	0.77	3.29	0.2113	
C-Phosphorus	2.60	1	2.60	11.13	0.0793	
AC	0.39	1	0.39	1.66	0.3268	
BC	1.14	1	1.14	4.88	0.1578	
Residual	0.47	2	0.23			
Cor Total	34.78	7				

The final equation in terms of the actual factors was described by:

$$Absorbance = -2.72028 + 2.28417 \times carbon + 9.63248 \times nitrogen + 288.24289 \times phosphorus - 34.10853 \times carbon \times phosphorus - 600.27827 \times nitrogen \times phosphorus \quad (7.3)$$

The model for Isolate 10 was significant (Table 7.9). There was only a 3.45% chance that the large F-value of 492.33 could occur due to noise. Carbon and nitrogen as well as their interaction were all significant model terms. Similarly to Isolate 8, the factor with the greatest effect was carbon. The effect of the phosphorus component was the least. The residual was representative of ABC.
The final equation in terms of the actual factors was described by:

$$Absorbance = 9.68431 - 0.39792 \times carbon + 16.56838 \times nitrogen + 210.91731 \times phosphorus - 7.47436 \times carbon \times nitrogen - 45.93023 \times carbon \times phosphorus - 105.34685 \times nitrogen \times phosphorus \quad (7.4)$$

Table 7.9: Results of ANOVA analysis of the 2^3 factorial design experiments for absorbance values obtained from Isolate 10.

Source	Sum of Squares	DF	Mean Square	F Value	p value Prob > F	
Model	45.23	6	7.54	492.33	0.0345	Significant
A-Carbon	35.49	1	35.49	2317.73	0.0132	
B-Nitrogen	3.71	1	3.71	242.47	0.0408	
C-Phosphorus	1.04	1	1.04	68.18	0.0767	
AB	4.25	1	4.25	277.46	0.0382	
AC	0.70	1	0.70	45.85	0.0933	
BC	0.035	1	0.035	2.29	0.3716	
Residual	0.015	1	0.015			
Cor Total	45.25	7				

7.2.2.3.2 Biomass

The model for Isolate 1 was found to be significant when the interactions between A and C and the three effects (ABC) were excluded (Table 7.10)

Table 7.10: Results of ANOVA analysis of the 2^3 factorial design experiments for biomass values obtained from Isolate 1.

Source	Sum of Squares	DF	Mean Square	F Value	p value Prob > F	
Model	21.86	5	4.37	33.58	0.0292	Significant
A-Carbon	17.32	1	17.32	133.04	0.0074	
B-Nitrogen	0.074	1	0.074	0.57	0.5293	
C-Phosphorus	0.17	1	0.17	1.31	0.3702	
AB	2.77	1	2.77	21.30	0.0439	
BC	1.52	1	1.52	11.70	0.0759	
Residual	0.26	2	0.13			
Cor Total	22.12	7				

The model F-value of 33.58 implies the model is significant. There is only a 2.92% chance that a model F-value this large could occur due to noise. In this case A and AB are significant model terms. A is the most significant component for biomass. B is only significant for biomass accumulation when A is present in the medium and the significance for C is dependent of the presence of B. In order to understand this further, a three dimensional (3D) plot was generated and presented in Figure 7.6.

Figure 7.6 shows the dominant effect of carbon versus that of nitrogen. However, when the concentration of nitrogen in the medium is high, carbon has less of an effect. When nitrogen is low,

carbon has more of an effect, but increasing the nitrogen at low carbon has an inhibitory effect. At high carbon, the opposite applies – an increase in nitrogen increases the biomass concentration. This may support the decrease in biomass during Run 3 and increases in Runs 7 and 8. The highest biomass is achieved at low carbon and low nitrogen. Run 1 was found to produce the highest biomass, but without absolute certainty.

The final equation in terms of the actual factors was described by:

$$Biomass = 20.24639 - 2.45250 \times carbon - 24.58547 \times nitrogen - 90.05168 \times phosphorus + 6.03846 \times carbon \times nitrogen + 693.69907 \times nitrogen \times phosphorus \quad (7.5)$$

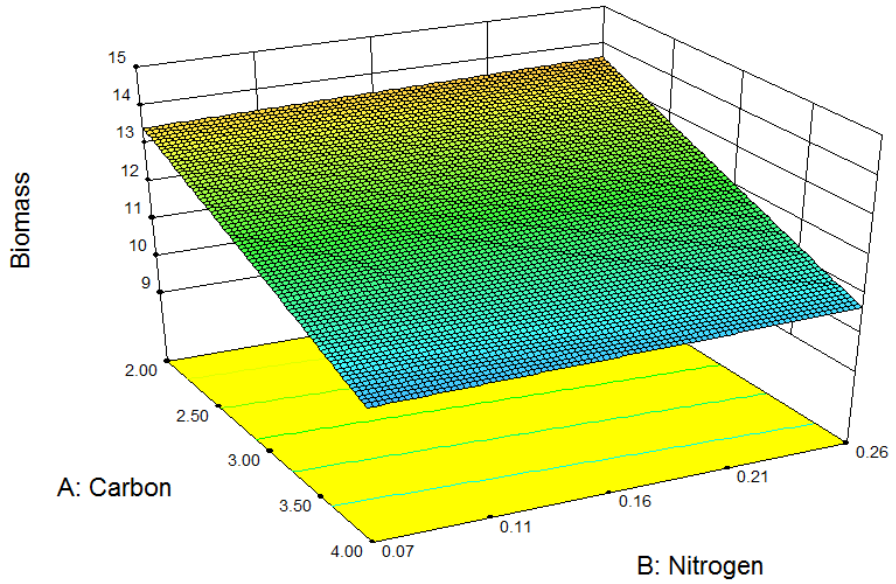


Figure 7.6: A 3D surface plot illustrating the effect of carbon and nitrogen on biomass accumulation in Isolate 1. Phosphorus was set to a value of 0.01.

The effect of phosphorus on biomass accumulation was then investigated. Both low (0.0043) and high (0.02) phosphorus did not influence the effect of carbon and nitrogen on biomass (Figure 7.7). Thus, the optimisation of this component in this system is not necessary for maximal biomass accumulation as the addition of any amount in the medium has no significant effect for this isolate.

A correlation of absorbance and biomass with the factors which contribute to these responses revealed that whilst phosphorus was the main factor for absorbance, it was not significant for biomass. This causes the absorbance readings to be biased by the presence of especially high phosphorus concentrations in the medium. The presence of phosphorus in the medium may lead to a higher OD measurement, possibly due to the formation of a precipitate or this factor causing the prevelence of bigger cell sizes.

A high absorbance reading does not necessary translate to high cell concentration, as was found in the results discussed in Section 7.2.1.

Isolate 7 did not produce a significant model for biomass (Table 7.11). Interestingly, nitrogen was the factor which displayed the greatest individual effect. Carbon showed the least effect. The interaction between carbon and phosphorus (AC) influenced biomass the most.

The model for Isolate 8 was also not significant. Carbon was the greatest influencer for biomass in this isolate, whilst the effect of phosphorus was the least. The interaction between carbon and phosphorus was more beneficial for biomass than nitrogen and phosphorus. A larger improvement in effect for biomass accumulation was predicted for phosphorus when this component interacts with both carbon and nitrogen (ABC). The presence and interaction of carbon and nitrogen was also important. The results can be found in Table 7.12.

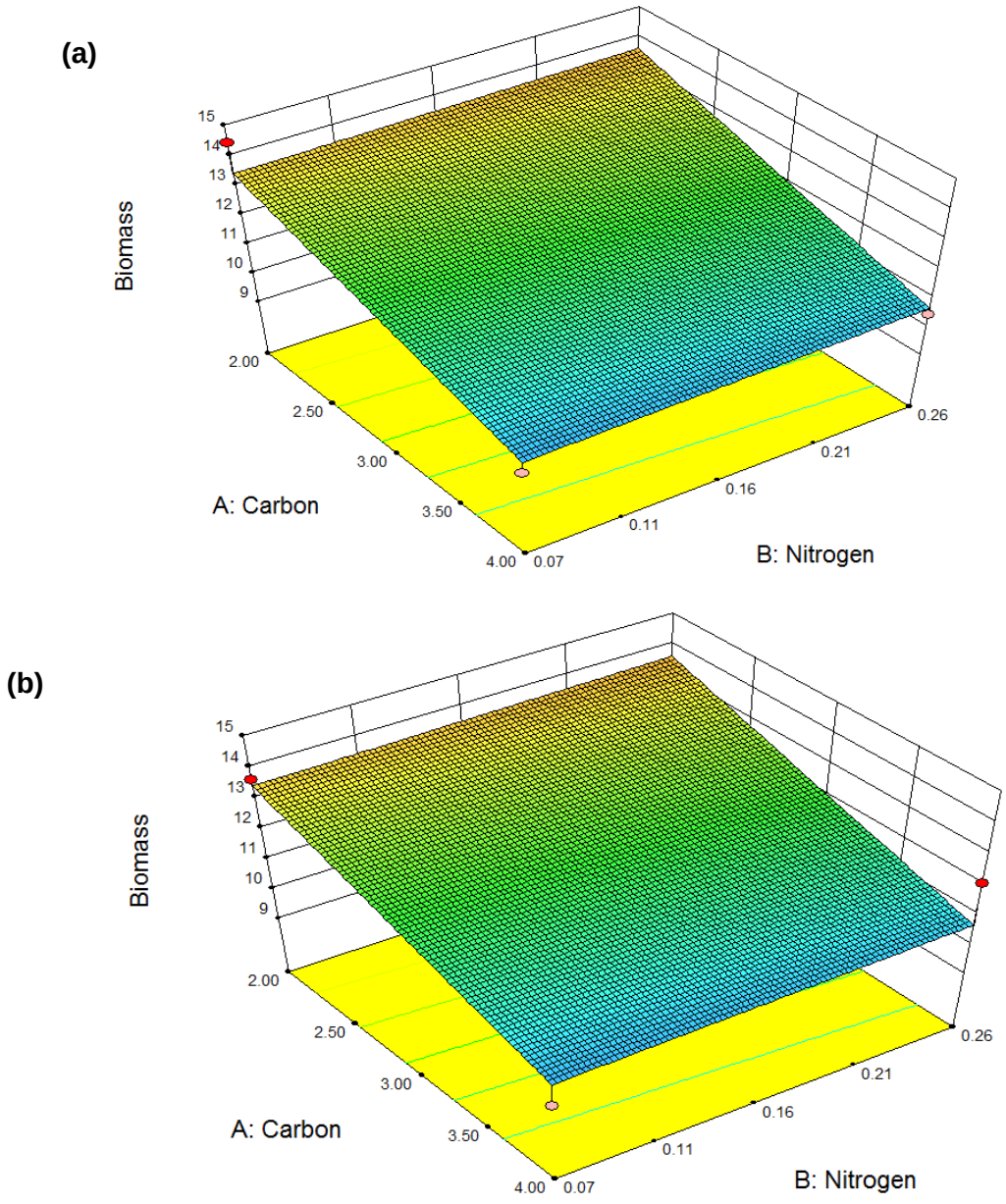


Figure 7.7: A 3D surface plot illustrating the effect of carbon and nitrogen on biomass accumulation in Isolate 1 at low (a) and high phosphorus (b).

Table 7.13 shows that A, B, C and BC yielded a significant model for Isolate 10. The most significant factor was carbon. Phosphorus was also a significant model term. The medium interaction between nitrogen and phosphorus did not significantly improve the effect of nitrogen on biomass in the system.

The final equation in terms of the actual factors was described by:

$$Biomass = 8.74042 + 0.54875 \times carbon + 1.10256 \times nitrogen + 96.12403 \times phosphorus - 220.63208 \times nitrogen \times phosphorus \quad (7.6)$$

Table 7.11: Results of ANOVA analysis of the 2^3 factorial design experiments for biomass values obtained from Isolate 7.

Source	Sum of Squares	DF	Mean Square	F Value	p value Prob > F	
Model	55.30	6	9.22	0.51	0.7909	Not significant
A-Carbon	0.72	1	0.72	0.039	0.8751	
B-Nitrogen	13.21	1	13.21	0.72	0.5511	
C-Phosphorus	4.68	1	4.68	0.26	0.7015	
AB	15.35	1	15.35	0.84	0.5275	
AC	21.13	1	21.13	1.16	0.4767	
BC	0.22	1	0.22	0.012	0.9307	
Residual	18.24	1	18.24			
Cor Total	73.54	7				

Table 7.12: Results of ANOVA analysis of the 2^3 factorial design experiments for biomass values obtained from Isolate 8.

Source	Sum of Squares	DF	Mean Square
Model	32.74	7	4.68
A-Carbon	13.65	1	13.65
B-Nitrogen	0.23	1	0.23
C-Phosphorus	0.05	1	0.05
AB	11.26	1	11.26
AC	1.63	1	1.63
BC	0.466	1	0.466
ABC	5.462	1	5.462
Pure Error	0.00	0	
Cor Total	32.74	7	

Table 7.13: Results of ANOVA analysis of the 2^3 factorial design experiments for biomass values obtained from Isolate 10.

Source	Sum of Squares	DF	Mean Square	F Value	p value Prob > F	
Model	3.89	4	0.97	26.31	0.0113	Significant
A-Carbon	2.41	1	2.41	65.09	0.0040	
B-Nitrogen	0.12	1	0.12	3.31	0.1664	
C-Phosphorus	1.21	1	1.21	32.66	0.0106	
BC	0.15	1	0.15	4.16	0.1341	
Residual	0.11	3	0.04			
Cor Total	4.01	7				

7.2.2.3.3 Maximum specific growth rate

The model of μ_{max} for Isolate 1 was not significant, but Table 7.14 shows that the nitrogen source was the greatest influence. This effect was however, also not significant as the p value was equal to 0.48. The cumulative effect of the ABC interaction is also great, but less than the individual effect of B. This is possibly due to the presence of the low sum of squares attributed by the carbon and phosphorus components.

The model for Isolate 7 was only significant when nitrogen was selected (Table 7.15). This further supports the importance of the presence of a nitrogen source for efficient bacterial growth. A further analysis of the effect of nitrogen showed that this component can be inhibitory at excessive concentrations. Figure 7.8 shows that an increase of nitrogen to the highest ratio of 0.26, results in a decrease in maximum specific growth. This was observed in Runs 3, 4, 7 and 8. The media which contained lower nitrogen (1, 2, 5 and 6) allowed for more fastiduous growth of the microorganism.

The final equation in terms of the actual factors was described by:

$$\mu_{max} = 0.28600 - 0.42692 \times nitrogen \qquad (7.7)$$

Table 7.14: Results of ANOVA analysis of the 2^3 factorial design experiments for μ_{max} values obtained from Isolate 1.

Source	Sum of Squares	DF	Mean Square
Model	0.035	7	0.005
A-Carbon	0.002	1	0.002
B-Nitrogen	0.014	1	0.014
C-Phosphorus	1.25E-05	1	1.25E-05
AB	0.001	1	0.001
AC	0.004	1	0.004
BC	0.002	1	0.002
ABC	0.012	1	0.012
Pure Error	0	0	
Cor Total	0.035	7	

Table 7.15: Results of ANOVA analysis of the 2^3 factorial design experiments for μ_{max} values obtained from Isolate 7.

Source	Sum of Squares	DF	Mean Square	F Value	p value Prob > F	
Model	0.014	1	0.014	7.00	0.0382	Significant
B-Nitrogen	0.014	1	0.014	7.00	0.0382	
Residual	0.012	6	0.002			
Cor Total	0.026	7				

Both Isolates 8 and 10 did not yield a significant model. The ANOVA analyses of these two isolates are found in Table 7.16 and Table 7.17.

Table 7.16: Results of ANOVA analysis of the 2^3 factorial design experiments for μ_{max} values obtained from Isolate 8.

