



Towards value from waste: Bioreactor selection for the reduction of nutrient load and production of Poly- γ -glutamic acid

***In fulfilment of the requirements for the degree of
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13 November 2022

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This thesis is dedicated to my two beloved grandmothers and angels, Ganna and Pom-Pom, who sadly passed away in 2020 before they could see me graduate.

Ganna leaves behind a legacy of strength, hard work, overcoming life's hardships and a fierce love for her family. Ganna, thank you for all you did for me in helping to believe in myself and in raising strong women whose limits know no bounds.

Pom-Pom was one of the most incredibly positive, kind and loving women I've ever met. She taught me the joy of laughing at the little things, the meaning of a loving prayer and the power in believing in yourself wholeheartedly. My Pom, I wish more than anything I could have hugged you one more time.

I know you are both smiling proudly from Above and wish I could have shared this achievement with you.

Acknowledgements

I would like to express my huge gratitude to the funders of this research, who made this research possible; The Water Research Commission (Project number K5/2380) and the National Research Foundation (SARChI in Bioprocess Engineering, Fund Number UID64778).

Professor Sue Harrison, who has been a role model and inspiration since my dream to pursue a career in Bioprocessing Engineering began during my undergraduate degree. Thank you for always believing in me, even when I didn't, for your patience through this tough postgraduate journey and for the tough love when I needed it. You have given me the very best education, in more than just Bioprocess Engineering. I will forever be grateful that one of the most brilliant minds has taught me, and that you entrusted this research to me. It has been the key to unlocking so many of my wildest dreams, and I hope to one day leave a legacy to be proud of, like you have.

Dr. Madelyn Johnstone-Robertson, whose influence on my life, postgraduate journey and early career is difficult to put in words. From day one, you were always available to listen, guide, advise and give me to tools to solve my own problems. Your kindness, integrity, empathy and honesty have helped shape my own personal development. I will forever be grateful that our paths crossed, and that our journey continued at Biovac. Thank you for encouraging me to chase my dreams and never settle for less.

To the incredible team on the K5/2380 project. Thank you, Lesley Mostert, Sharon Rademeyer, Shilpa Rumjeet, Madelyn Johnstone Robertson and Sue Harrison for making this project so much fun. I am incredibly proud to be part of a team of powerful women doing incredible work.

Sharon Rademeyer, for being the most wonderful friend, late night lab buddy and confidant through the great times and the not-so-great times. I will always treasure the fun that we had in the lab, the laughter until tears, the tears, the terrible singing. Thank you for looking after my reactor babies so beautifully during the day when I started my first job and couldn't be there during the day.

Bernelle Verster, whose hard work and vision for Wastewater Biorefineries helped lay the groundwork for this research. Thank you for helping me through a few panic attacks, heart break and for being such an incredible friend who was always willing to listen, come up with a plan and encourage me.

Tich and Emmanuel, for always going above and beyond to make sure I had all the equipment I needed, working miracles when equipment failed and always brightening up my day.

Joachim Macke and Peter Dobias from the Chemical Engineering Workshop, who patiently brought the design of my MBBR to life so beautifully.

Justin Pead, one of my best friends. Thank you for the countless cups of tea, interesting conversation, reality checks, brutal honesty, keeping me company (and safe) on late night reactor runs, during the protests and support during difficult moments. For staying up late on Discord and keeping me company virtually when I moved to the UK, in the last push to finish. Your influence on my life during this journey is difficult to describe. I am forever grateful that I went on that hike with a total stranger.

To my Deep Branch family, with special mention to Pete, for your patience, encouragement and understanding during the last big push to finish and final hurdle I had to overcome. Being a part of this company and working alongside so many brilliant minds in my dream career is an honour and privilege that I do not take for granted.

Lastly, most importantly, my family. Mum, Dad, Kayla and Richie, I don't think I will ever be able to thank you enough for support you have given me on this journey. It has been a marathon, and you were always there encouraging me, giving me the pep talks and reality checks I needed. You have supported me unconditionally and your enthusiasm and shared joy when things go well makes it all worth it. I am blessed with the most incredible family. None of this would have been possible without you.

Abstract

Humanity is reaching a critical point in history where the re-use of resources previously classified as waste is becoming a necessary strategy to long term sustainability, through concepts such as circular economies. The re-use and reimagination of wastewater through the concept of a wastewater biorefinery (WWBR) is one such potential opportunity. It is the merging of integrated wastewater processing with bioproduction of valuable products from a waste stream, whilst still achieving clean water as an equally important and valuable product. These value-add products need to have sufficient value, and fulfil a market need to ensure that the WWBR is economically viable.

South Africa's wastewater treatment plants, whilst faced with the challenges of rapidly growing populations, limited financial investment in infrastructure, maintenance and skilled operators, have the potential to achieve the goals of the bioeconomy and wastewater treatment through the implementation of the wastewater biorefinery concept.

One of the main challenges facing the implementation of WWBR is the dilute and non-sterile nature of the wastewater. In traditional bioprocessing, this does not favour product formation due to the high flowrate and dilute streams. However, through optimisation of bioreactor design and careful selection against a set of design and operational criteria, a suitable reactor technology can be chosen that will facilitate bioproduction from these dilute streams and overcome the tensions. This project investigated the current wastewater treatment technologies used in a South African context and selected a reactor technology that meets the bioreactor selection criteria to address the challenges of bioproduction.

To test its concept, the study focuses on an example product and microorganism from wastewater. One such product that has high potential for application in a WWBR is poly- γ -glutamic acid (γ -PGA.) It is a naturally occurring biopolymer with potential for application in medical, food, agricultural, wastewater treatment and cosmetic industries. A known γ -PGA-producing *Bacillus subtilis* strain was isolated (referred to as Isolate 1) from a wastewater treatment facility in Mitchells Plain, South Africa by Madonsela (2013).

The kinetics of Isolate 1 were studied in stirred tank reactors (STRs) with a dilute minimal media under ideal temperature conditions (37°C) as well as uncontrolled room temperature to mimic environmental fluctuations that could be seen in a WWBR. The biomass productivity and maximum specific growth rates were estimated. Fed-batch room temperature cultivation was used to investigate if the biomass and PGA productivities could be maintained over extended time by feeding at the maximum glucose-utilisation rate seen during the batch cultivations. The maximum specific growth rates determined were used to inform the critical dilution rate expected in the continuous experiments.

A detailed review of the existing reactor technologies used in South Africa's wastewater treatment plants was contrasted against the criteria for WWBR reactor selection, and through further literature review and refinement of the criteria, a SWOT analysis was done. The Moving Bed Biofilm Reactor or MBBR fulfilled the key criteria of a WWBR. With its reputation as a simple, yet robust technology with the ability to be retrofitted into existing wastewater treatment plant infrastructure (Odegaard, 2006; Wang et al., 2006; van Haandel & van der Lubbe, 2012), it was identified as the most promising reactor technology to investigate the aims of this research.

A lab-scale MBBR was designed and constructed to demonstrate the continuous production of γ -PGA and the impact of biomass retention on productivities and nutrient removal, under continuous and non-sterile conditions. The dilution rate was increased beyond the calculated critical dilution rate to confirm that biomass retention would allow operation at higher dilution rates, whilst still maintaining or improving biomass and γ -PGA yields.

The results from the STR batch cultivations compared the growth and productivity of Isolate 1 (*Bacillus subtilis*) at room temperature (RT) and 37 °C. The overall and maximum biomass productivities in the room temperature batch cultivations were an average of 0.071 ± 0.007 g/L/h and 0.425 ± 0.108 g/L/h respectively. These increased to 0.174 g/L/h and 1.246 g/L/h at 37 °C. The maximum specific growth rates under the

RT conditions achieved average values of $0.150 \pm 0.049 \text{ h}^{-1}$ and 0.376 h^{-1} at 37°C . Based on the maximum specific growth rates, a range of critical dilution rates were calculated to guide the process design and operating parameters in the continuous cultivation studies.

Duplicate fed-batch experiments were conducted with the feed-rate set to the maximum glucose utilisation rate calculated from the batch experiments to achieve a final glucose loading of 2.86 g/L/h . Overall biomass productivities were increased from batch to fed batch phase from $0.092 \pm 0.004 \text{ g/L/h}$ to $0.189 \pm 0.008 \text{ g/L/h}$. The average overall yield and productivities of γ -PGA ($Y_{P/S}$) in the fed-batch cultivations were calculated to be $0.681 \pm 0.066 \text{ g}_P/\text{g}_S$ and $0.667 \pm 0.801 \text{ g/L/h}$.

Following the design and construction of the MBBR, optimal operating parameters such as the loading of carrier and aeration rate needed to be found. Mass transfer studies using the static gassing in-out method were conducted to determine the preferred biofilm carrier loading (percentage of carrier volume in the reactor working volume) allowing the highest oxygen mass transfer. It was found that the optimal filling percentage was 40% and aeration rates of 3 to 5 L/min with volumetric mass transfer coefficient range of 17.23 to 35.64 h^{-1} . Hydrodynamic studies conducted at three different retention times (24h, 12 and 6 hours) and fixed aeration rate of 4 L/min. An increase in the liquid flowrate through the reactor resulted in shortened mixing times. No dead zones were observed. The mixing times of 40.5 ± 5.2 minutes (24 h), 24.5 ± 3.8 minutes (12 h) and 12.5 ± 2.7 minutes (6 h) found to be significantly smaller than the retention times, and thus the system was well-mixed with no visible dead zones or poor mixing.

Biofilm attachment of Isolate 1 was demonstrated on the K3 Annox Kaldnes9® carriers. After a 4-week acclimation period, SEM imaging confirmed a thin and robust biofilm layer consisting of rod-shaped *Bacillus*-looking cells on the carriers.

The MBBR continuous studies were commenced at a 24 h retention time (0.042 h^{-1} dilution rate) to ensure adequate biofilm retention and attachment onto the carriers. SEM imaging of the carriers again confirmed the presence of attached biofilm and dominance of rod-shaped bacteria as expected from *Bacillus subtilis*. The dilution rate was gradually increased until it reached the critical dilution rates calculated from the RT batch experiments. To test the robustness of the system the dilution rates were doubled twice thereafter until a decrease in substrate utilisation was observed at dilution rate 3-fold higher than the critical dilution rate. The retention times tested were 24, 21, 15, 10, 6.6, 4 and 2 hours (corresponding dilution rates of 0.042, 0.048, 0.037, 0.100, 0.152, 0.250 and 0.500 h^{-1}).

The steady-state results showed an increase in biomass and γ -PGA productivities with an increase in dilution rate. The biomass productivity increased from 0.156 g/L/h at 24 h (0.042 h^{-1}) to 0.839 g/L/h at 2 h (0.500 h^{-1}). The substrate utilisation (total carbon fed) decreased from 100% at a retention time of 0.042 to 95% at a retention time of 0.500 h^{-1} , while γ -PGA productivity increased from 0.367 g/L/h to 7.519 g/L/h . At dilution rates $> 0.152 \text{ h}^{-1}$, the OD_{600} of the planktonic cell started to decrease. This is a significant result as, in a suspension culture, it would signify that cell washout was occurring and there would be a decrease in productivity. This is, however, not the case with productivity increasing; this demonstrates that significant biomass retention and biofilm development within in the MBBR and the uncoupling of hydraulic and biomass retention times drive productivity up, with decreasing dilution rates.

One of the key objectives of this research was to demonstrate the proof of concept of the MBBR following its selection against key criteria required for the WWBR bioreactor and prove that bioproduction is possible. The MBBR was able to achieve increasing productivities of biomass and γ -PGA and high substrate utilisation at dilution rates higher than the critical dilution rate. This result demonstrates that WWBR using existing wastewater reactor technologies have great potential. Alongside this, the MBBR is easily retrofitted into existing activated sludge infrastructure that is widely used across South Africa. It is recommended that further study at a larger scale and conditions closer mimicking those of a WWTP be investigated to further test the potential of a WWBR in a South African context in fulfilling the sustainable future we all hope to achieve.