Source	Sum of Squares	DF	Mean Square
Model	0.054	7	0.008
A-Carbon	0.013	1	0.013
B-Nitrogen	0.015	1	0.015
C-Phosphorus	0.004	1	0.004
AB	0.008	1	0.008
AC	0.006	1	0.006
BC	0.005	1	0.005
ABC	0.002	1	0.002
Pure Error	0.000	0	
Cor Total	0.054	7	

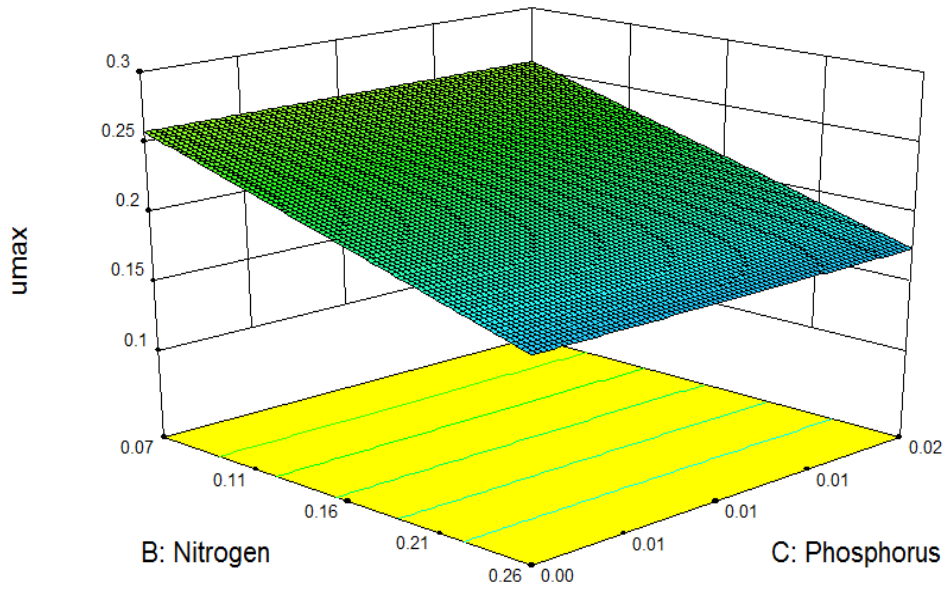


Figure 7.8: A 3D surface plot illustrating the effect of nitrogen and phosphorus on μ_{max} for Isolate 7. Carbon was set to a value of 3.00.

Table 7.17: Results of ANOVA analysis of the 2^3 factorial design experiments for μ_{max} values obtained from Isolate 10.

Source	Sum of Squares	DF	Mean Square
Model	0.053	7	0.008
A-Carbon	0.012	1	0.012
B-Nitrogen	0.021	1	0.021
C-Phosphorus	0.005	1	0.005
AB	0.005	1	0.005
AC	0.003	1	0.003
BC	0.004	1	0.004
ABC	0.002	1	0.002
Pure Error	0.000	0	
Cor Total	0.053	7	

For both these isolates, the individual effect of nitrogen was not significant (Figure 7.9), but effect of this component on maximum specific growth rate was greatest compared to carbon, phosphorus and all the combined interactions. The results obtained from all the isolates indicate that this medium component is the most important for $\mu_{max.}$

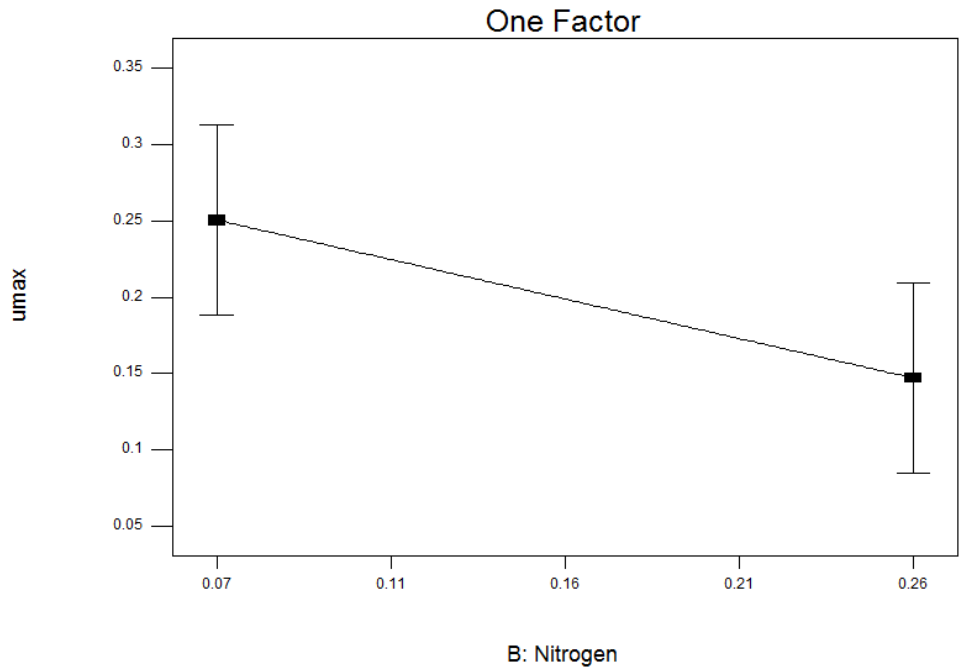


Figure 7.9: A one factor model graph illustrating the overlapping error bars of nitrogen for the μ_{max} response of Isolate 8. Carbon was set to a value of 3.00 and phosphorus to 0.01. A similar graph was obtained for Isolate 10.

7.2.2.3.4 Regression analysis

The effect of the factors on the responses can be based on the regression coefficient values. The effects for on the maximum specific growth rate and biomass production are presented in Figure 7.10 and Figure 7.11. Statistical analysis of the data was performed using Minitab 16 software (LEAD Technologies Inc., North Carolina, USA) in order to produce these graphical interpretations.

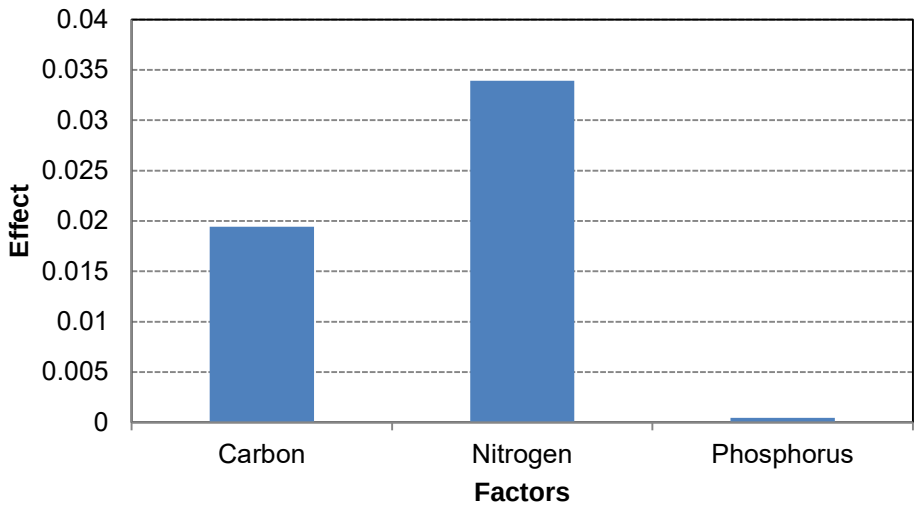


Figure 7.10: Effect of carbon, nitrogen and phosphorus on the maximum specific growth rate (μ_{max}).

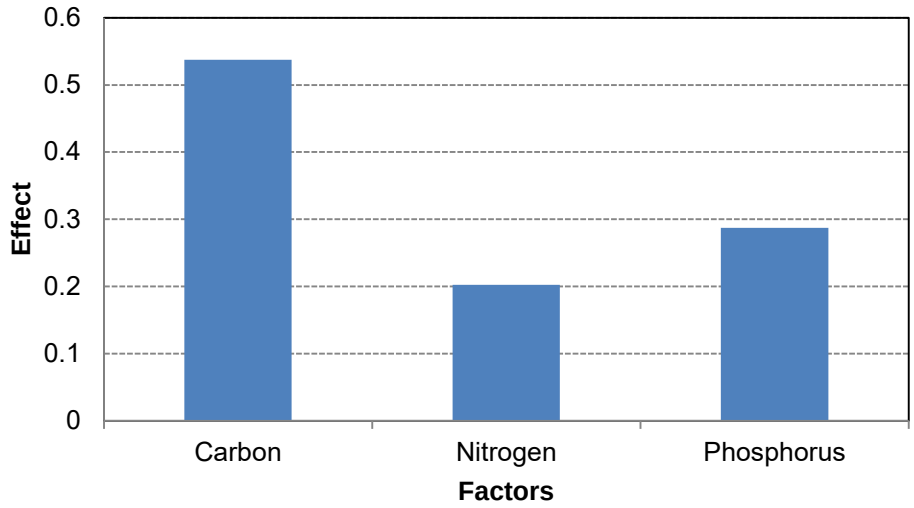


Figure 7.11: Effect of carbon, nitrogen and phosphorus on biomass production.

Whilst Design Expert uses the terms “effect” and “factor” interchangeably, Minitab distinguishes between the two in order to quantify the effect of the factor.

The main factor affecting the specific growth rate is the nitrogen source whereas carbon is the main effecting factor in biomass production. These results further support the findings discussed in Section 7.2.2.3. The phosphorus has limiting or no effect on the specific growth rate. Higher biomass production at higher carbon concentrations was only observed for Isolate 8. Runs 7 and 8, which contained high nitrogen, were among the 3 highest maximum specific growth rates for Isolate 7.

7.2.2.3.5 Effect plots

The effect plots in Figure 7.12 and Figure 7.13 are adapted from Minitab. These plots are based on the actual experimental data and not the first order model. The first order model does not fit the specific growth rate data accurately and will therefore result in an inaccurate effect plot.

In order to get an effect plot for a factor, the low level values are averaged and plotted against -1 and the high values are averaged and plotted against +1. A line is drawn between these two points to determine the effect of the associated factor. The steeper the gradient of the line is, the greater the effect on the response.

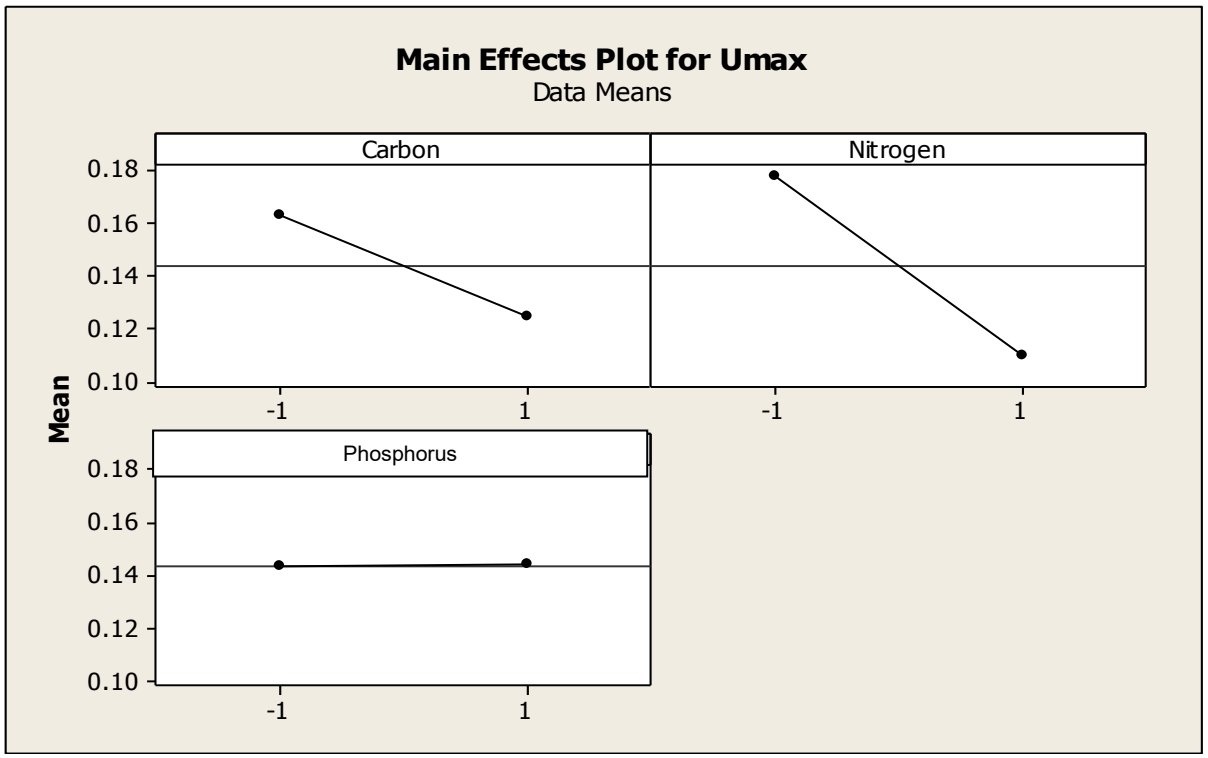


Figure 7.12: Effect plot for μ_{max} where -1 = low level ratios and 1 = high level ratios.

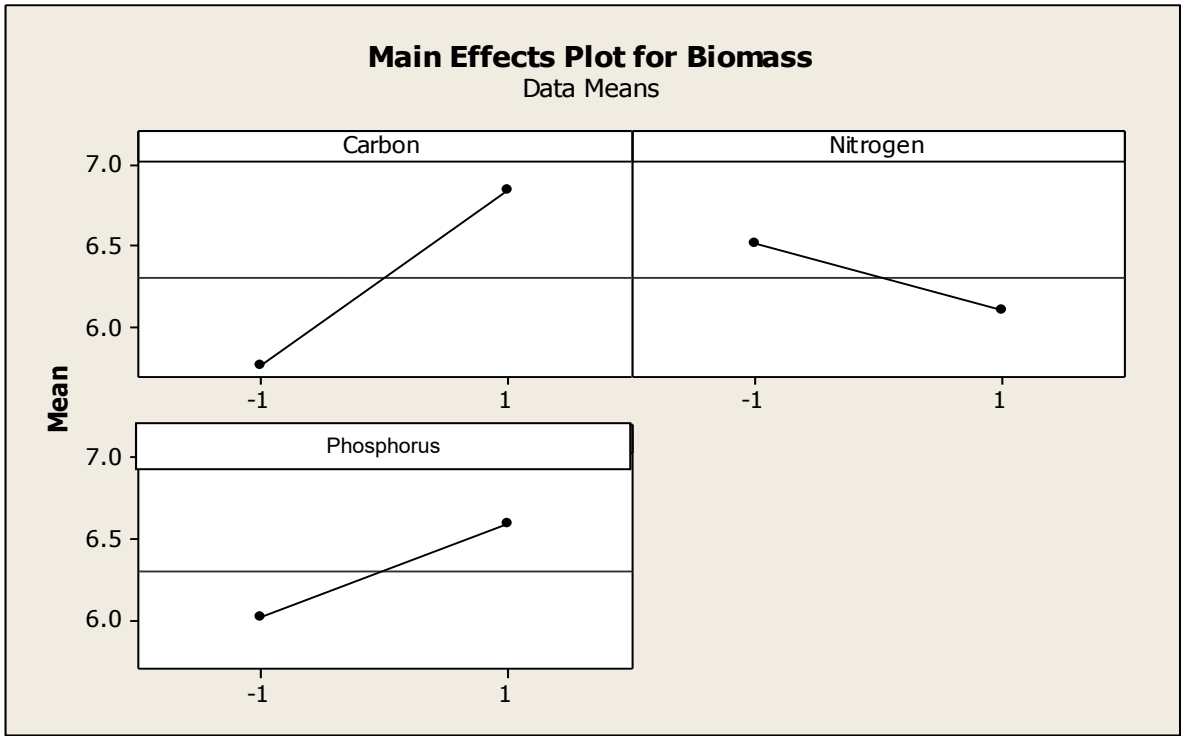


Figure 7.13: Effect plot for biomass production where -1 = low level ratios and 1 = high level ratios.

From Figure 7.12, the plots indicate that nitrogen has the biggest effect on the specific growth rate, whereas carbon has an intermediate effect and phosphorus has no effect. In Figure 7.13, carbon has

the greatest effect on biomass production whereas both nitrogen and phosphorus have intermediate effects. These effects have also been indicated by the regression coefficients (Section 7.2.2.3.4)

7.2.2.3.6 Interaction plots

The interaction plots below are also adapted from Minitab. These plots are also based on the actual experimental data and not the first order model. If the lines cross then there are interactions between the factors. If the lines do not across they are known to be parallel and therefore result in no interactions between factors.

In order to obtain an interaction plot, two plots are gathered: first plotting the values for the low independent factor against the dependant factor and then plotting the values for the high independent factor against the dependant factor. Using carbon versus phosphorus as an example, the first step was to calculate the mean of low phosphorus values at the relative low carbon values and plot these against the mean of low phosphorus values at the relative high carbon values. This will depict the first plot. The second plot was obtained by calculating the mean of high phosphorus values at the relative low carbon values and plotted against the mean of high phosphorus values at the relative high carbon values. The interactions between these two plots were then identified.

The interaction plots can be seen in Figure 7.14 and Figure 7.15. The dependant variable is the factor on the y-axis.