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Abbreviations

AFPB	Aerobic plant fibrous-bed bioreactor
AGSR	Aerobic granular sludge reactor
AS	Activated sludge
AU	Absorbance units
37B	37°C batch cultivation
BNR	Biological nutrient removal
BOD	Biochemical oxygen demand
BOD ₅	Five-day biochemical oxygen demand
CDW	Cell dry weight
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
DMSO	Dimethylsulphoxide
DO	Dissolved oxygen
DoHET	Department of Higher Education and Training
DSP	Downstream processing
DSI	Department of Science and Innovation (previously Department of Science and Technology)
DTI	Department of Trade and Industry
DWAFF	Department of Water Affairs Fishery and Forestry
FB1	Fed-Batch Repeat 1
FB2	Fed-Batch Repeat 2
FBBR	Fluidized bed biological reactor
GPC	Gel permeation chromatography
HMDS	Hexamethyldisilane
HPLC	High pressure liquid chromatography
HRT	Hydraulic retention time
MBBR	Moving bed biofilm reactor
MBR	Membrane bioreactor
MME	Minimal Media E
OD	Optical density
OTR	Oxygen Transfer Rate
PBR	Packed bed reactor
PCR	Polymerase chain reaction
RBC	Rotating biological contactor
RTB1	Room Temperature Batch Repeat 1
RTB2	Room Temperature Batch Repeat 2
RTB3	Room Temperature Batch Repeat 3
RTFB1	Room Temperature Fed-Batch Repeat 1

RTFB2	Room Temperature Fed-Batch Repeat 2
SALR	Surface area loading rate
SBR	Sequencing batch reactor
SDS-PAGE	Sulphate-polyacrylamide gel electrophoresis assay
SEM	Scanning Electron Micrograph
SOL	Soluble organic loading
STR	Stirred tank reactor
TBR	Trickle bed reactor
TF	Tricking filter
THL	Total hydraulic load
TOL	Total organic load
TSA	Tryptone soy agar
TSB	Tryptone soy broth
TSM	Tryptone soy media
UV	Ultra violet
VOC	Volatile organic compound
VSS	Volatile settleable solids
vvm	Volume of gas per volume liquid per minute
WWBR	Wastewater biorefineries
WWT	Wastewater treatment
WWTP	Wastewater treatment plant
WWTW	Wastewater treatment works
γ -PGA	Poly-gamma-glutamic acid
$[\gamma\text{-PGA}]_n$	Glutamic acid molecule (monomer of Poly- γ -glutamic acid)

Glossary

Bioeconomy	An economy based on the sustainable production and conversion of renewable biomass into a range of bio-based products, chemicals and energy. It is an entire innovation chain (from research and development through to commercialisation) that aims to ensure sustainable environment, social and economic development.
Biorefinery	A multi-stage and multi-functional concept that converts microbial biomass as a raw material into a variety of products in a sustainable manner, ensuring minimal waste production. (Harrison et al., 2017)
Biofilm	Multicellular structure adhering to a solid surface that is comprised of various components including microbial cells, extracellular polysaccharide substance (EPS), proteins, extracellular DNA and in case of <i>Bacillus subtilis</i> , also extracellular γ -PGA.
Biofilm carrier	A matrix to which biomass can form attachment and form an adhesive biofilm, facilitating higher biomass concentrations and retention in a reactor.
Carrier fill percentage	The volume of the carriers as a fraction of reactor volume
Floc	Microbial aggregates that form a specialised type of biofilm where cells are held together by a matrix of biopolymers, protein and EPS.
Flocculant	A substance (chemical or biological) which is added to bulk liquid to facilitate bonding between particles, creating larger particles that result in phase separation and act as a clarifying agent. A biological flocculant (or bioflocculant) refers to a type of flocculant that is extracted from a non-chemical source e.g., biopolymers from microorganisms
Green Drop Programme	An incentive-based programme launched by the Department of Water Services in South Africa, to facilitate compliance with regulatory standards and objectives through reward and motivation of positive behaviour. Their programme aims to improve the standard of wastewater management in South Africa (Ntombela et al., 2016)
Humectant	A hygroscopic substance used to prevent loss of moisture, typically found in skin lotions and food products.
Hydrolysate	A substance produced from the process of hydrolysis.
Macrophyte	An aquatic plant, large enough to be seen with the naked eye.
Wastewater Biorefinery	A biorefinery that functions using wastewater as the raw material to generate valuable products through the conversion of waste nutrients, while simultaneously producing water that is 'fit for purpose' as the priority product. Typically, the wastewater biorefinery consists of multiple unit operations and products to enable optimisation of both value recovery and water quality.

Nomenclature

Symbol	Meaning	Units
A	Arrhenius rate constant/frequency factor	-
F	Feed rate of MME in fed-batch and continuous cultivations	mL/h
C_{sat}	Saturated oxygen concentration	mmol/L
$S_{\text{CA},f}$	Concentration of citric acid fed	molC/L; g/L
$S_{\text{G},f}$	Concentration of glucose fed	molC/L; g/L
S_f	Concentration of total carbon fed	molC/L; g/L
k_{La}	Volumetric mass transfer coefficient	s^{-1}
M_w	Molecular Weight	(g/mol)
$[\text{PGA}]_n$	Monomer of γ -PGA (glutamic acid molecule)	-
N	Impeller speed	rpm
OLR_{CA}	Citric acid organic loading rate	molC/L/h
OLR_{G}	Glucose organic loading rate	molC/L/h
OLR	Total carbon organic loading rate	molC/L/h
P	Crude γ -PGA concentration	mol $[\text{PGA}]_n$ /L; g/L
R	Universal gas constant	J/mol.K
R_{G}	Glucose consumption rate (volume independent)	g/L/h
Q_{P}	Productivity of γ -PGA	mol/L/h
Q_{x}	Productivity of biomass	g/L/h
$S_{\text{CA},R}$	Residual citric acid concentration in bulk liquid	molC/L; g/L
$S_{\text{G},R}$	Residual glucose concentration in bulk liquid	molC/L; g/L
S_{R}	Total residual carbon concentration	molC/L
T	Temperature	$^{\circ}\text{C}$
t	Time	h
V_{R}	Volume of reactor	L
x	Biomass concentration	g/L
X	Mass of biomass	g
$Y_{\text{x/s}}$	Yield of biomass on substrate	g biomass / g substrate
$Y_{\text{P/S}}$	Yield of γ -PGA total carbon	mol/molC
$Y_{\text{P/SG}}$	Yield of γ -PGA on glucose	mol/molC

Greek Symbols

ρ	Density of fluid	kg/m^3
μ	Specific growth rate	h^{-1}
μ_{max}	Maximum specific growth rate	h^{-1}

1 Introduction

1.1 Rationale for new approaches to wastewater

As the global population and the demand for clean water increases and water scarce regions increase, the need for effective water management comes under continued pressure and remains a challenge, especially throughout South Africa and other water-scarce regions. The demand for clean drinking water has surpassed the supply on multiple occasions in South Africa during periods of low-rainfall, as well as globally in certain drought-stricken regions. It is critical to address the water scarcity crisis and find alternative ways of storing, using and supplying water. One approach to increasing the supply of water, is through increasing and optimising water reuse and recycle with appropriate use of water that is fit-for-purpose.

There is a strong movement globally to strive for a circular economy where biological systems and renewable raw materials are used to produce materials, chemicals, energy and fuels such that waste streams are minimised or recycled. South Africa was one of the early countries to adopt a “Bioeconomy Strategy”, now found in many countries. At the same time, policies have been implemented to reduce the use of fossil fuels and increase dependence on renewable resources, often processed through bioprocesses. While this shift is expected to ensure more sustainable use of raw materials and enhanced resource efficiency, it can still have a significant impact on water footprints. The growing constraints on water may fundamentally impact the feasibility of the bioeconomic growth (Rosegrant et al., 2013). This dissertation explores and demonstrates the potential for the application of biorefinery principles to wastewater treatment to reduce this water footprint while enhancing resource efficiency and delivering bioproducts, through developing the wastewater biorefinery (WWBR) concept. In this topic, wastewater is seen as a valuable raw material where the nutrients are converted into bioproducts and the water discharged meets legislated requirements for its specified use or recycled within the process at fit-for-purpose quality (Harrison et al., 2017).

To explore the potential of the wastewater biorefinery in general and in South Africa in particular, knowledge on wastewaters in South Africa is necessary. An analysis of South Africa’s Green Drop Programme conducted by Masangane (2000) showed that South Africa has a total of 152 water service authorities which operate 824 municipal wastewater treatment works (WWTW). The collective amount of wastewater being treated by these WWTW is approximately 5000 ML/day. These municipal waste streams contain ‘pollutants’ that provide sources of carbon, nitrogen, phosphorus and other nutrients to the microorganisms (Burton et al., 2009).

The wastewater provided to these WWTWs is a potential source of nutrients from which to form new products and treated, “fit-for-purpose” water. This provides a second treatment objective in line with industrial biotechnology objectives of product formation. To achieve this objective, it is necessary to merge these two approaches, thereby merging the process objectives of environmental biotechnology (minimising effluent concentrations) and industrial biotechnology (maximising productivity of the product). Through the integration of these process objectives, the wastewater biorefinery approach in which effective wastewater treatment is key and industrial biotechnology approach focussed on maximal bioproduct productivity may be achieved simultaneously in a multi-stage process, allowing sustainable production of bioproducts of marketable value, together with energy and clean water.

By adapting the reactor design of the wastewater treatment plants, one has potential to exploit nutrients in the wastewater and reactor conditions to enable the development of niche conditions for bioproduct-forming micro-organisms in a mixed-culture environment. This process, of producing a valuable (primary) bioproduct from dilute waste streams and achieving nutrient depletion, especially depletion of the organic carbon, is the core of a wastewater biorefinery. To enable optimisation of both this product and water quality, the wastewater biorefinery exists as a multi-stage process to allow sequential depletion of specified

nutrients with product formation targeted at each stage to result in the optimised production of 'fit-for-purpose' water for re-use, as a non-negotiable product.

One such primary bioproduct, polyglutamic acid (γ -PGA), is a high value biodegradable biopolymer that can be produced by cultivating *Bacillus* species (Shih et al., 2001). In this study, it is selected as a case study to demonstrate the feasibility of its bioproduction from a dilute nutrient environment i.e. low strength wastewater. An investigation into the reactor technologies used in conventional wastewater treatment plants in South Africa was carried out with the aim of exploring potential for their re-purposing into wastewater biorefinery unit operations. Based on the results of this investigation, one reactor design, the moving bed bioreactor, was selected to reduce the challenges faced in production of bioproducts from wastewater, was commissioned at lab-scale to investigate the feasibility of producing γ -PGA from a low-strength nutrient stream under continuous, room temperature conditions.

1.2 Scope of the dissertation

This dissertation investigated the feasibility of the wastewater biorefinery concept through the first stage operation of a continuous reactor for the removal of major carbon-based nutrients and some nitrogen. To achieve this, reactor technologies appropriate for this type of operation were investigated. The production of a biodegradable polymer, poly- γ -glutamic acid (γ -PGA), from *Bacillus* species in the selected reactor, is used to demonstrate the feasibility at lab scale, as a case study.

Cultivation in the stirred tank reactor was used to investigate and quantify the growth kinetics (maximum specific growth rate, biomass productivity and biomass yields) under batch and fed-batch operation mode, at 37°C and room temperature conditions to provide design data for the continuous process. The impact of operating at room temperature without temperature control was used to demonstrate the microbial response to varying temperature in a wastewater biorefinery application (e.g., Outdoor reactors on a wastewater treatment plant with exposure to fluctuating temperatures). The productivity of γ -PGA was not fully investigated in this part of the study due to the proven growth association of γ -PGA with the selected *Bacillus* isolate (Madonsela, 2013; Rademeyer, 2018). However, γ -PGA was quantified at the start and end of the room temperature fed-batch phases to demonstrate increased productivity in biomass and product formation as a result of constant feed-rate and declining growth rate.

Through a critical review of the current wastewater treatment reactor technologies currently applied in a South African context, an appropriate reactor technology that most adequately address the challenges presented by wastewater biorefineries, is selected, designed and commissioned for the continuous reactor case study.

The operational aspects of the reactor required for combined water treatment and bioproduct formation led to the selection of the moving bed bioreactor (MBBR). MBBR studies focused on investigating the substrate utilisation and γ -PGA product formation rates under non-sterile conditions and at varying dilution rates in the MBBR to establish the dominance of the γ -PGA-producing *Bacillus* isolate, native to wastewater treatment systems, and its potential for retention in the bioreactor system through attachment and biofilm formation.