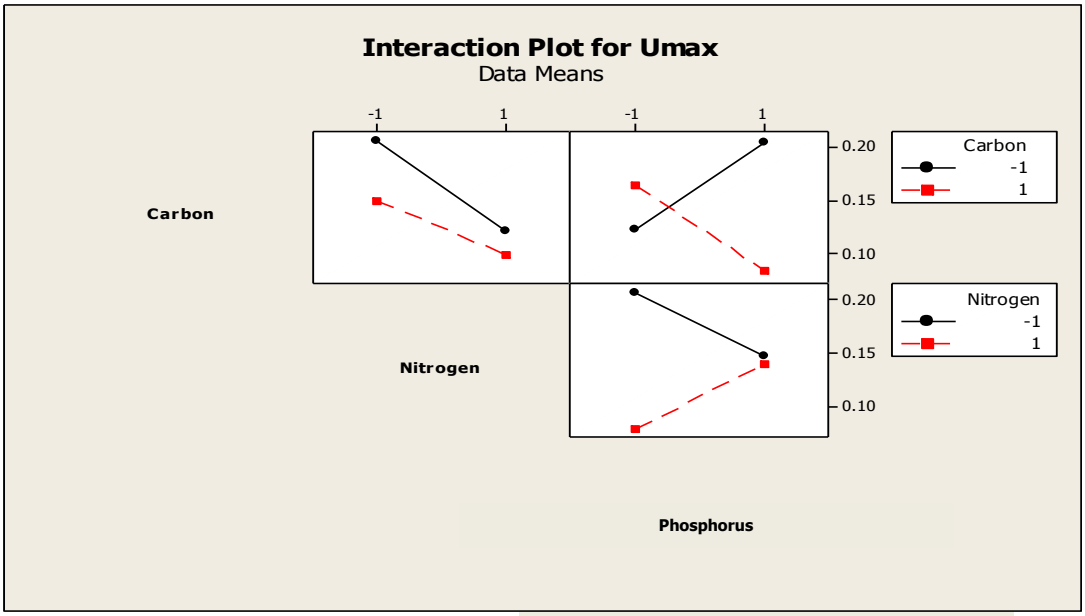


Figure 7.14: Interaction plot for μ_{max} where -1 = low level ratios and 1 = high level ratios.

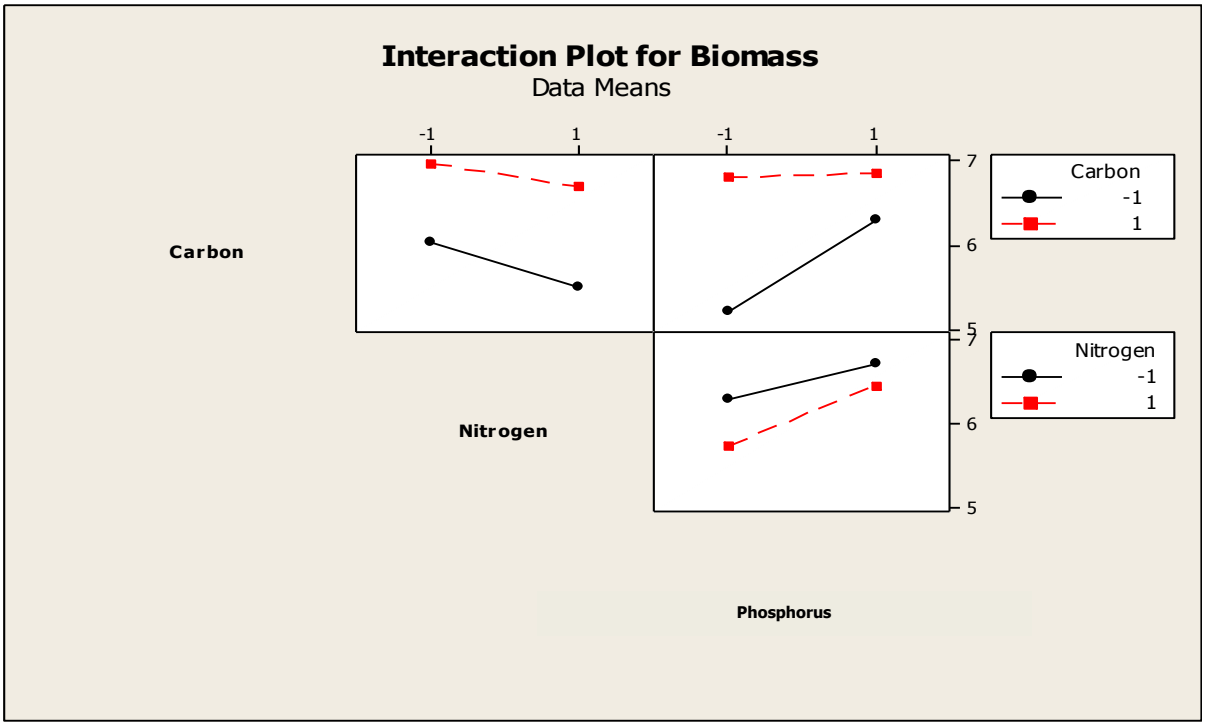


Figure 7.15: Interaction plot for biomass production where -1 = low level ratios and 1 = high level ratios.

The only notable interaction is between carbon and phosphorus when measuring the growth rate. This relationship may have been responsible for the higher growth rate experienced by Run 6 of Isolate 7.

7.3 Medium carbon source optimisation

Isolates 1 and 7 were grown in deep well plates for further carbon source optimisation using various combinations of lower total initial concentrations. The five substrate concentrations used for the different optimisation runs are described in Section 3.7.2. The media for Runs 12 and 13 which contained 1 $g.L^{-1}$ of citric acid were not homogenous after autoclaving and produced a precipitate (Figure 7.16). Citric acid acts as a chelating agent, thus binding the metals in the media. The low concentration of this chelator in media 12 and 13 resulted in poor binding and the metals precipitating out of the solution. Ethylenediaminetetraacetic acid (EDTA), which is a powerful chelating agent (Reynolds, 1963), was added to these media to a final concentration of 0.5 $g.L^{-1}$. The metals remained in solution, but both isolates were unable to grow in these two media. Consequently, these were excluded from the further optimisation experiments.

Figure 7.16: The shake flasks containing media for Runs 9 to 13 (from left to right).

The results from Runs 9 to 11 can be found in Table 7.18 and Table 7.19 for Isolates 1 and 7 respectively. For both isolates, the measured average OD and biomass concentrations after 48 hours were highest in Run 9. Isolate 1 grew at an impressive 0.5 hr^{-1}, the highest recorded specific growth rate thus far. A μ_{max} of 0.3 hr^{-1} was obtained during all three runs for Isolate 7. This value is similar to the fastest growth observed for this isolate during the preliminary C:N:P optimisation experiment Runs 6 and 7, which both contained a higher proportion of carbon. Run 9 contained the same citric acid, glucose, and other medium component concentrations as described for un-optimised MME, but 1 $g.L^{-1}$ of glycerol. There was 5 $g.L^{-1}$ of each carbon source in Run 10. The same amount of glycerol (1 $g.L^{-1}$), 10 $g.L^{-1}$ glucose and 5 $g.L^{-1}$ citric acid were used in Run 11 (Table 3.3).

Table 7.18: Carbon source optimisation study results for Isolate 1 after growth for 48 hours in the various media in microtitre deep well plates. The averages of triplicate experiments are shown.

Run	Average OD_{600}	Maximum OD_{600}	Biomass ($g.L^{-1}$)	μ_{max} (hr^{-1})
9	5.06 ± 2.13	7.76 ± 0.48	8.36 ± 0.57	0.493 ± 0.01
10	1.80 ± 1.15	7.88 ± 0.77	2.33 ± 0.38	0.293 ± 0.02
11	4.22 ± 0.98	8.18 ± 0.58	7.32 ± 0.36	0.274 ± 0.05

Table 7.19: Carbon source optimisation study results for Isolate 7 after growth for 48 hours in the various media in microtitre deep well plates. The averages of triplicate experiments are shown.

Run	Average OD_{600}	Maximum OD_{600}	Biomass ($g.L^{-1}$)	μ_{max} (hr^{-1})
9	9.28 ± 0.64	11.81 ± 0.25	13.32 ± 0.25	0.270 ± 0.01
10	6.687 ± 1.09	8.47 ± 0.29	8.64 ± 1.65	0.290 ± 0.07
11	5.625 ± 0.44	7.11 ± 0.85	9.76 ± 0.72	0.314 ± 0.03

Table 7.20: The maximum absorbance results for Isolates 1 and 7 after growth over a 48 hour cultivation period in the first eight optimisation media in microtitre deep well plates. The averages of triplicate experiments are shown.

Run	Isolate 1	Isolate 7
1	11.14 ± 0.84	13.07 ± 1.25
2	15.48 ± 0.75	13.04 ± 2.24
3	10.10 ± 0.06	11.62 ± 0.52
4	12.46 ± 0.65	11.78 ± 0.09
5	9.82 ± 0.71	13.65 ± 0.95
6	12.10 ± 2.55	12.87 ± 0.83
7	9.83 ± 2.62	14.60 ± 1.30
8	8.82 ± 1.18	2.84 ± 0.06
*MME	3.21 ± 0.05	3.47 ± 0.02

The maximum absorbances are also recorded and presented in Table 7.18 and Table 7.19 for these two isolates. Table 7.24 was included for comparative purposes. The highest overall maximum OD 11.81 obtained for Isolate 7 during cultivation in medium 9 was lower than Runs 5, 6 and 7, but similar to the second highest group comprising of Runs 1 to 4 which were media that also contained lower carbon concentrations. Isolate 1 showed much lower maximum OD values in Runs 9, 10 and 11 (Figure 7.17).

It is also important to note that the maximum absorbances for these isolate runs were reached 40 hours after beginning the cultivation (Runs 1 to 3), after 32 hours (Runs 4 to 8) and after 24 hours (Runs 9 to 11).

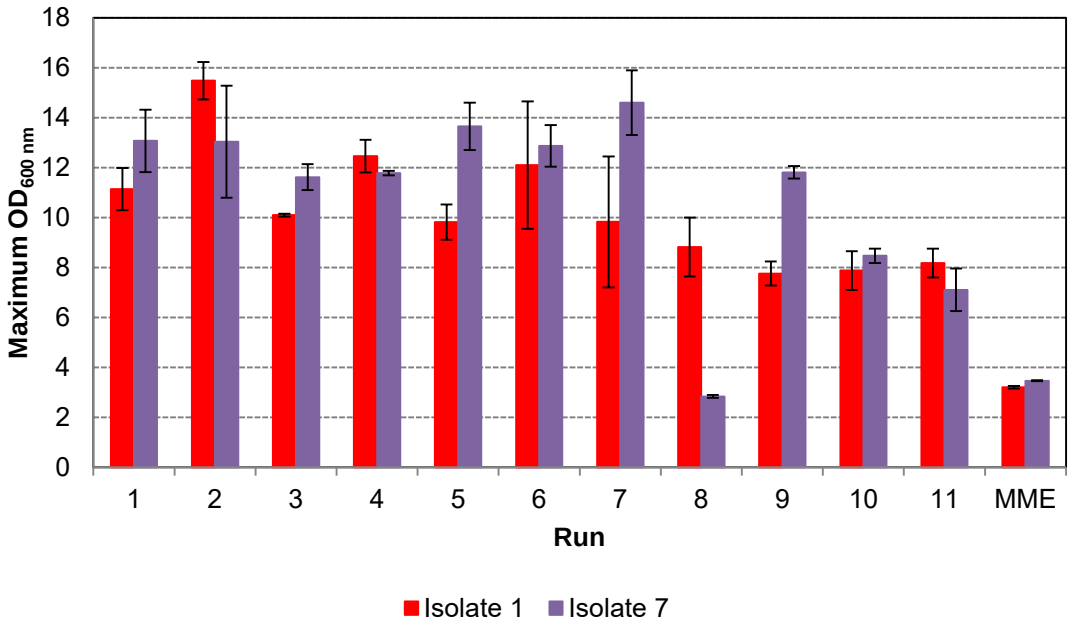


Figure 7.17: The maximum absorbance obtained for Isolates 1 and 7 in MME and the eleven media used in this study. The experiments were conducted over a 48 hour period. The average of triplicate experiments is shown.

Figure 7.19 illustrates that the μ_{max} values for Runs 9 to 11 were high, even at lower concentrations of carbon sources. This was especially the case for Isolate 1, which grew most rapid during Runs 9 to 11. Decreasing the glucose input to 5 $g.L^{-1}$ (Run 10) had a negative effect on both isolates as this caused a decrease in the cell concentration with lower absorbances and corresponding biomass after 48 hours of cultivation (Figure 7.18). Isolate 1 could not maintain the growth rate and was most affected, experiencing a death phase 40 hours into the cultivation (data not shown) and a low biomass concentration at 48 hours (Figure 7.18). With an increase in glucose concentration in Run 11 to half of that contained in the baseline medium and Run 9 (10 $g.L^{-1}$), the isolate's growth steadily recovered.

The decrease in glycerol concentration from 80 $g.L^{-1}$ (in MME) to 1 $g.L^{-1}$ improved the growth of the isolate in the medium. These three final optimisation runs did not produce the same high levels of OD readings and biomass compared to the media with higher glycerol, but the results of these experiments indicate that glycerol is not a critical component and not detrimental to bacterial growth.

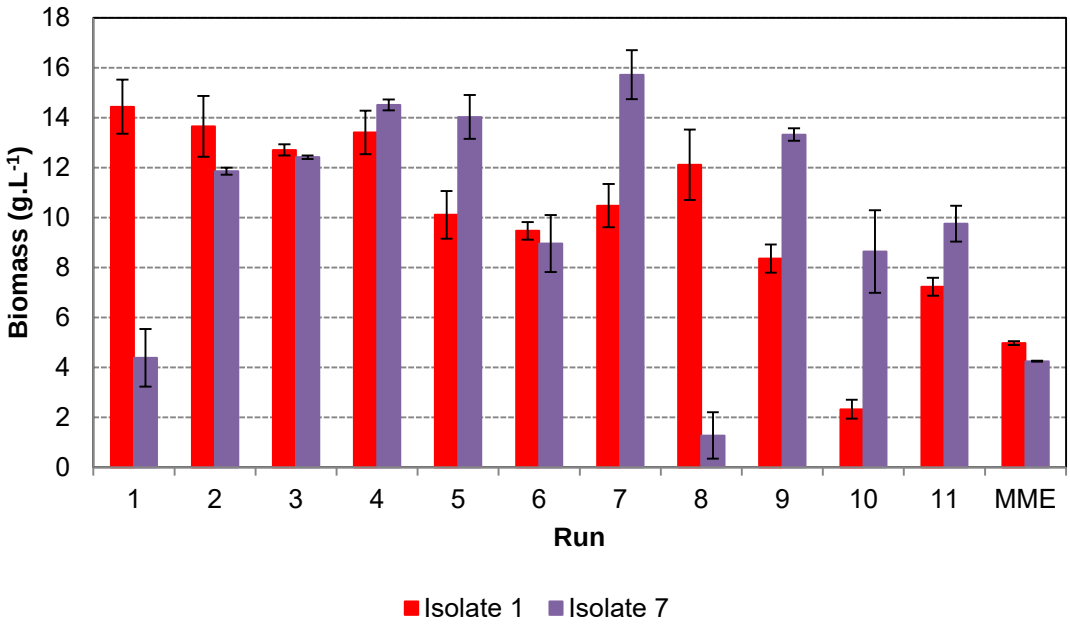


Figure 7.18: The biomass concentrations obtained for Isolates 1 and 7 in MME and the eleven media used in this study. The experiments were conducted over a 48 hour period. The average of triplicate experiments is shown.

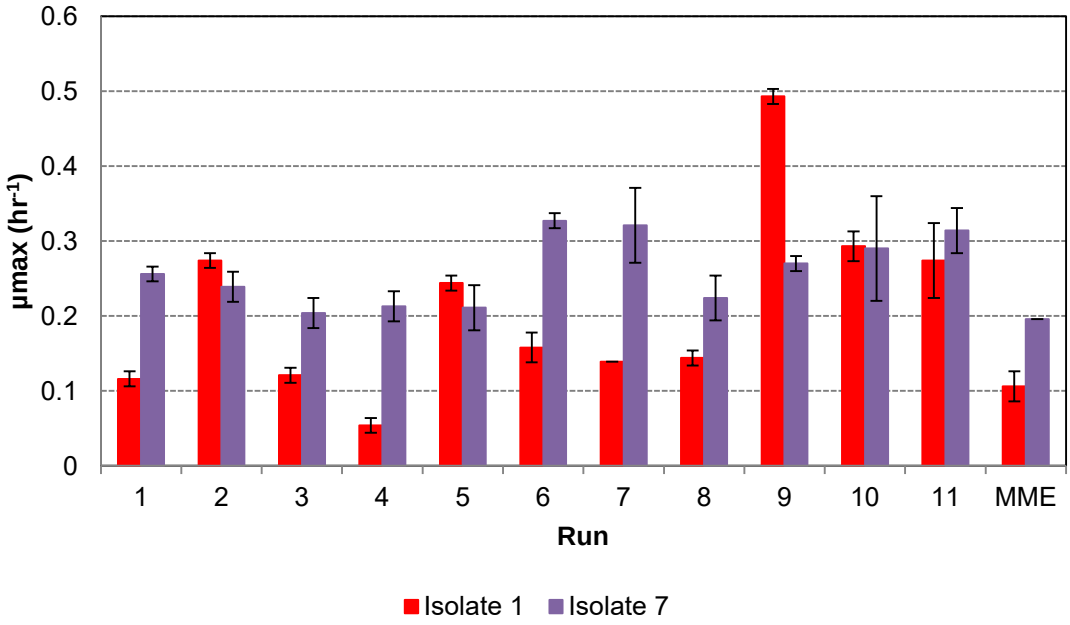


Figure 7.19: The maximum specific growth rates obtained for Isolates 1 and 7 in MME and the eleven media used in this study. The experiments were conducted over a 48 hour period. The average of triplicate experiments is shown.