This study did not investigate the treatment of actual industrial wastewater or a synthetic industrial wastewater. A dilute minimal medium was used as a form of synthetic wastewater across all experiments. Varying concentrations of the media was not investigated.

1.3 Dissertation Structure

To address the objectives of this dissertation, seven chapters are presented. The 1st section of the literature review in Chapter 2 investigates the motivation behind why WWBR's are worth investigating in South Africa, the key requirements of a wastewater biorefinery and the challenges surrounding the reactor design for bioproduction and the key factors for consideration to achieve proof-of-concept. In the 2nd part of the literature review, the microbial production of γ -PGA by *Bacillus* is reviewed. This includes discussion of γ -PGA as a valuable bioproduct and the motivation for its investigation as the product of choice for the case study in this dissertation. The selection of *Bacillus* as microorganism of choice is reviewed as are the culture conditions for it to thrive and key factors maximising PGA production. The analytical methods for identifying PGA produced are summarised and the most suitable methods identified. The aims and objectives, key questions and hypotheses are presented in Sections 2.3 at the close of this chapter.

In Chapter 3 the material and methods used in this dissertation are presented. This includes the biological and chemical assays used in the analysis of cultivation samples, method optimisation for the UV-spec method for the identification and quantification of γ -PGA and summary of the experimental approach. The methods and experimental approach for the reactor studies are presented in detail in Chapter 4 and 6.

In Chapter 4, batch and fed-batch cultivation of *B. subtilis* in a STR bioreactor, at both 37 °C and room temperature are investigated. The work presented is compared to previous work done by Madonsela (2013) using the same isolate, as well as to literature studies. The growth kinetics (maximum specific growth rate, glucose utilisation rates, biomass yields and productivity) are investigated to provide data to inform the approximated dilution rates used for application in Chapter 6.

Chapter 5 provides an in-depth review of the current wastewater treatment reactors to determine the requirements for a reactor design suitable for application in a WWBR. Existing reactor technologies are then contrasted against these criteria to address challenges of bioproduction from dilute and variable streams and the potential of each reactor technology of interest to achieve this. From this assessment, a short-list of reactors with potential for implementation into a WWBR application is created. Further elimination by prioritisation of the WWBR criteria developed by Verster et al. (2014) results in a list of three potential reactor types. These were further analysed using a SWOT analysis. Recommendations of the most suitable reactor technology arrived at, the MBBR, are discussed.

Chapter 6 demonstrates the feasibility of γ -PGA production under continuous conditions, similar to those expected on application in a WWBR, using a 7 L scale MBBR. This chapter discusses the design, commissioning and operational aspects of this reactor, as well as the experimental study into the hydrodynamics and mass transfer characteristics at specific dilution and aeration rates. Following this, the results obtained on growth of the *Bacillus* species in the MBBR, with cell retention on biofilm carriers, and associated γ -PGA production are presented and considered in terms of yield and productivity. These results from the continuous bioreactor are discussed, and recommendations made.

Chapter 7 provides key conclusions from the research. Recommendations are made to further the investigation of value from waste using MBBR technology and to obtain further insight into the operation of this reactor under different, potentially optimised, conditions.

2 Literature Review

To address the scope of this thesis, an introduction to the concept and necessity of a wastewater biorefinery in a South African context is discussed. An understanding is required surrounding the challenges that this new-concept biorefinery could face, specifically surrounding the bacterial reactor and an introduction to how they can be addressed. In the second part of this chapter, an understanding of the existing knowledge around the value of γ -PGA as the selected bioproduct, the production of γ -PGA from *Bacillus* species and organisms isolated from municipal wastewater, as well as the current knowledge of γ -PGA production in bioreactors under different operational modes is presented. The analytical methods for the quantification of γ -PGA are summarised.

Following this review, the research motivation is outlined as well as the key questions, aims and objectives and hypotheses that drives the focus of the research.

2.1 Introducing the Wastewater Biorefinery Concept

2.1.1 Bioeconomy strategies in a South African context

Globally, countries such as Sweden, Norway, the Netherlands, Ireland, Canada, Germany, the USA and the European Union, have committed to developing advanced bioeconomy strategies that have an increased focus on value generation that is biotechnology-based. It is an important step in the transition towards sustainable development and removing the focus from fossil-fuel based economies (de Besi & McCormick, 2015).

South Africa launched a bioeconomy strategy in 2013, building on its National Biotechnology strategy that was launched in 2001. The key vision for this strategy is to be a significant contributor to the country's economy (specifically the GDP) by 2030 (DST, 2013). This strategy, which was drafted by the Department of Science and Technology (DST), now known as Department of Science and Innovation (DSI) but has the buy-in and cooperation from multiple government ministries including Department of Trade and Industry (DTI), Department of Higher Education and Training (DoHET), Department of Water Affairs Fishery and Forestry (DWAF) and others. Close cooperation between industry, science councils, government departments and universities were called upon, to ensure that the bioprocesses, biotechnology, and bio-related innovations are relevant for easy application in a South African context.

Health, Agriculture and Industrial Bioprocesses and the Environment are the three focal points of South Africa's Bioeconomy Strategy. All three of these sectors heavily involve water use and treatment. Water and waste management are specifically mentioned under the Industrial Bioeconomy section, where the major strengths and opportunities lie in the use of micro-organisms in the manufacture of bio-based products such as chemicals, feed, detergents etc and water treatments and waste minimisation (DST, 2013). It is specifically mentioned in the strategy document that "alternative use of wastewater streams for other products" is an area requiring research, development and innovation to minimise waste generation and create effective waste-management systems.

With the goals of this strategy in mind, and the growth of South Africa's green economy it hopes to achieve, the opportunity for a merge between the production of biobased products and water treatment arises as an emerging concept and is defined in Section 2.1.3.

2.1.2 The biorefinery concept

A biorefinery can be characterised as an over-arching, integrative and multifunctional concept that makes use of biomass or bio-based raw materials as the feedstock for the production of a range of products (bioenergy, chemicals, fuels and materials). Biomass is used as a raw material to the fullest extent possible to maximise resource productivity as well as minimising waste products (Kamm, et al., 2006). Subsidiary products can also be used in feed for animals. These multi-pronged objectives require

the integration of different methods and technologies (Verster et al., 2014). The main steps within the biorefinery process chain are initially pre-treatment and preparing the biomass, separation of the biomass components (primary refining), conversion into products using a conversion process (secondary conversion), followed by separation steps (de la Fuente, 2014).