Yoon *et al.* (2000) also found poor utilisation of glycerol and excluded this polyol compound from their γ-PGA production medium.

Therefore, the relatively high baseline concentration of 80 $g.L^{-1}$ glycerol was not critical for the bacterial metabolism and maintaining good growth.

7.4 General discussion

The growth matrix was optimised in two manners. Firstly, by using two level factorial design to investigate various high and/or low carbon, nitrogen and phosphorus ratios. Second was investigating initial carbon source input using different combinations of citric acid, glycerol and glucose. The aim of these experiments was to find a medium or media which supported good growth of the isolate, at a lower nutrient concentration than what is found in MME.

The initial optimisation experiments comprised of eight media. The first four comprised a low carbon ratio, whilst the fifth to eighth contained higher carbon. The nitrogen and phosphorus ratios were incorporated in either ratio throughout the eight media to give a final of eight different combinations. As expected, the four runs with higher carbon allowed for overall improved growth compared to the lower carbon containing media. This was especially the case for Isolates 7 and 8. Isolate 1 was the only isolate which grew better in the lower carbon media. ANOVA analysis of the data obtained from these initial optimisation experiments revealed that carbon was the main contributing factor for biomass production for all isolates with the exception of Isolate 7.

The maximum specific growth rates appeared to be independent of the runs which produced the highest biomass for the isolates. This means that the media which produced the highest biomass did not automatically allow for the highest maximum specific growth rates. This was either an indicator for discrepancies in the initial biomass contained in the inocula or a different factor in the medium which influenced the growth rate. The nitrogen source was found to be the main factor affecting the specific growth rate.

Carbon source substrate analysis for Isolates 1 and 7 showed that efficient use of some or all carbon sources did not translate to an increased quantity of biomass or maximum specific growth rates. The results also indicated that glycerol was leased efficiently utilised. MME contains 80 $g.L^{-1}$ of this substrate.

These experiments indicated that media containing lower carbon concentrations could be used effectively as a lower nutrient medium option.

Deceasing the glycerol concentration to 1 $g.L^{-1}$ (Runs 9 and 11) produced the highest biomass of 8.36 $g.L^{-1}$ and 13.32 $g.L^{-1}$ for Isolates 1 and 7 respectively. These concentrations were lower than the 14.44 $g.L^{-1}$ (Isolate 1) and 15.72 $g.L^{-1}$ (Isolate 7) obtained in previous experiments. However, these biomass values were obtained when 43.27 $g.L^{-1}$ and 86.53 $g.L^{-1}$ initial glycerol was used in the media respectively. Furthermore, Run 9 produced the highest maximum growth rate for Isolate 1. The growth rate of 0.314 hr^{-1} for Isolate 7 during Run 11 was similar to the highest value of 0.327 hr^{-1} (Run 6).

Although the OD and biomass values of the carbon input optimisation Runs 9 to 11 were lower than some obtained during Runs 1 to 8, the maximum absorbances were reached 24 hours post inoculation. Runs 1 to 3 required 40 hours and Runs 4 to 8, 32 hours. The economics of a process

are greatly affected by the cultivation times. Thus, it would be more beneficial for a process to reach the highest possible biomass and product formation sooner with a faster turnaround time. The differences between the highest biomass and maximum specific growth rates obtained for these two isolates cannot adequately justify prolonging the cultivation times to 32 or 40 hours, instead of 24 hours.

Overall, the isolates produced more biomass in all 11 media compared to the un-optimised MME, with the exclusion of only two runs: Run 8 (Isolate 7) and Run 10 (Isolate 1). This supports the potential of dilute wastewater as a substrate use.

8. BIOREACTOR STUDIES

8.1 Introduction

Based on the results in the previous chapter, an optimal media combination which supported high a growth rate by the microorganisms was used for final screening experiments. Run 2 had a low ratio of nitrogen, important for the maximum specific growth rates. Run 2 produced higher growth rates for all isolates, compared to Runs 3 and 4 (Section 8.2.1). This highlighted the ability of these isolates to maintain relatively good growth at low carbon, nitrogen and phosphorus concentrations. Run 9, which contained a lower glycerol concentration of 1 $g.L^{-1}$, was also shown to support good growth of Isolates 1 and 7. Isolates 1 and 7 were able to achieve a μ_{max} 0.5 hr^{-1} and 0.3 hr^{-1} respectively in this medium. Isolate 7 was also able to produce a relatively high biomass concentration of 13 $g.L^{-1}$. Additionally, these two isolates were able to achieve maximal absorbance 16 hours earlier in this medium compared to the preliminary low carbon-containing Runs 1 to 4. A new medium, which was denoted as Run 14, consisted of the carbon concentrations of Run 9 and nitrogen and phosphorus concentrations of Run 2 was prepared. The detailed composition is described in Section 3.8.1. These two runs produced the best growth results for Isolate 1.

Both Isolates 1 and 7 were screened in this new medium in deep well plates, but due to time constraints, only Isolate 1 was grown in the bioreactor. The batch and continuous culture bioreactor experiments were also only repeated once. The results of both the deep well plate experiments and bioreactor cultivation in Run 14 are presented in this chapter.

8.2 Results of microtitre plates runs on optimised medium and discussion

Figure 8.1 shows that both isolates experienced a lag phase of approximately three hours for adaptation to the medium, with Isolate 1 experiencing a slow transition into the exponential phase. The growth then occurred exponentially until stationary phase was reached after approximately 12 hours. The fluctuation in the growth profile for Isolate 1 during the stationary phase could indicate the presence of diauxic growth resulting from a change in metabolism in response to a particular nutrient being depleted in the medium followed by a death phase from 42 hours. This is a remote possibility that requires further elucidation, as the error bars show that there is not a significant difference in growth during the stationary phase between 12 hours and 48 hours. The substrate analysis results will be discussed in Section 8.3.

The maximum absorbance and biomass values for both isolates were generally lower than in the previous experiment (Table 8.1). However, the maximum specific growth rates were similar to those obtained for Run 2 (0.274 hr^{-1} for Isolate 1 and 0.239 hr^{-1} for Isolate 7).

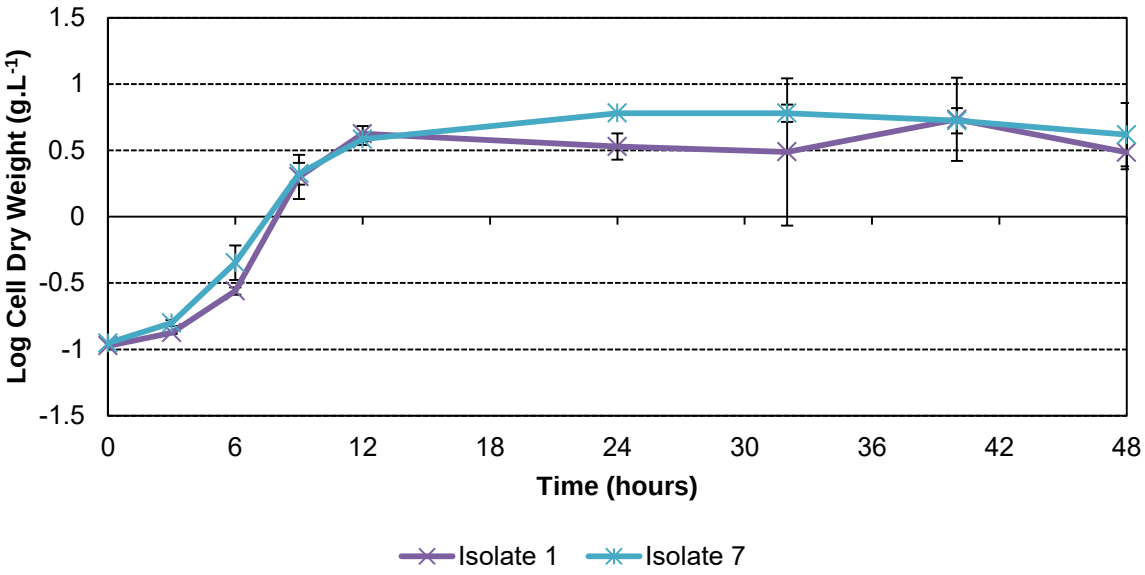


Figure 8.1: The growth curves of Isolates 1 and 7 in the final MME. The average of the triplicate runs was corrected using the volume and evaporation rate over the cultivation period to obtain the final values.

Table 8.1: A summary of the maximum specific growth rate, maximum biomass and absorbance values obtained for Isolates 1 and 7 after a 48 hour cultivation period.

Factor	Isolate 1	Isolate 7
μ_{max} (hr^{-1})	0.287 ± 0.01	0.224 ± 0.01
Max Biomass ($g.L^{-1}$)	7.85 ± 0.12	8.74 ± 0.09
Max Absorbance (OD_{600})	5.42 ± 0.31	6.03 ± 0.07

8.3 Batch lab-scale bioreactor results and discussion

Isolate 1 was grown in the Sixfors® bioreactor (Infors, Bottmingen, Switzerland) with a 0.25 L working volume of the final medium (Run 14). The cultivation conditions were similar to those previously used (37°C, initial pH of 6.5, agitation rate of 200 rpm). However, unlike previous experiments, Sixfors® software allowed for monitoring and control of pH, temperature and dissolved oxygen.

The growth curve is illustrated in Figure 8.2. The microorganism experienced a lag phase of three hours in the bioreactor, which was similar to the growth displayed in the microtitre plate. However, the transition into the exponential phase, which was achieved by six hours, was more rapid. Isolate 1 was also able to maintain exponential growth over a longer period of time, which was double that obtained in the microtitre plate. Although the growth declined after nine hours, the isolate did experience a slight sustained growth rate until 32 hours. The maximum absorbance (5.78) and biomass (8.38 $g.L^{-1}$) values obtained were slightly higher than those of the deep well plate.

Although the glucose was the most efficiently utilised substrate in the medium, consumption by the bacterium only began after 10 hours of cultivation, and was utilised to a lesser degree overall than seen previously in Runs 1 to 8. Only 6% of the glycerol and citric acid were utilised by the end of the cultivation. The lower overall biomass obtained at the end of the cultivation could be an indicator of the lower extent of substrate utilisation. The prolonged growth phase until 32 hours could be due to the steady glucose and citric acid utilisation. When the isolate was grown on the deep well plate using Run 2 as shown in Figure 7.4, utilisation of 100% glucose and 72% of the glycerol was achieved.

The μ_{max} obtained was 0.143 hr^{-1} which was lower than in the deep well plates without pH control or sparging. This can be indicative of high shear stress caused by the increase in agitation to 600 rpm during cultivation, which was aimed at increasing the oxygen transfer when dissolved oxygen in the bioreactor decreased below 50%.

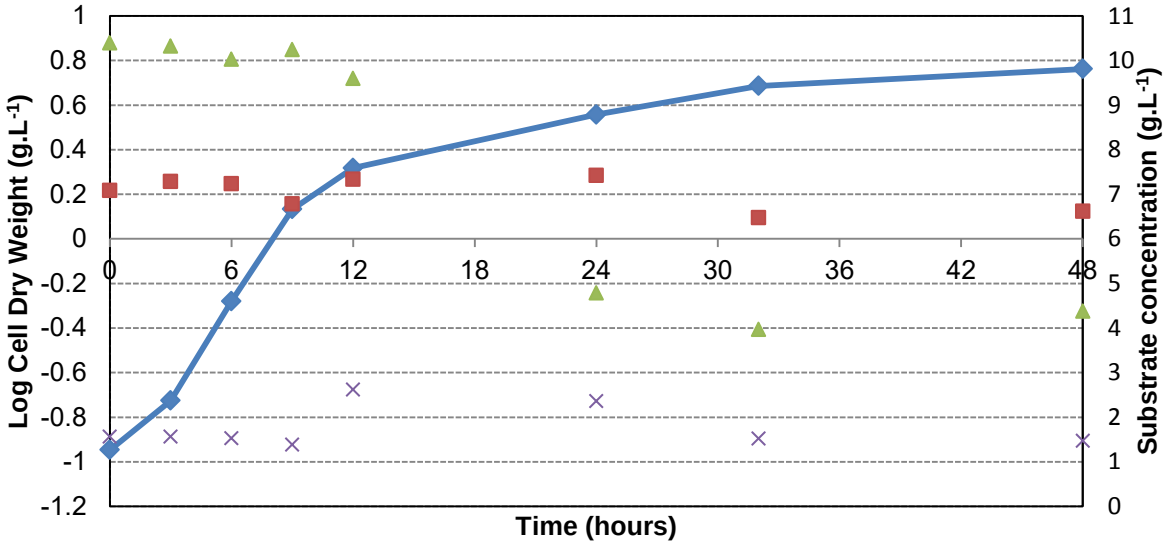


Figure 8.2: The growth curve (◆) of Isolate 1 in the final MME over a 48 hour cultivation period in the Sixfors® bioreactor. The citric acid (■), glucose (▲) and glycerol concentrations (X) are also shown. The experiment was correctly completed once.

The Monod equation (Monod, 1942) is a kinetic model which describes the homogenous and balanced growth of microorganisms as a functional relationship between the specific growth rate and the limiting substrate concentration. Mathematically, it is described as follows:

$$\mu = \frac{\mu_{max} \cdot S}{K_s + S} \qquad (8.1)$$

where:

μ is the specific growth rate of the microorganism [hr^{-1}]

μ_{max} is the maximum specific growth rate of the microorganism [hr^{-1}]

S is the limiting substrate concentration [$g.L^{-1}$]
K_s is the substrate saturation constant [$g.L^{-1}$]

In a batch reactor, the growth rate of the microorganism changes due to the variation in metabolic state and phase of growth as well as the substrate concentration in the medium. As the cells consume the substrate, the change in concentration results in a proportional increase of biomass with respect to time. This relationship is expressed as the biomass yield coefficient, $Y_{X/S}$:

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \qquad (8.2)$$

where:
X is the biomass concentration [$g.L^{-1}$]

The overall $Y_{X/S}$ obtained for Isolate 1 during batch growth using glucose as the limiting substrate in this study was 1.36 $g.g^{-1}$. Typical values for aerobic processes based on carbohydrate carbon sources lie in the range of 0.38 to 0.55 $g.g^{-1}$ (Shuler and Kargi, 1992).

The value of the saturation constant, K_s, in the Monod model is described as the limiting substrate concentration which gives a growth rate half its μ_{max}. Initially, in batch cultivation, the substrate concentration is much higher than the K_s. The rapidly growing cells utilise this substrate, maintaining a fairly constant growth rate, μ. A plot of the specific growth rate versus the substrate concentration may be used during this exponential phase to obtain the value of K_s. This would infer that the K_s is equivalent to 0.072 $g.L^{-1}$ in this experiment. As the substrate concentration decreases over the cultivation period, cell growth follows a first order reaction rate and s becomes more comparable to K_s (Villadsen *et al.*, 2011). Pirt (1975) found the typical K_s values of microorganisms grown with glucose as the limiting nutrient to range from 0.004 to 0.025 $g.L^{-1}$. Whilst determining μ_{max} is a straight-forward exercise in batch growth, the determination of K_s in this system is not advisable. The relatively small K_s value, especially when using glucose as a limiting substrate, is difficult to determine accurately. Continuous culture is recommended for better estimation (Doran, 2013; Villadsen *et al.*, 2011).

8.4 Continuous culture results and discussion

The Infors-HT Sixfors® was converted into a continuous stirred tank bioreactor to determine the growth kinetics of Isolate 1 under the same conditions as the batch culture. The biomass concentration was determined by periodic removal of approximately 2 mL samples for five dilution rates over a total of three residence times each to ensure steady state conditions. The inverse relationship between dilution rate and residence time is denoted as follows:

$$D = \frac{1}{\tau} \qquad (8.3)$$

where:

D is the dilution rate [hr^{-1}]

τ is the residence time [hr]

The dilution rate used, which is the quotient of the flow rate ($L.hr^{-1}$) and vessel volume, was calculated taking cognisance of the μ_{max} of 0.143 hr^{-1} to avoid cell washout. Figure 8.3 denotes these biomass concentrations expressed as cell dry weight.