Examples of biomass that could be used as a biorefinery feedstock include lignocellulosic type feeds such as trees, grass, agricultural crops and residues, animal waste or municipal solid waste (Fernando et al., 2006; Kamm et al., 2006). Through a series of unit operations, the main goal of a biorefinery is the production of high-value-low-volume (HVLV) products and low-value-high-volume (LVGV) products with maximisation on the feedstocks extractions and minimisation on waste generated. The HVLV products ensure the profitability, whilst the LVHV products are converted into energy for the process, and ensure the energy demand and cost is minimised (Fernando et al., 2006).

2.1.3 The wastewater biorefinery concept

The concept of the wastewater biorefinery can be simply defined as the integration of wastewater treatment and traditional bioprocessing to simultaneously produce products of value, including “clean” water (sufficiently remediated wastewater effluent). This is achieved through the multi-stage approach of a biorefinery with integrated process steps, and products of sufficient value to ensure the economic viability of the wastewater biorefinery. The wastewater biorefinery provides a means to close material flows and contribute towards the concept of circular bioeconomies discussed in Section 2.1.1.

The Centre for Bioprocess Engineering (CeBER) at the University of Cape Town, has played a leading role in helping to define and develop the wastewater biorefinery approach, most notably through two Water Research Commission (WRC) reports (Verster et al., 2014; Harrison et al., 2017) and a book chapter (Pott et al., 2017). Verster et al., (2014) introduced the wastewater biorefinery concept through merging wastewater treatment and traditional bioproduction. The report outlined the characteristics of the wastewater biorefinery and highlighted the key process constraints that inherently arise when merging wastewater treatment (large volumes of dilute and complex streams) and industrial biotechnology (maximising resource productivity and products of value) to achieve bioproduction from municipal wastewater streams. These challenges and tensions are discussed in Section 2.1.4. γ -PGA produced from *Bacillus* was used as an example product and organism to develop the concept of the WWBR. The selection of this product and organism for further research in this dissertation are discussed in Section 2.2. This report highlighted that the reactor selection and design are a key component of the wastewater biorefinery, and that the reactor selection needs to address the tensions and challenges of a WWBR. The requirement for an integrated approach to the bioreactor and process design of WWBR to maximise resource productivity and water treatment was highlighted.

The WRC report by Harrison et al., (2017) presented this integrated approach towards WWBR. Extensive research was conducted on the various aspects of the WWBR through interaction with industry stakeholders. A detailed review was conducted on the wastewater streams available in South Africa. A review of potential products was examined, and the bioreactors required to produce these were reviewed and selected. A generic flowsheet and mass balance model was developed along with the features of the integrated biorefinery unit operations to facilitate the production of a valuable bioproduct and water that is compliant with regulations. The findings from this report allow for the potential value for WWBRs in a South African context to be considered.

The wastewater biorefinery concept operates within the arena of wastewater treatment, with respect to the key objective of reducing nutrient load in wastewater, and that both WWBR and WWTWs processes have similar multiple stages (pre-treatment or settling, primary treatment, secondary treatment and polishing), each with the objective of enhancing process performance and objectives. Where the WWBR deviates from WWT, is that these unit processes are adapted for commercial production of valuable bioproducts from the wastewater, depending on the composition of incoming wastewater and market demand.

The potential WWBR process flowsheet that was developed by Harrison et al., (2017), using municipal wastewater as a raw material, is shown in Figure 2-1. It illustrates the simplified process steps with the different bioreactors being bacterial and algal for primary treatment, the macrophyte (plants) reactor to assist with polishing and a solid bioreactor to deal with settled fractions.

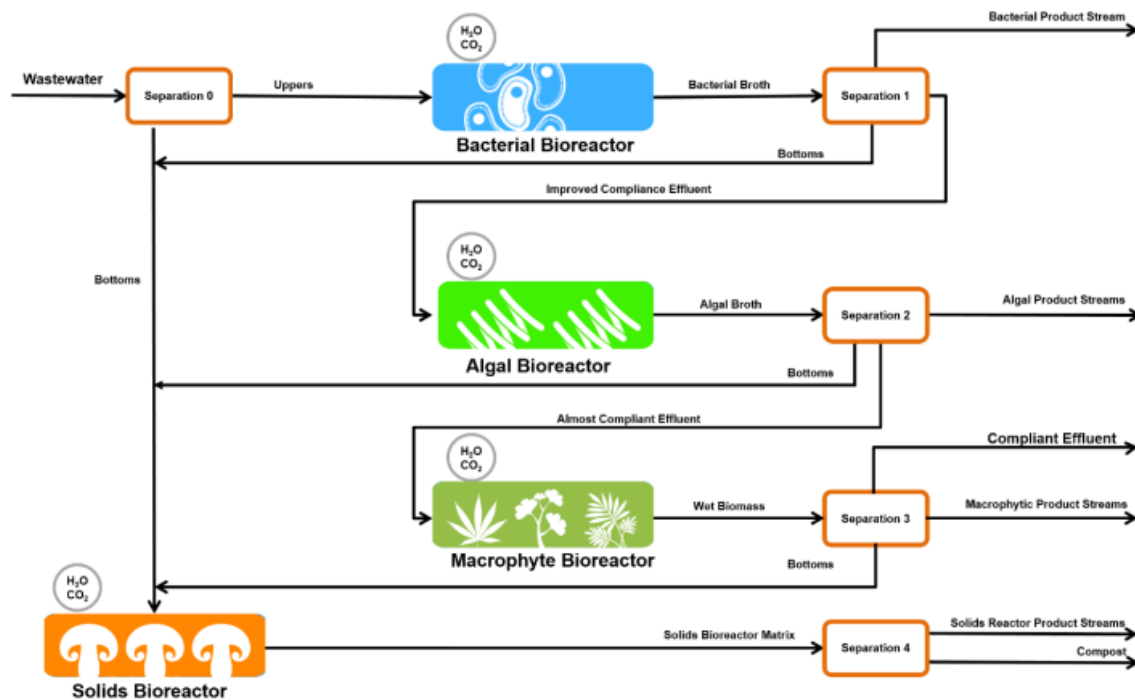


Figure 2-1: Simplified flowsheet of a potential WWBR (Harrison et al., 2017)

Harrison et al., (2017) describes that there is a significant difference between wastewater treatment bioreactors, and those of conventional bioprocessing; while wastewater treatment relies on mixed and dynamic cultures, conventional bioprocessing usually relies on defined and controlled microbial populations. Within the WWBR process flowsheet, the reactors, although named bacterial, algal, macrophyte and solids, would realistically be operated under mixed or enhanced biological environments. The bacterial bioreactor, due to its reactor design and operational requirements, would inherently exclude certain organisms from being dominant e.g., high stirring or shearing would exclude larger organisms. This bioreactor could also be described as a “heterotrophic microbial reactor”. Being the primary reactor, its main function is to remove organic carbon and nutrients through well understood microbial cultivation processes.

The algal bioreactor is predominantly designed for nitrogen and phosphorus removal, with focus on ensuring that the reactor design allows for sufficient light and photosynthetic activity. The macrophyte reactor is predominately designed using plants, however bacteria and other organisms are associated with nutrient removal through their symbiotic relationship with plants. Lastly, the solids bioreactor is anticipated to be dominated by fungal species. (Harrison et al., 2017; Verster, 2019).

2.1.4 Bioproduction challenges from wastewater

Wastewater treatment plants are well designed to achieve high levels of nutrient removal from wastewater with the primary goal of ensuring that “clean” water (treated effluent) meets regulatory requirements as the product. A well understood and globally employed wastewater treatment technology, Activated Sludge (AS), has throughout the years been optimised to handle large volumes of continual flows. The activated sludge process used in wastewater treatment is shown in Figure 2-2.

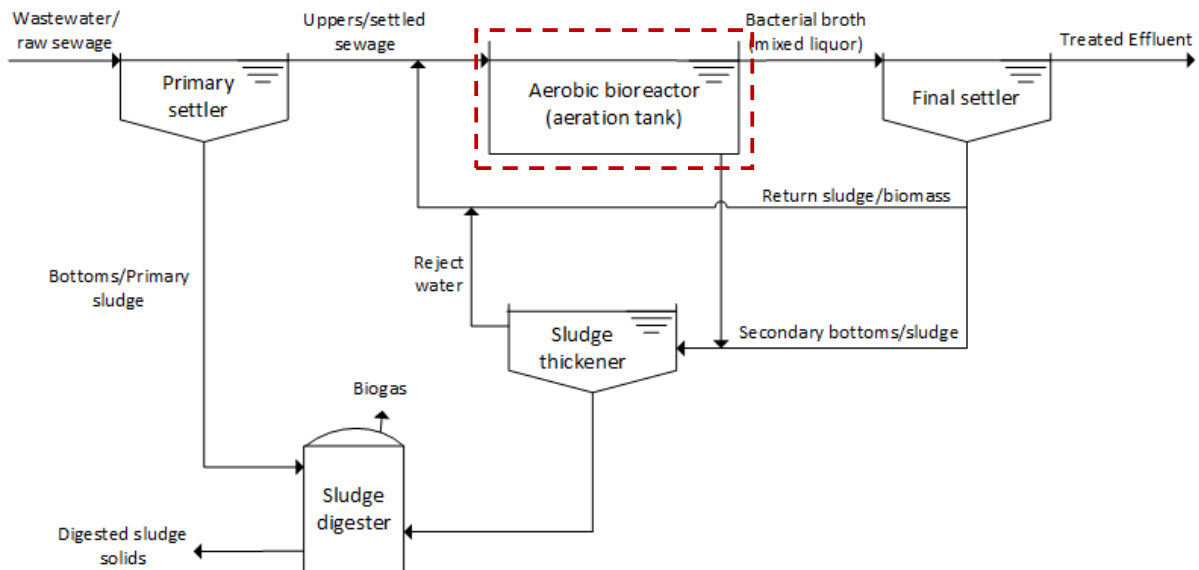


Figure 2-2: Representation of a basic activated sludge system flowsheet (primary and secondary wastewater treatment). Adapted from van Haandel & van der Lubbe (2012).

The aerobic bioreactor (or aeration tank) in Figure 2-2 is the process step in which the majority of the nutrient removal from wastewater is achieved, however the design of this reactor is limited with respect to product formation and recovery. From a traditional bioprocessing perspective, the bioreactor optimisation is maximised for productivity at controlled substrate concentrations, through strategies such as volume reduction to reduce overall energy requirements (Harding, 2009). Another strategy is through increasing biomass concentrations, which eases the cost per unit product on downstream processing (Richardson, 2011). Within the context of a WWBR, the bacterial bioreactor in Figure 2-1 would play the same function of the aerobic bioreactor in the activated sludge system. Implementing the wastewater biorefinery concept into the existing WWTP (such as an activated sludge system), requires intentionally innovative reactor design and selection, that will address that challenges and tensions associated with using wastewater as a low cost, yet readily available and valuable resource for bioproduction (Harrison et al., 2017).

The type of products that are selected for production, need to be suited to utilising organics from high flowrates and enhance the ecological function to drive the competitive advantage of the microorganism (Kleerebezem & van Loosdrecht, 2007; Mooij et al., 2015). This can be achieved by creating ecological niche environments through modifications to bioreactor design, the organism that is producing the valuable bioproduct, will have a competitive advantage over the consortium of other organisms within the bioreactor.

Each unit operation in the WWBR system requires adherence to key principles in order for bioproduction to be feasible, and the bacterial bioreactor selection is a crucial element. These key principles provide the framework for converting organic nutrients into a commodity product (Verster et al., 2014) and were established and assimilated by Harrison et al., (2017). These key principles include:

- Decoupling the solid (biomass) residence time with the hydraulic (wastewater) residence time by retaining and/or recycling biomass.
- High biomass retention enables higher biomass concentrations, resulting in increased volumetric conversion of larger volumes.
- Ecological niche is created through the careful selection of product and chosen reactor. This is applied in a non-sterile environment.
- Ensure that adequate mass transfer and nutrient requirements of the cells are met, without excessively increasing energy requirements.