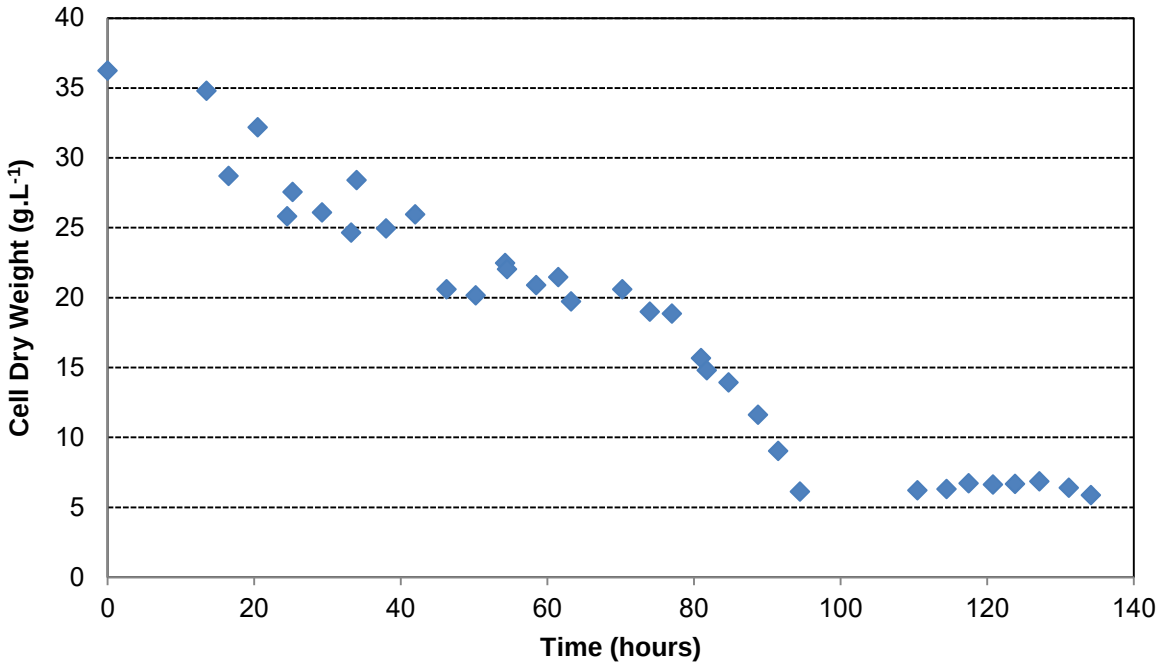


Figure 8.3: The biomass concentration of Isolate 1 in the final MME over a 140 hour continuous cultivation period in the Sixfors® bioreactor. The experiment was correctly completed once.

During continuous cultivation, specific growth rate of the cells remains constant due to the steady addition of substrate. When steady state conditions are reached, this specific growth rate, μ, equals the dilution rate, D where cell death and maintenance is negligible and a sterile feed is used. Hence, Equation 8.1 can be written as

$$D = \frac{\mu_{max} \cdot S}{K_S + S} \qquad (8.4)$$

The relationship between D and residual substrate concentration S is given in terms of kinetic constants μ_{max} and K_s:

$$S = \frac{D.K_S}{\mu_{max} - D} \quad (8.5)$$

In this study, the cell maintenance requirements were assumed to be negligible. Steady state conditions were reached when the cell concentration displayed a variation less than 10%. According to Figure 8.3, three steady states were observed between 16 and 42 hours, 46 and 70 hours and finally from 94 to 140 hours. A plot of the average cell concentration during these steady states given as a function of dilution rate can be found in Figure 8.4. The cell concentration decreased over time as the dilution rate was increased. The three observed steady states were used to determine the K_s value more accurately and estimate μ_{max}. The substrate concentration was determined using Equation 8.6.

$$X = (S_i - S).Y_{X/S} \quad (8.6)$$

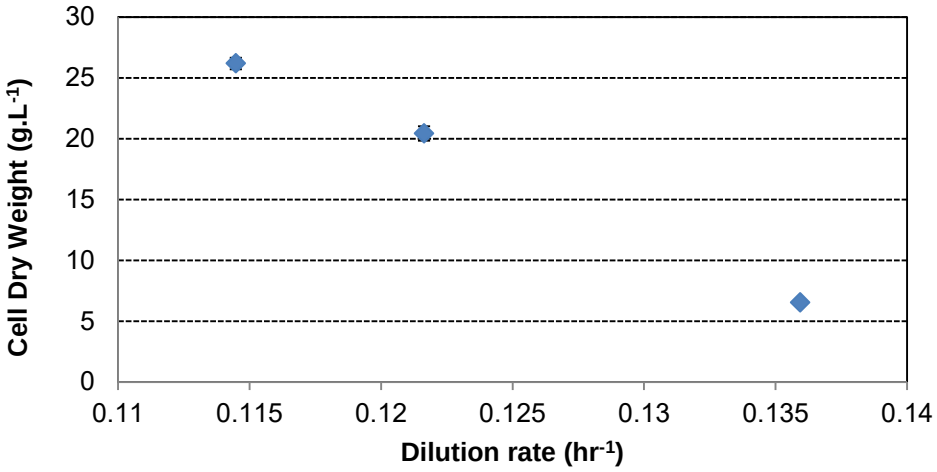


Figure 8.4: The steady state biomass concentration of Isolate 1 in the final MME as a function of dilution rate during continuous cultivation period in the Sixfors® bioreactor.

The Lineweaver-Burk plot is a widely used method in enzyme kinetics. This equation linearizes the Michaelis-Menten equation, and can also be used for the Monod equation. Inverting Equation 8.4 yields:

$$\frac{1}{D} = \frac{K_S}{\mu_{max}.S} + \frac{1}{\mu_{max}} \quad (8.7)$$

A plot of 1/D versus 1/S (Figure 8.5) was used to determine the K_s value of 21.15 $g.L^{-1}$ and μ_{max} of 0.149 hr^{-1}.

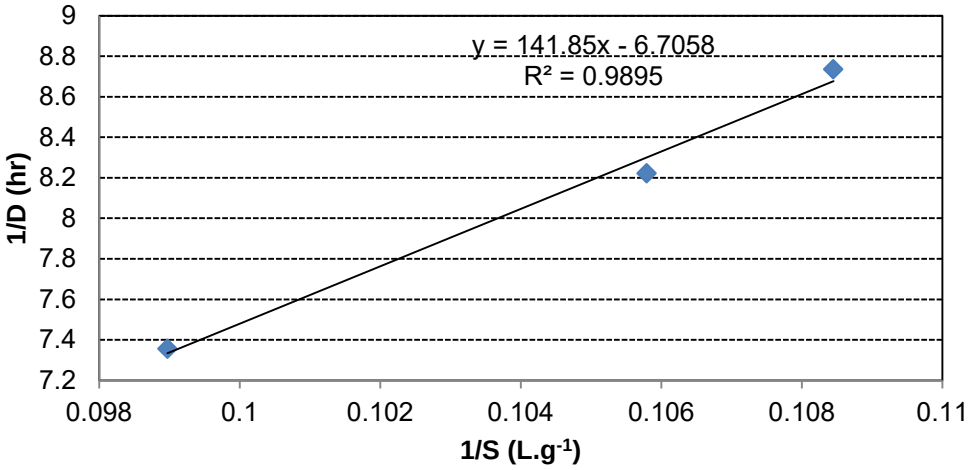


Figure 8.5: The Lineweaver-Burk plot used for the determination of the cultivation Monod kinetic parameters.

Both the K_s and μ_{max} differed from those obtained during batch culture. Even though the μ_{max} value of 0.143 hr^{-1} obtained during batch growth was slightly lower, the two values are comparable. More rapid growth was favoured when the system was converted from batch to continuous culture. The dissolved oxygen decrease during the exponential growth phase of batch culture was greater than in the continuous culture experiment, reaching below 5% in some cases (data not shown). A determination of the oxygen volumetric coefficient (K_La) and oxygen uptake rate (OUR) in these two systems can be used to assess the oxygen transfer rate of the microorganism in this specific medium.

The K_s value obtained in this work was much higher than the range proposed by Pirt (1975). This is an indication of a low glucose affinity of this isolate and the dependence for higher substrate concentrations for fast growth. Interestingly, by using the observed batch growth μ_{max} of 0.143 hr^{-1} and Equation 8.8 at the various steady states, the theoretical K_s value average is 1.50 ± 0.5 $g.L^{-1}$, which is much lower than 21.15 $g.L^{-1}$ obtained using just continuous culture data. The assumption that half of μ_{max} yields the K_s value is not true for this work. More recent growth kinetic work based on *Bacilli* by various researchers is presented in Table 8.2.

$$K_S = \frac{S(\mu_{max} - D)}{D} \qquad (8.8)$$

Although Feng and colleagues (2003) determined all growth kinetics in batch culture and using a complex carbon source, the K_s and μ_{max} values compare closely to those obtained in this work using a chemically-defined medium. Rowe and Howard (2002) also used complex media to study the K_s and μ_{max} of *B. subtilis*. The reported μ_{max} was especially higher for the medium containing varying concentrations of the polyurethane Impranil DLN™. The K_s values followed the same trend. All the K_s

values reported in Table 8.2 were in an overall lower range of between 0.04 – 3.1 $g.L^{-1}$. The theoretically determined K_s fell within this range.

Table 8.2: A summary of the *Bacillus* species growth kinetic studies available in the literature.

Species	System	Carbon source	X ($g.L^{-1}$)	$Y_{x/s}$ ($g.g^{-1}$)	K_s ($g.L^{-1}$)	μ_{max} (hr^{-1})	Reference
B. licheniformis	Batch	Corn step liquor	8	0.51	1.91	0.1	Feng *et al.* (2003)
B. subtilis	Batch	Yeast extract tributyrin	ND	ND	0.04	0.46	Rowe and Howard (2002)
B. subtilis	Batch	Yeast extract Impranil DLN™	ND	ND	0.09	0.76	Rowe and Howard (2002)
B. thuringiensis	Batch	Glucose	0.55	0.51	ND	0.78	Rivera (1999)
B. thuringiensis	Fed batch	Glucose	5	1.2	3.1	0.38	Rivera (1999)
B. thuringiensis	Fed batch	Glucose	6.5	0.64	3.1	0.46	Rivera (1999)
B. thuringiensis	Continuous	Glucose	various	ND	3.1	1.1	Rivera (1999)
B. thuringiensis	Continuous	Glucose	various	0.43	0.56	0.54	Rodriguez-Monroy and de la Torre (1996)

ND denotes not determined

Rivera (1999) who studied the growth kinetics of *B. thuringiensis* in batch, fed batch as well as continuous culture, also found differences with the graphical calculation of K_s and μ_{max} using the Lineweaver-Burk plot and by solving the numerical equations. The Lineweaver-Burk method has been described to lack accuracy. An alternative to this method is the Langmuir plot, which is also a linearized form of the Monod equation, but unlike Lineweaver-Burk, this method is able to minimise experimental error distortions. Langmuir plots S/D versus S to evaluate K_s and μ_{max} using Equation 8.9 as the basis for this (Doran, 2013).

$$\frac{S}{D} = \frac{K_S}{\mu_{max}} + \frac{S}{\mu_{max}} \qquad (8.9)$$

The Langmuir plot method yielded similar values of 21.25 $g.L^{-1}$ and 0.150 hr^{-1} for K_s and μ_{max} respectively.

Richard and Margaritis (2004) proposed an empirical kinetic model for batch growth of γ-PGA from *B. subtilis* IFO 3335 that could be used to fit kinetic data from other bacterial strains. Although this model is highly applicable to this study, the biopolymer was a component of the extended biomass (X'), and this requires the monitoring of γ-PGA concentration during the cultivation.

Rivera (1999) verified the μ_{max} by conducting an independent continuous culture experiment and monitored the dilution rate at which the washout of all bacterial cells in the bioreactor occurred. The μ_{max} of 1.16 hr^{-1} which was found by using this approach was in agreement with the continuous steady state $\mu_{max.}$ This is possibly an alternative experiment which could be employed in future studies as a good confirmatory tool. The K_s and μ_{max} obtained by Rivera (1999) and Rodriguez-Monroy and de la Torre (1996) with *B. thuringiensis* grown under continuous culture conditions using glucose as the limiting nutrient in the medium were all higher than the values obtained in this study. However, both utilised complex medium components such as yeast extract which could have contributed additional carbohydrates for improved biomass growth. The maximum specific growth rate increase from 0.78 to 1.1 hr^{-1} from batch to continuous cultures illustrates the potential that a continuous stirred tank bioreactor operating efficiently has in improving productivity on limited substrate. These preliminary studies also report on some growth kinetic information for *Bacillus*, which can be used as a benchmark.

8.5 Implications of growth kinetics on bioreactor selection for biorefinery

The growth of Isolate 1 was studied in greater detail in a newly composed medium. The biomass yield coefficient obtained during the batch cultivation was 1.36 $g.g^{-1}$, which was higher than those reported for *Bacillus* strains in the literature. The maximum specific growth rate of 0.143 hr^{-1} was in the lower end of the range of typical values reported for *Bacillus* in the literature.

The microbial isolates used in this study were selected for product formation in a wastewater biorefinery. Wastewater contains a much lower COD concentration, approximately 30 fold less compared to the final medium composition of this study. Due to this low substrate concentrations, the growth rate (μ) of the cells and biomass concentration supported is low, resulting in low productivity. When the dilution rate (D) is greater than the maximum specific growth rate (μ_{max}), cell washout occurs. In order to prevent washout and maintain satisfactory productivity through maintaining a suitable biomass concentration at very low substrate concentrations, a setup is required in which the biomass is retained in the reactor. Cell retention in a bioreactor can be achieved by cell separation and recycle, maintaining the superficial liquid velocity lower than the particle settling velocity (Nicolella *et al.*, 2000) or immobilising the biomass e.g. as a biofilm. In WWT, the retention of the biomass as a biofilm is preferred to minimise the unit operations required. A biofilm can be defined as a complex structure of cells and cellular products which are either formed spontaneously as large dense granules or grown attached to a solid surface (Heijen, 1984). Apart from providing bacteria with a supporting medium to attach, efficient contact time and controlled growth is required to prevent clogging (Henze *et al.*, 2002).

The formation of a biofilm on a support matrix helps to retain the cells in a reactor when the dilution rate is increased to values where cell washout would normally occur if the cells were not retained on a

support matrix i.e. hydraulic and biomass residence times can be separated. Such increased dilution rates are required to provide nutrients at a sufficient rate, given their low concentration.

Three major consequences result from the biofilm and culture medium constituents (Nicolella *et al.*, 2000):

i. Biomass concentration without adequate cell retention depends only on the substrate concentration in the feed, and consequently large retention times are required in the presence of dilute feeds.
ii. The substrates (carbon, nitrogen, phosphorus, oxygen) have to cross the liquid interface and be transported through the aggregate to reach the cells and be consumed. The penetration depth of the substrates in the biofilms mainly depends on the porosity of the biofilm, substrate concentration in the bulk liquid, mass transfer at the biofilm-liquid interface and reaction rate in the biofilm.
iii. Due to diffusional substrate concentration gradients, a growth rate gradient also exists within the aggregate. The organisms with the highest growth rate will be found on the outside of the biofilm, whereas slower growing organisms will be found inside (Heijenn et al., 1989). As a result, slower growing organisms will be protected from external shear forces, and are less likely to be lost due to detachment and wash-out. In this case the maximum growth rate under reactor conditions should be considered and not the maximum growth rate of the organisms.

The application of particulate biofilm reactors are relatively recent technology and are used in full scale applications. The most effective reactor for large scale applications is the rotating biological contractor (RBC) (Water Treatments, 2008-2011). Figure 8.6 shows an example of the structure of a RBC.

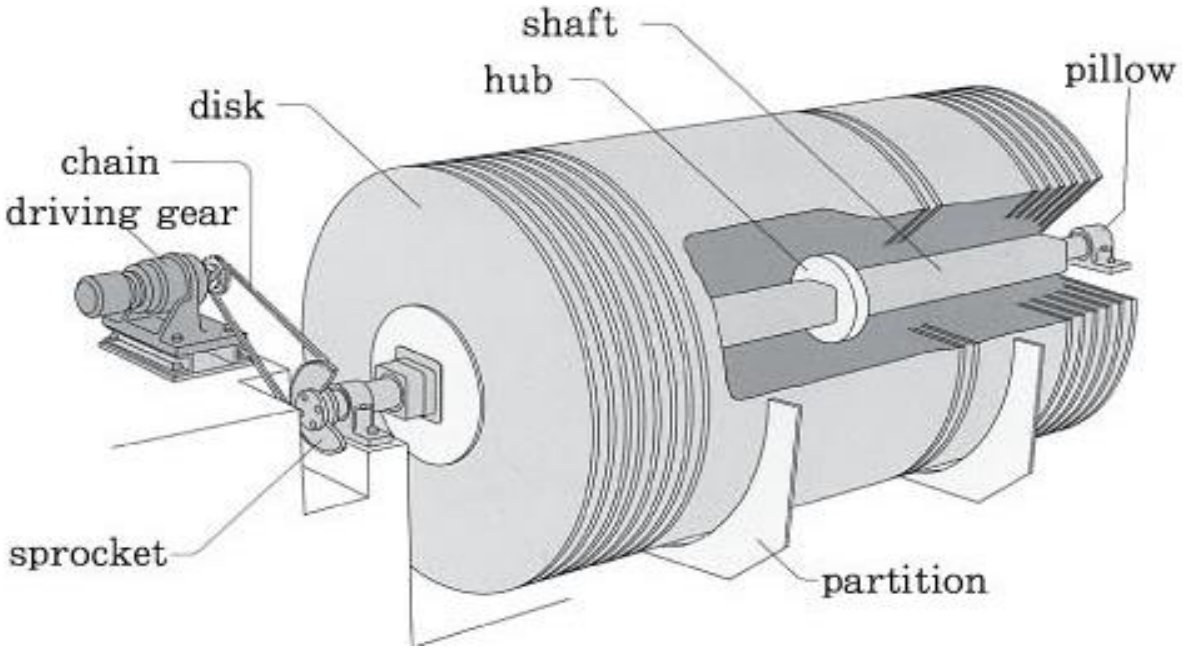


Figure 8.6: The structure of a Rotating Biological Contactor (Water Treatments 2008-2011).

The RBC is a series of large discs in which the microorganisms are housed, and these discs are supported on a single shaft which is slowly rotated through the wastewater. The RBC is covered by a removable fiberglass housing which has access portals at each end. The discs are covered with a thick coating of slime of microorganisms, which treat the wastewater. RBCs act much like a trickling filter in that the contactors perform well at removing about 85% BOD (Water Treatments, 2008-2011).

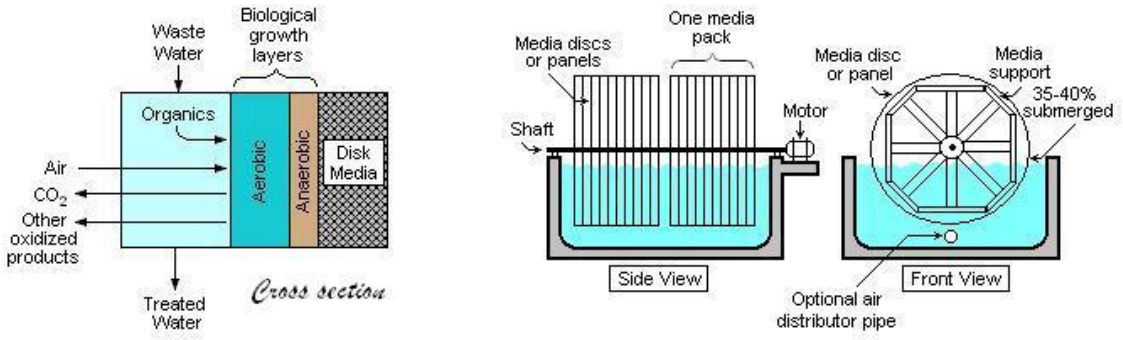


Figure 8.7: Cross Sectional Views of the Rotating Biological Contractor (Water Treatments 2008-2011).

A biofilm grows on the surface of a series of rotating discs mounted on a shaft and placed in a tank conforming to the general shape of the discs (Figure 8.7). The discs are commonly corrugated plastic media, but a mesh type is used in the Hybrid Bacillus Activated Sludge (HYBACS) process, giving the biofilm better stability and providing an environment for denitrification (Henze *et al.*, 2008). The corrugations increase stiffness, increase available surface area, improve mass transfer, and define spacing between individual disks. With a mature biofilm much of this mesh will be occupied, but a significant increase in active biomass is still expected. (Henze *et al.*, 2008).The shaft is rotated at constant speed, providing a shear force as well as aeration to the biofilm through the turbulence created (Water Treatments, 2008-2011).

The use of a rotational biological contactor is effective in biofilm formation and cell retention. This increases the contact time between cells and substrate which allows for better substrate utilisation. The RBC is a suitable bioreactor that can be placed in the aerated section of the wastewater treatment process, as the wastewater will be treated whilst simultaneously producing biomass and γ-PGA.

Following confirmation of the potential for these isolates to form a biofilm, the values of K_s and μ_{max} obtained can be used together with typical WWTW substrate concentrations, and estimation of mass transfer in the biofilm to predict the accumulation of biomass in the RBC, the associated substrate conversion and finally the production of γ-PGA.

9. γ-PGA PRODUCTION

9.1 Introduction

The majority of γ-PGA-producing strains do so extracellularly by secreting this biopolymer into the cultivation medium. This enables the recovery of the γ-PGA to follow a straightforward approach which utilises precipitation by cold ethanol, drying and weighing. The most common method cited by various researchers in the literature was proposed by Ashiuchi *et al.* (1999). This method was also applied for γ-PGA extraction and purification for the work undertaken during this project and is described in more detail in Section 3.12.

Depending on the targeted potential application, this purified γ-PGA is sometimes characterised for product homogeneity, structure and molecular weight. Previously, only the γ-PGA concentration would be determined by hydrolyzing the γ-PGA at high temperatures, followed by measuring the change in glutamic acid concentration (Bovarnick, 1942). Amino acid analysis or thin layer chromatography is now more commonly used to characterise the composition of the polymer. Homogeneity, in terms of the D- and L-glutamic acid ratio, and structure is elucidated by proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance spectroscopy (NMR). Molecular weight distribution and polydispersity measurements were perfomed using gel permeation chromatography (GPC) (Birrer *et al.*, 1994; Goto and Kunioka, 1992; Perez-Camero *et al.*, 1999; Yokoi *et al.*, 1995).

Following the conclusion of cultivation, γ-PGA was extracted, purified and analysed by gel permeation chromatography (GPC) using HPLC. When an aqueous mobile phase is used to elute, this method is referred to as gel filtration chromatography (GFC). This rapid and convenient chromatographic method separates particles based on their hydrodynamic volume or molecular size, and is used for large molecules such as proteins and polymers.

There is currently no standard methodology which is employed or recommended for γ-PGA analysis. Consequently, most researchers either quantify γ-PGA produced at the end of the cultivation using a gravimetric approach or monitor cultivations using GPC with a previously purified γ-PGA sample as the standard for the calibration curve. The preceding characterisation of the extracted and purified γ-PGA is optional.

In addition to GPC, protein and amino acid analyses as well as residual carbohydrate and lipid quantifications were carried out to further assess the quality of the obtained biopolymer. The results of these will be presented and discussed in this chapter.

9.2 Quantification and characterisation of γ-PGA results and discussion

9.2.1 Quantification of γ-PGA

Gravimetric extraction of the polymer by ethanol precipitation from shake flask liquid cultures of Isolates 1 and 7 grown in un-optimised MME for 48 hours yielded 22.5 $g.L^{-1}$ and 19.3 $g.L^{-1}$ respectively. This corresponded to a product yield coefficient ($Y_{p/x}$) of 7.7 g polymer per g biomass and 12.8 $g.g^{-1}$ for Isolates 1 and 7 respectively.

The un-characterised, purified γ-PGA was used as a standard for quantification by GPC. Samples were removed periodically during the various cultivations for OD and biomass measurements. The same samples were then centrifuged and filtered and used for both the substrate and γ-PGA analyses using the respective HPLC and GPC columns. An example of a characteristic GPC chromatogram obtained for most of the samples is illustrated in Figure 9.1. The second, larger peak corresponded with the purified γ-PGA standard. The difference in the molecular size of the polymer produced by MME and ME was negligible, as MME samples eluted at 24.0 minutes compared to ME which eluted at 23.99. A more detailed analysis of the molecular weights obtained is described in Section 9.2.2. The medium was most viscous when using ME, and lighter brown in colour. All samples produced a large tailing peak, as well as an additional smaller peak (Figure 9.1). When using the generated standard curve, sample peak area and the dilution factor, some samples were calculated to contain a final concentration which was as much as ten times the 100 $g.L^{-1}$ standard - the highest γ-PGA standard used.

The use of extracted and purified γ-PGA which has not been assessed in terms of composition and purity is commonly described in the literature. However, these results indicate that prior characterisation and further purification of the polymer is required.

9.2.2 Molecular size verification

Lyophilized extracted γ-PGA from *B. licheniformis* grown in both ME and MME, Isolate 1 and Isolate 7 grown in MME and used previously as GPC standards were used for this size exclusion HPLC analysis. All cultivations were performed in shake flasks to allow for sufficient culture volume for use in the extraction. The extraction method proposed by Ashiuchi *et al.* (1999) as described in Section 3.12 was used. Gel filtration chromatography by HPLC analysis of the extract was carried out as described in Section 3.13.6.2.

All the samples produced four peaks on average, between approximately 900 and 1200 daltons (Figure 9.2). The multiple peaks could be a function of sample purity.

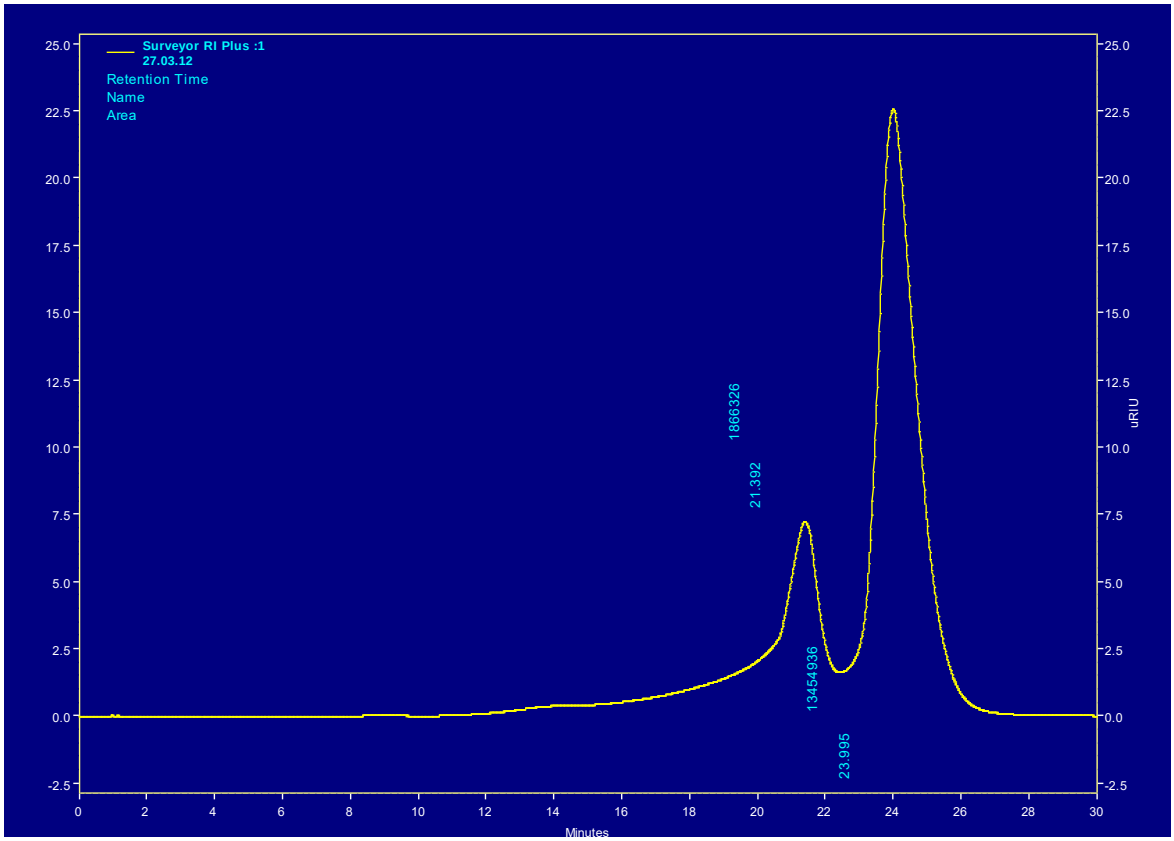


Figure 9.1: The gel filtration chromatogram obtained for Isolate 10 after 48 hours when grown in medium E.

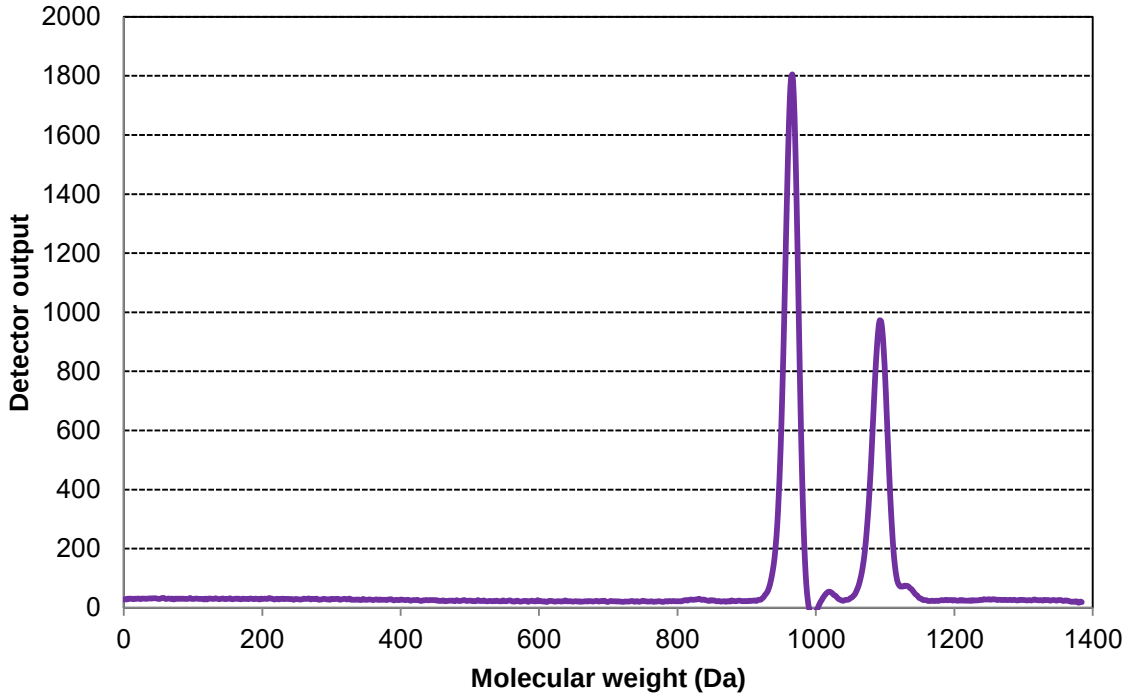


Figure 9.2: An example of a size exclusion chromatogram. This chromatogram was obtained for the purified γ-PGA sample of Isolate 1 after growth in MME for 48 hours.

9.2.3 Analysis of purity - protein, carbohydrate, lipid content

The same purified γ-PGA samples from *B. licheniformis* and Isolates 1 and 7 were hydrolyzed at high temperature in the presence of 6 N HCl and 15% phenol in under vacuum pressure and the total crude protein was quantified as a function of nitrogen. The results are found in Table 9.1.

The sample from *B. licheniformis* grown in MME produced a polymer of the highest protein content, followed by *B. licheniformis* grown in ME. The Isolates 1 and 7 produced 0.035 and 0.072 g protein per g sample. These protein contents were unexpectedly low.

Table 9.1: Protein determination of the extracted γ-PGA.

Sample name	Weight (g)	% Protein
B. licheniformis ME	0.1000	12.13 ± 0.23
B. licheniformis MME	0.1059	16.42 ± 0.06
Isolate 1 MME	0.1022	3.49 ± 0.06
Isolate 7 MME	0.1025	7.20 ± 0.08

The purity of these extracted samples was also analysed by determining the carbohydrate concentration by the phenol-sulphuric acid method using glucose as a standard. Lipid content was determined using a laboratory protocol which hydrolyses fats present and derivatizes the resultant fatty acids in the samples to volatile fatty acid methyl esters quantifiable by gas chromatography. Additionally, the moisture content of the sample was determined by measuring a change in mass after drying in an oven in pre-weighed Eppendorf tubes.

The results in Table 9.2 indicate that the extracted polymer from all the samples was not solely composed of γ-PGA. The sample from *B.licheniformis* grown in MME contained the highest residual carbohydrate content of 22%. Although this medium contained glucose as one of the carbon sources, HPLC analysis after cultivation did not detect any residual sugar (Figure 4.2). *B.licheniformis* grown in ME yielded less carbohydrate (12%), whilst Isolate 1 grown in MME contained the least with 7%. Dominant polysaccharide after cultivation using glucose was also found by Feng *et al.* (2007). There was no detectable lipid in all the samples. The moisture content was generally low, especially for Isolate 7 and *B.licheniformis* ME which both contained 3%. Isolate 1 contained the highest moisture at 8% followed by the *B.licheniformis* MME sample which was composed of 5% water. These results indicate that the cultivations produced by-products not detected by these assays, and other unknown substances. Further it is noted that in three of the four samples, the protein and carbohydrate content was similar, whereas in the sample from Isolate 1, the carbohydrate content was double the protein content.

Table 9.2: Carbohydrate, lipid and water content of the extracted γ-PGA

Sample name	% Carbohydrate	% Lipid	% Water
B. licheniformis ME	11.69 ± 0.10	0	2.81 ± 1.64
B. licheniformis MME	21.60 ± 0.15	0	4.59 ± 0.54
Isolate 1 MME	7.24 ± 0.03	0	7.75 ± 3.29
Isolate 7 MME	8.63 ± 0.17	0	3.04 ± 0.29

9.2.4 Amino acid analysis

The hydrolysed samples were used to analyse the actual amino acid composition. This is found in Table 9.3.and Table 9.4. Serine and histidine were the two amino acids which accounted for over half the composition. Interestingly, glutamic acid only formed 3.7 and 5.2% of the hydrolysate from Isolate 1 and *B. licheniformis*. Both were grown in MME. A significant proportion (over 70%) of the polymer obtained from these two samples consisted of the amino acids serine and histidine.

Table 9.3: Amino acids expressed as a % of the total.

	Isolate 1 ME	Isolate 7 ME	*B. lich* ME	*B. lich M*ME
His	38.597	6.535	42.174	21.329
Ser	36.558	56.235	15.854	51.840
Arg	2.472	1.275	1.595	4.037
Gly	1.458	0.689	0.494	1.320
Asp	2.083	3.554	1.246	2.349
Glu	3.705	24.294	33.146	5.171
Thr	1.636	0.598	0.473	1.341
Ala	2.802	0.895	1.203	1.894
Pro	1.002	0.480	0.380	1.218
Cys	0.000	0.000	0.000	0.000
Lys	2.075	0.831	0.765	1.174
Tyr	1.510	0.452	0.386	1.054
Met	0.350	0.185	0.141	0.450
Val	1.606	1.978	0.445	1.616
ILe	1.128	0.473	0.313	1.400
Leu	1.909	1.091	1.128	2.845
Phe	1.111	0.436	0.257	0.964

The results show that the extracted γ-PGA is in fact not only composed of glutamic acid units. Instead, this is a polymer composed of a mixture of various amino acids. A more γ-PGA-specific purification method needs to be investigated. Additionally, a direct method such as its hydrolysis and measurement of glutamic acid monomers is required to improve the accuracy of the analysis.

A new method which has the potential for rapid quantification of γ-PGA has been proposed by Ashiuchi (2011). This method is a spectrophotometric assay that utilises cetyltrimethylammonium bromide (CTAB) to bind very specifically to γ-PGA, forming a water-insoluble, highly dispersed micelle-like complex, which results in an increase in turbidity. The turbidity-based calibration curve of γ-PGA concentration compiled by the author showed a good linearity over at least the range of 0.2 – 2 $\mu g.mL^{-1}$ γ-PGA, regardless of the polymer stereochemistry.

A sample extracted from the culture of Isolate 1 grown in the final optimised medium in the bioreactor using batch cultivation was also analysed for protein and amino acids. This sample contained 3% protein of which glutamic acid was the dominant amino acid at 17%. The controlled environment provided by the bioreactor was able to increase the glutamic acid content by 13%, in a more dilute medium.

Table 9.4: Amino acid content expressed in g per 100g.

	Isolate 1 ME	**Isolate 7 ME**	***B. lich* ME**	***B. lich M*ME**
His	0.204	0.047	4.355	1.474
Ser	0.194	0.404	1.637	3.581
Arg	0.013	0.009	0.165	0.279
Gly	0.008	0.005	0.051	0.091
Asp	0.011	0.026	0.129	0.162
Glu	0.020	0.175	3.422	0.357
Thr	0.009	0.004	0.049	0.093
Ala	0.015	0.006	0.124	0.131
Pro	0.005	0.003	0.039	0.084
Cys	0.000	0.000	0.000	0.000
Lys	0.011	0.006	0.079	0.081
Tyr	0.008	0.003	0.040	0.073
Met	0.002	0.001	0.015	0.031
Val	0.009	0.014	0.046	0.112
ILe	0.006	0.003	0.032	0.097
Leu	0.010	0.008	0.116	0.197
Phe	0.006	0.003	0.027	0.067

9.3 General discussion

The isolates selected in this study were able to produce relatively high yields of biopolymer measured gravimetrically: 22.5 $g.L^{-1}$ from Isolate 1 and 19.3 $g.L^{-1}$ from Isolate 7. The $Y_{p/x}$ obtained were 7.7 $g.g^{-1}$ and 12.8 $g.g^{-1}$ for Isolates 1 and 7 respectively.

Wastewater treatment applications do not have stringent requirements for polymer composition, molecular weight or specific stereochemistry. The polymer produced by the isolates grown in shake flasks using un-optimised medium and extracted using the protocol defined was not pure γ-PGA. The extracted polymer samples were shown to contain similar amounts of protein and carbohydrate with no lipid present. Moisture contents of 3 to 8% were found. In the protein fraction, the amino acids serine, histidine and glutamate were most prevalent.

Glutamic acid-independent strains generally require high concentration of glucose in the medium of approximately 50 - 80 $g.L^{-1}$, which is not completely utilised. According to the literature, the highest concentration of γ-PGA obtained from a microorganism grown on glucose was 28 $g.L^{-1}$. These isolates compared well. The initial glucose and citric acid concentrations were 80 $g.L^{-1}$ and 20 $g.L^{-1}$ respectively. Only 33% of the glucose was consumed. This study was able to produce a 13% higher glutamic acid concentration in the polymer in a medium which contained only 20 $g.L^{-1}$ glucose and 12 $g.L^{-1}$ citric acid. A 58% consumption of glucose was measured.

While Isolate 1 is a good polymer producer, its polymer requires further characterisation. The conditions which positively influence the increased accumulation of γ-PGA also need optimisation. Minimal process optimisation can potentially further improve the yield and purity. It is noted that Isolate 7 also shows the potentially efficient γ-PGA-producing ability. These isolates show great promise as efficient γ-PGA producers in a nutrient limited environment such as a WWTW.

It is reported that weight average molecular weight (Mw) and polydispersities (PD) changes with the strain and culture conditions used for γ-PGA production (Bajaj and Singhal, 2011). Although the polymer produced in this study from the various strains varied in protein and glutamic acid content, the measured molecular weights fell within a similar region of approximately 1000 Daltons. Care must be taken when recovering γ-PGA by ethanol precipitation as this method is less selective, and may simultaneously precipitate other proteins and some polysaccharides (Buescher and Margaritis, 2007). An analysis of the homogeneity of the extracted γ-PGA is critical following quantification by gravimetric methods or prior to use of this as a standard for quantification of samples by GPC.

10. IDENTIFICATION OF ISOLATES

10.1 Introduction

Following sampling from Mitchell's Plain WWTP, culture enrichment, screening and selection, the microbial identity of the five most promising samples, Isolates 1, 6, 7, 8 and 10, was determined. Genomic DNA was extracted from the selected five strains and the *B. licheniformis* JCM 2505 reference strain. Following PCR, the *16S rRNA* amplifications were sequenced as specified in Section 3.9. NCBI BLAST analysis was performed on the *16S rRNA* gene sequences to confirm the identities. Uncultured and environmental samples were included in the search as the sludge sample from which the stains were isolated was from an environmental source and could contain uncultured microorganisms. The identity of these strains as well as a comparison of the various homologies is discussed in this chapter.

10.2 Results and discussion

The BLAST results obtained from the forward and reverse primer aligned consensus sequences are found in Table 10.1 below.

Table 10.1: The *16S rRNA* gene sequencing BLAST results for the identification of the isolates

Isolate	Identity	Accession number (s)	Query coverage %	Max identity %
Reference	*Bacillus licheniformis*	HE804781.1	100	99.9
1	*Bacillus* sp. RacNa2.50/ *Bacillus subtilis* strain DmB55	HQ606094.1/ HQ111354.1	100	99.5
6	*Enterobacter cloacae*	FP929040.1	99	87.5
7	*Serratia* sp.	A total of 100	100	99.6
8	*Klebsiella* sp	EF645654.1	100	99.7
10	*Enterobacter* sp. PXG15	JQ396392.1 JQ396391.1 JQ396389.1 JF681666.1	100	99.8

Similarly to the *pgs* results, the search output E values obtained for all the isolate query sequences were equal to zero. The query coverage is the percent of query sequence that is aligned and matches the database entry. In these results, all sequences were 100% aligned, except for Isolate 6. Although there are no universally agreeable defined threshold values for *16S rRNA* identification criteria, it is generally accepted that:

- Identical sequences show ≥ 99.5% sequence similarity
- closely related sequences are 98.5 to 99.4% similar, and
- unique species are < 98.5% similar to another sequence in the database (Janda and Abbott, 2007).

Isolate 6 was re-sequenced, but the second sequence chromatogram obtained was similar to the original. DNA was then re-extracted from this isolate, amplified, purified and sequenced. This was still unsuccessful in improving the BLAST score (data not shown). Therefore, it can be concluded that Isolate 6 potentially represents a unique genus. DNA-DNA hybridization, which is much more cumbersome and costly, but also the most definitive method, will be required to confirm this finding.

The reference strain was confirmed to be *B. licheniformis* with a 99.9% match. However, the accession number with the highest score belonged to a strain annotated as “isolate 3.47” which was deposited into the GenBank. Isolate one was identified as belonging to the *Bacillus* genus. Another hit with equal scores identified the query sequences as matching *B. subtilis* strain DmB55. This result is consistent with the observed cell morphology of isolate one. *Bacillus subtilis* is a generally accepted as safe (GRAS) Gram positive bacterium. This naturally-occurring, ubiquitous bacterium can be found in soil, water, air and decomposing plant material. It has the ability to form endospores and is commonly used as a model and reference organism by researchers (Barbe *et al.*, 2009). Amongst many applications, *B.subtilis* is also used in industry as a plant disease control agent, but is non-pathogenic to humans and animals (CPL, 2002).

The remaining three isolates were identified to be Gram negative, rod-shaped bacteria, all belonging to the Enterobacteriaceae family. These characteristics of Isolate 7 are consistent with the morphology described in Section 5.2. The Gram stain and microscopic analysis of both Isolates 8 and 10 also revealed rod-shaped bacteria, but these isolates appeared to display staining characteristics of Gram positive bacteria. This further highlights the inconclusive nature of Gram stains.

Enterobacteriaceae are generally facultatively anaerobic, which enables the organism to switch its metabolism to respire in environments where oxygen is both present and absent. Along with *Steptococci* and *Staphylococci*, these bacteria are common culprits in human infections and diseases. *Serratia*, *Klebsiella* and *Enterobacter* are well known in hospital settings as they are all opportunistic pathogens frequently responsible for nosocomial infections in patients. The natural habitat for these coliforms is the intestinal tract of humans, but they can also be found free-living in soil, water, fruit, vegetables, grains, flowering plants, trees or in insect, animal and plant hosts (Holt *et al.*, 1994).

Prior to the widespread use of antibiotics, *Enterobacter* were rarely found as opportunistic pathogens. These usually cause burn, wound and urinary tract infections, septicaemia and meningitis (Grimont and Grimont, 2006). Musil *et al.* (2010) and Paauw *et al.* (2006) report on two example cases of *E. cloacae* outbreaks.

Serratia are straight rods and are usually motile. They grow best at temperatures between 30°C and 37°C and are found naturally in soil, water and plant surfaces. They also occupy rodent, insect and

worm digestive tracts. Some species of this genus produce a characteristic red pigment The top two hits from the BLAST results were *Serratia nematodiphila* strain HBEB1 (accession number KF036188.1) and *Serratia marcescens* strain KtPC3-9 (accession number KF017546.1). *S. marcescens* is the most common species and is also an opportunistic pathogen responsible for nosocomial infections. It is also the cause of mastitis in cows, which is the inflammation of the udder tissue (Grimont and Grimont, 2006).

Short chains of straight rods often characterise *Klebsiella*. This bacterium forms capsulated cells, is non-spore forming and grows optimally at 37°C. *Klebsiella* distinguishable from other Enterobacteriaceae as it is the only genus which is not motile (Holt *et al.*, 1994). Human faeces, soil, water, grains and fruit are some of the places where this bacterium can be found. *Klebsiella* is an important opportunistic pathogen as it commonly colonizes the gut of humans and animals and is easily transmitted from a wide range of species. *K. pneumonia* and *K. oxytoca* are the two most common strains and are responsible for bacteraemia, pneumonia and other nosocomial infections in patients (Brisse *et al.*, 2006). When uncultured samples were included in the BLAST search, the 26 highest hits, all the same score, included 13 uncultured and 13 *K.oxytoca* strains.

For further analysis, the phylogenetic relationship between the isolates was investigated. The maximum likelihood method was used. Maximum likelihood searches for an evolutionary model that has the highest likelihood of producing the observed data, based on nucleotide or amino acid substitutions (Felsenstein, 1981).

One of the models maximum likelihood is able to use on the DNAMAN software is the Poisson or Jukes-Cantor model. This model is the simplest, and states that all substitutions occur with equal frequency. This probability matrix (M) is denoted by:

$$\mathrm{M} = \begin{pmatrix} 1-\delta & \delta/3 & \delta/3 & \delta/3 \\ \delta/3 & 1-\delta & \delta/3 & \delta/3 \\ \delta/3 & \delta/3 & 1-\delta & \delta/3 \\ \delta/3 & \delta/3 & \delta/3 & 1-\delta \end{pmatrix} \qquad (10.1)$$

where δ is the number of nucleotide substitutions per site per unit time interval, taken at δ=0.01.

The relative frequency (R) of substitution can be derived from M, and for this model, it is represented by:

$$\mathrm{R} = \begin{array}{c} \\ T \\ A \\ C \\ G \end{array} \begin{array}{c} \begin{array}{cccc} T & A & C & G \end{array} \\ \begin{pmatrix} 0 & \alpha & \alpha & \alpha \\ \alpha & 0 & \alpha & \alpha \\ \alpha & \alpha & 0 & \alpha \\ \alpha & \alpha & \alpha & 0 \end{pmatrix} \end{array} \qquad (10.2)$$

where α = 1.

On average, transitional substitution events for nucleotides are greater than transversions from purines to a pyrimidines and vice versa (Brown *et al.*, 1982). The Hasegawa, Kishino and Yano model takes this into account, extending the Poisson model. The relative substitution rate matrix for the Hasegawa, Kishino and Yano model is,

$$\mathrm{R} = \begin{array}{c} \\ T \\ A \\ C \\ G \end{array} \begin{array}{c} \begin{array}{cccc} T & A & C & G \end{array} \\ \begin{pmatrix} 0 & \alpha & \beta & \beta \\ \alpha & 0 & \beta & \beta \\ \beta & \beta & 0 & \alpha \\ \beta & \beta & \alpha & 0 \end{pmatrix} \end{array} \qquad (10.3)$$

where α and β are relative substitution rates of transition and transversion events, respectively.

Using the more robust Hasegawa, Kishino and Yano model, the un-rooted and rooted phylogenetic trees in Figure 10.1 and Figure 10.2 were obtained.

Both trees were bootstrapped 1000 times to ensure the accuracy of the topology final tree. Bootstrapping is random resampling of the tree evaluation method that works with distance-based & parsimony-based methods of phylogenetic analysis.

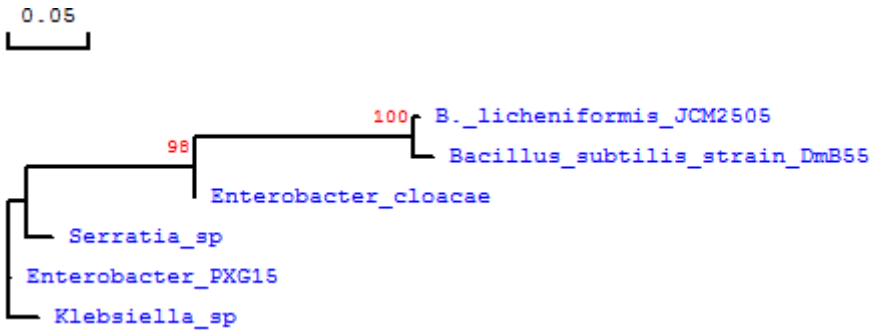


Figure 10.1: The un-rooted phylogenetic tree measuring the molecular evolutionary distances between the isolates with 1000 bootstrap trials.

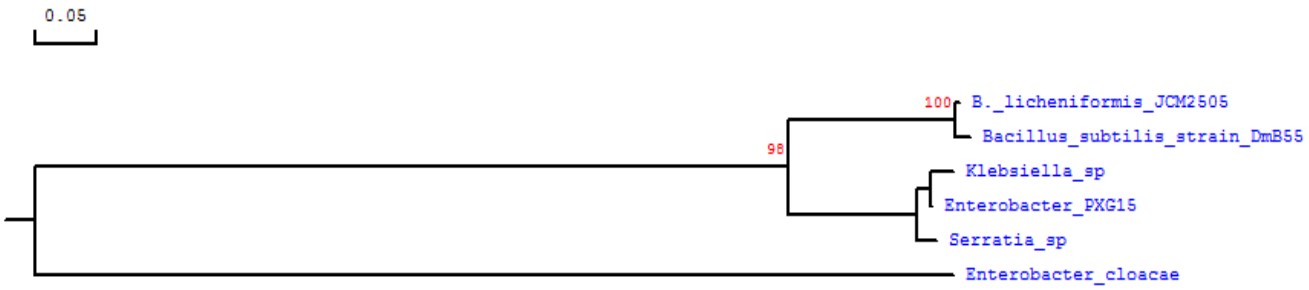


Figure 10.2: The rooted phylogenetic tree measuring the molecular evolutionary distances between the isolates (1000 bootstrap trials).

The software automatically used *E. cloacae* as the out-group to root the tree. The close relationship of the Enterobacteriaceae is clearly demonstrated as the *Klebsiella*, *Enterobacter* and *Serratia* all form a clade in both dendrograms. *Klebsiella* and *Enterobacter* are most similar, as *Serratia* forms another separate branch. The *E. cloacae* strain was 98% similar to this clade. *B. licheniformis* JCM2505 and *B. subtilis* (or Isolate 1) formed another clade together, which was expected since these two strains belong to the *Bacillus* genus. When aligned, the sequences for these were 95.95% similar.

A drawback that researchers have found with using *16S rRNA* gene sequencing is the low phylogenetic resolution at the species level and the poor discriminatory power for some genera. Biochemical tests or DNA-DNA homology studies then become necessary. *Bacilli* are one of the most problematic genera for species differentiation, as large numbers of species exist with some newly described reported incompletely (Holt *et al.*, 1994). Recent diverges in species also pose taxonomic resolution issues, as *16S rRNA* gene sequence data cannot distinguish between these (Janda and Abbott, 2007).

11. CONCLUSIONS AND RECOMMENDATIONS

Poly (γ-glutamic acid) (γ-PGA) is a naturally occurring biopolymer which consists of D- and L-glutamic acid monomers connected by amide linkages between the α-amino and γ-carboxyl groups (Jeong *et al*., 2010). It is anionic, biodegradable, water soluble, edible and non-toxic to humans, animals and the environment. This homo-polyamide has potential applications in the medical, food, cosmetic, wastewater treatment, plastic and agricultural and textile industries (Shih and Van, 2001).

To design and optimise a process for γ-PGA production, it is important to understand the growth kinetics of the microorganisms able to synthesize this biopolymer. The *Bacillus* genus is comprised of many species which are ubiquitous in the environment, including wastewater, and form a large proportion of the Proteobacteriaceae family (Bramucci and Nagarajan, 2000; Buthelezi *et al.*, 2009). *Bacillus* species are the most widely reported γ-PGA-producing organisms in the literature. Additionally, an improved level of understanding of the challenges of using minimal media as a source of nutrients combined with understanding the key bioprocess parameters influencing γ-PGA production will allow for efficient optimisation of this system, providing the information required to design of a cost-effective and sustainable process with nutrient sources such as dilute wastewater streams (Richard and Margaritis, 2004).

In the present study, the base case for growth, substrate utilisation and γ-PGA production has been reported for the *Bacillus licheniformis* JCM2505 culture strain. In presenting this, a complete experimental methodology using both shake flasks and deep well plates is developed and appropriate analytical protocols selected.

Following enrichment, 18 isolates were obtained from the Mitchell's Plain domestic wastewater treatment plant. These microorganisms were initially characterised in terms of morphology related to γ-PGA production ability. Further, using DNA sequence data for the enzymes involved in γ-PGA synthesis, primers were designed to identify strains carrying γ-PGA synthesis potential through PCR studies. Isolates showing reproducible growth and genetic evidence for polymer production ability were selected for further screening in terms of their growth kinetics. Growth was assessed in terms of rate and extent in both Medium E and modified Medium E. Based on this, six isolates were chosen for more detailed screening. In shake flask cultures, maximum biomass concentrations varied from 2 to 8 $g.L^{-1}$ while maximum specific growth rates varied from 0.11 to 0.27 hr^{-1}. Culture growth in deep well plates was slower, with maximum specific growth rates ranging between 0.07 hr^{-1} and 0.20 hr^{-1}. Maximum biomass concentrations were generally higher in this screening system than in shake flasks, ranging from 5 to 9 $g.L^{-1}$.

It was apparent from the data obtained that even though the strains were isolated from the same environmental source, each isolate responded differently to the laboratory environment and that the growth characteristics of the strains differed greatly under identical culture conditions.

Cultivation conditions were optimised for improved growth rates, substrate utilisation and biomass concentrations for the isolates, in terms of the ratio of carbon, nitrogen and phosphorus provided. Preliminary media optimisation was undertaken to identify optimal C:N:P ratios in the growth matrix and to assess the impact of medium concentration. Two-level factorial design was used to investigate eight different C:N:P ratios. The media containing higher carbon were favoured by most of the isolates for improved growth. Isolates 7 and 8 grew best in the high carbon media, producing the highest biomass concentrations of 15.72 $g.L^{-1}$ and 15.27 $g.L^{-1}$ respectively. Regression analysis of the data obtained confirmed that carbon was the main contributing factor for biomass production. Isolate 1 grew better in the lower carbon media, producing a maximum biomass of 14.44 $g.L^{-1}$. This suggests its preference for growth in dilute wastewaters.

The isolates which produced the highest biomass did not necessarily also show the highest maximal specific growth rates. When Isolate 7 was grown in the medium annotated as Run 6, it produced the third least biomass amongst the eight runs, but the highest growth rate of 0.33 hr^{-1}. Similar findings were observed for Isolate 1, which produced the highest biomass concentrations in runs displaying the lowest maximum specific growth rates. The nitrogen component in the medium was found to be the main factor affecting the specific growth rate.

Using Table 2.5 as a basis, a typical C:N:P ratio found in wastewater is 10:1:0.4 when using only the total carbon, ammonia and inorganic phosphorus as was used in the experiments. The ratios used in this study ranged from 7.7:1:0.02 to 61.5:1:0.26. It is recognised that the optimal C:N:P ratio will need to be validated at lower nutrient concentrations if wastewater is to be used as a substrate for cell growth and γ-PGA production.

Further medium optimisation was conducted by investigating the effect of media containing lower carbon concentrations on the growth of two isolates. Deceasing the glycerol concentration to 1 $g.L^{-1}$ produced high biomass concentrations, with the maximum OD readings at 24 hours. Maximum OD was reached between 32 to 40 hours in the media containing 43.27 $g.L^{-1}$ and 86.53 $g.L^{-1}$ initial glycerol. However, these earlier maximum absorbances were lower than those obtained when the higher initial glycerol concentrations were used. The highest biomass concentrations obtained when 1 $g.L^{-1}$ initial glycerol was added into the media also did not reach the maxima obtained when higher concentrations were used. The maximum specific growth rates of 0.31 hr^{-1} for Run 11 and 0.33 hr^{-1} Run 6 were similar for Isolate 7. Run 11 consisted of 85.53 $g.L^{-1}$ less glycerol and less than half the concentration of glucose and citric acid found in Run 6. Isolate 1 showed the fastest reported growth rate of 0.49 hr^{-1} during Run 9 which contained 1 $g.L^{-1}$ glycerol and the same concentrations for the remaining components as found in the un-optimised medium.

A quantitative substrate analysis showed that the isolates utilised glucose much better than citric acid and glycerol. However, efficient use of some or all carbon sources did not translate to an increased quantity of biomass or maximum specific growth rates. There was better substrate utilisation at lower concentrations. It is recommended that this analysis be carried out for all isolates and runs in order to

accurately compare and further understand the behaviour of the different isolates in response to the various media compositions.

Isolate 1 was subjected to growth in a batch bioreactor in the optimised medium which contained lower carbon, with the aim to gain a better understanding of the growth of this particular organism in a dilute wastewater environment. The maximum absorbance and biomass concentrations were similar to those obtained in the deep well plates when the same medium was used. However, the maximum specific growth rate of 0.143 hr^{-1} was lower. The quantification of the aeration efficiency and the effects of the operating variables on the provision of dissolved oxygen for sufficient microorganism growth can allow for better experimental design for the scale-up process from miniature bioreactors to larger reactors, such as those installed in the WWTW.

A continuous culture experiment was conducted to verify the growth kinetics of Isolate 1. The widely used Lineweaver-Burk plot method of the Monod equation was applied. A K_s value of 21.15 $g.L^{-1}$ and μ_{max} of 0.149 hr^{-1} were determined. The μ_{max} values obtained during batch and continuous culture were comparable.

For generally slower growing microorganisms such as Isolate 1, at low substrate concentrations and high flow rates cell retention in a continuous flow bioreactor is essential for appropriate productivities. This could be achieved by using a static biofilm reactor such as a RBC. Static surfaces have low shear forces and therefore it is useful for retaining weakly attached biofilms. An example of this proven technology is known as the HYBACS RBC reactor which is currently used by plants throughout the world to treat both industrial and municipal wastewater. Since RBC reactors are aerated, the reactor can be placed within the activated sludge treatment step in a WWTW such as Mitchell's Plain, treating the water whilst producing biomass and the biopolymer. The elastic morphology of Isolate 1 is indicative of an efficient biofilm former and a suitable microorganism for colonisation of a static biofilm bioreactor.

Analysis of extracted γ-PGA from *Bacillus licheniformis* revealed a polymer which contained residual carbohydrate. The polymer produced by the bacterium grown in MME consisted of a higher proportion of protein compared to the widely utilised ME. The extracts obtained from growth of the isolates in MME using shake flasks consisted of a similar proportion nitrogen to carbohydrate. In general, the percentage of glutamic acid was lower compared to serine and histidine for both Isolates 1 and 7 as well as *B. licheniformis* grown in MME. An extract obtained from Isolate 1 grown under more controlled conditions in a bioreactor in the final optimised medium, which consisted of a decrease in overall substrate concentration, resulted in a 13% increase in glutamic acid content by Isolate 1.

The limitations due to the currently reported γ-PGA extraction and quantification methods are acknowledged. This project has highlighted the need for more rigorous methodology, which not only emphasises the quantitative nature, but also the quality of the final product. An approach which stringently selects for γ-PGA during extraction and purification followed by an in-depth analysis of the composition and purity of this extract prior to use as a quantification standard is recommended.

Identification of the selected isolates by *16S rRNA* gene sequencing revealed three Enterobacteriaceae and one Proteobacterium. The identity of Isolate 1 was confirmed to match that of *Bacillus subtilis.* Although many γ-PGA-producing *Bacilli* have been reported in the literature, this is a first such report involving this particular *Bacillus* strain. Isolate 7 was identified as *Serratia,* Isolate 8 as *Klebsiella* and Isolate 10, *Enterobacter*. No Enterobacteriaceae have yet been reported to have the ability to produce γ-PGA. This work has shown that the best growth kinetics was displayed by two contrasting bacteria - a ubiquitous GRAS *Bacillus* strain and the opportunistic pathogen, *Serratia.* These two strains both also possessed the *pgs* gene complex, indicating their natural ability to produce γ-PGA. This is a novel and most interesting finding.

The use of Isolate 1, the *Bacillus subtilis* strain, presents an exciting opportunity for a biological process which can be utilised for the purification of wastewaters, whilst producing a valuable biopolymer which can be enriched in γ-PGA. This bacterium has shown the ability to produce high biomass concentrations and good growth, even at low substrate concentrations. The latter is particularly important when processing wastewater. The highly mucoid morphology of this particular bacterium also favours good colonisation and biofilm formation. A rotational biological contactor is effective in biofilm formation and cell retention. This increases the contact time between cells and substrate which allows for better substrate utilisation. The RBC is a suitable bioreactor that can be placed in the aerated section of the wastewater treatment process, as the wastewater will be treated whilst simultaneously producing biomass and γ-PGA.

It is further recommended for studies which mimic the environment in a wastewater treatment plant to be conducted. This includes the use of mixed cultures in order to determine the interactions that occur between the microorganisms and how this will affect the performance of the selected and enriched *Bacillus subtilis* strain. The use of synthetic wastewater which is reflective of typical concentrations in domestic wastewater and finally the use of actual non-sterile wastewater will also be beneficial further research.

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APPENDIX A

Organic acids by HPLC

- Prepare 0.01 M H_2SO_4 solution using dH_2O. Filter and de-gas.
- Prepare standard solutions: 2, 5, 20, 25 and 30 $g.L^{-1}$ of citric acid and glucose and 10, 20, 40, 60, 80 and 100 $g.L^{-1}$ of glycerol by performing dilutions on using 0.01 M H_2SO_4 as a solvent.
- Filter samples and standards using 0.22 µm membranes.
- Place samples and standards (in 1 mL vials) in auto-sampler.
- Inject 10 µL samples using 0.01 M H_2SO_4 as a mobile phase at 0.5 $mL.min^{-1}$.
- Run using Bio-rad Aminex® HPX-87H organic acid column set at an oven temperature of 60°C on Thermo Scientific Finnigan Surveyor Plus HPLC machine, detected by the Finnigan Surveyor Refractive Index Plus.

Analysis using ChromQuest 5.0 software:

- Export chromatographs.
- Use Microsoft Excel to enter data.
- Plot a standard curve using peak areas against concentration.
- Obtain sample concentrations using the standard curve.

APPENDIX B

Carbohydrates by the phenol-sulphuric acid method

Prepare standards:

- Make up a solution of 1 $mg.mL^{-1}$ glucose in dH_2O.
- Dilute this 1:4 to get a 0.2 $mg.mL^{-1}$ solution (40 µg glucose in 200 µL).
- Dilute further to make 32 µg, 24 µg, 16 µg and 8 µg.

Procedure:

- Aliquot 200 µL of the standards/samples into test tubes.
- Add 200 µL of 5% phenol and mix by vortexing.
- Carefully add 1 mL of concentrated H_2SO_4 straight onto the standard/sample and mix immediately by vortexing.
- Leave at room temperature for 10 min.
- Incubate at 30°C for 20 min
- Read absorbance at 490 nm.

Notes:
Phenol is toxic – steps which involved the addition of phenol and H_2SO_4 were performed in a fume hood.
It is vital that the acid is added quickly in a directly into the liquid in the test tube.
Dilute samples further and repeat if absorbance reading does not fall within the standard curve.

APPENDIX C

Direct transesterification method for lipid sample analysis by gas chromatography

Procedure:

- Add 500 µL of toluene to sample.
- Add 100 µL C17-TAG internal standard.
- Add 1 mL of 0.5 N NaOH basic catalyst in methanol.
- Vortex and incubate at 80°C for 20 min, shaking at 300 rpm.
- Leave at room temperature for 5 min to cool.
- Add 1 mL of 5% HCl acid catalyst in methanol.
- Vortex and incubate at 80°C for 20 min, shaking at 300 rpm.
- Leave at room temperature for 5 min to cool.
- Add 400 µL dH_2O to stop the reaction.
- Add 300 µL hexane and 100 µL C19-methyl ester internal standard in hexane to extract the FAME.
- Vortex well.
- Centrifuge at 4000 rpm for 1 min to separate the layers.
- Transfer the upper layer, containing the FAME, to vials for gas chromatography.

Note: Solvents and catalysts are toxic – use fume hood and wear gloves.

Gas Chromatography:

- Use glass inserts in the GC vials and fill with 200 µL sample.
- GC: Varian 3900.
- Detector: flame ionisation detector (FID).
- Injection volume: 1 µL.
- Column: SupelcoWax 10 column (30m x 320mm x 1.0µm film thickness).
- Carrier gas: Nitrogen (2 $mL.min^{-1}$)
- Injection: Standard split/splitless injection was used with a split of 100.
- Injector temperature: 270°C.
- Oven: The column temperature program was increased from 180°C to 260°C at 2°$C.min^{-1}$
- Detector temperature: 260°C

- Peak identification: Identified by retention time using Supelco 37 Component FAME and C14:0 to C22:0 FAME mixtures.
- Peak quantification: Peak areas were used to quantify each FAME relative to the internal standards. Total fatty acid content was obtained by adding all individual FAME peak areas.