- Considering the DSP requirements when designing the bioreactor and selecting the biological system. Owing to the very dilute, high-volume streams that need to be processed, many DSP methods would be too costly. The product should be present in a different phase to the bulk liquid, or in a phase that is easily and rapidly recovered. Biomass retention through bioreactor design facilitates easier DSP if product is cell-associated.
- Producing clean water, as well as product formation are achieved without competing, through the integration and optimisation of the WWBR as a whole system.

In the following subsections, further insight into the challenges of bioproduction from wastewater, researched by Harrison et al (2017), is given as well as providing deeper understanding the impact that these key principles have on the bioreactor selection.

Large volumes of dilute wastewater

A significant challenge of the WWBR concept is the dilute nature of the feedstock, with both nutrients and products present at very low concentrations. When utilising wastewater streams like municipal wastewater, which can be up to a thousand-fold more dilute than conventional feed streams for bioproduction, this aspect is particularly challenging. Pre-concentrating the feed stream could be an option, however the impact on addition of energy, chemicals and infrastructure requirements needs to be strongly considered. A better approach is careful reactor design to allow for increasing biomass concentrations. This facilitates higher conversion of nutrients, the intensity of the process increases, and thus smaller reactors can be utilised.

With regards to product recovery and ensuring cost and energy efficient downstream processing (DSP), ensuring that the product is accessible and localised in a high concentration (e.g., a difference phase) is preferable to removing a product of the same phase as the bulk liquid. Many downstream processes are optimised post-production, however focusing on bioreactor design optimisation has the potential to impact DSP and overall process optimisation (Richardson, 2011).

Aeration is also an important factor to consider, because in high-volume and low concentration systems such as wastewater treatment, it can account for up to 70% of the operating costs (Tchobanoglous et al., 2003). Oxygen is sparingly soluble in water and its provision often controls reaction rate and stoichiometric limitations (Bailey & Ollis, 1986). Biofilm can also present additional challenges to oxygen delivery to biomass deep within the biofilm. This is because the large density population of bacteria that form the biofilm, require a higher oxygen demand to achieve the high biodegradation productivity, as well as reducing the efficiency of oxygen penetrating deeper depths of the biofilm resulting which can result in certain areas of deep biofilm becoming anaerobic.(Barwal & Chaudhary, 2015)

The importance of biomass retention

To both produce and recover bioproducts from dilute waste streams, while achieving fit-for-purpose water as a priority, achieving high biomass retention is essential to intensify conversion rates. This requires a decoupling of the solid and hydraulic residences.

Activated sludge is the most commonly employed process by WWTW. However, the flocs formed require large settling ponds, due to their inherently poor settling capability (Nicoletta et al., 2000). To facilitate the de-coupling of hydraulic and biomass residence times, the two approaches highlighted (with orange) in Figure 2-3 are most promising for WWBR bioreactor design i.e. to generate conditions suitable for static biofilms, and particle biofilms. These two approaches have increased tolerance for higher flowrates and substrate concentrations relative to the activated sludge approach.

Figure 2-3 shows that when substrate concentrations are high ($>10 \text{ kg/m}^3$) the microorganisms tend towards being a suspended culture. There is no need for biomass retention as the biomass proliferates rapidly and achieves the desired nutrient removal, due to fast growth rates ($> 0.1 \text{ h}^{-1}$). Here, sufficient biomass or product may be formed to justify traditional bioprocess approaches without biomass retention (Harrison et al., 2017). At lower substrate concentrations ($<10 \text{ kg/m}^3$, dilute wastewater),

biomass retention is used to decouple the solid and hydraulic residence times, thereby increasing the reactor volumetric conversion capacity (Nicolella et al., 2000).

Biomass retention can be achieved in a variety of ways; introducing recycling loops, immobilising cells through biofilm formations, using selective membranes to retain biomass, granulation or employing a combination of these. As an example, this be using selective membranes, and instead of suspended cells, including cell immobilisation matrices to further increase biomass retention (Harrison et al., 2017).

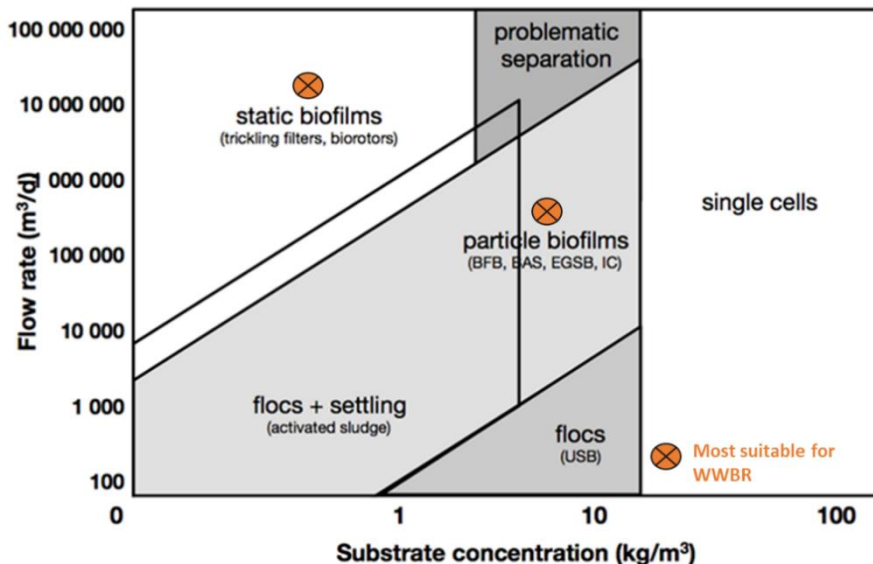


Figure 2-3: Substrate concentration flowrate diagram for application of floc and biofilm bioreactors, indicating the most suitable areas for a WWBR reactor design (adapted from (Nicolella et al., 2000)).

Downstream process design considerations

A fundamental consideration in the feasibility of bioprocessing from dilute streams, from both an economic and environmental standpoint, is in the approach to DSP. In many processes, the downstream processing is often only considered and optimised after production, however there is scope within the bioreactor design to ensure that overall, the process is optimised (Richardson, 2011). In dilute systems such as WWBRs, recovery and quality of both the product and the water must be prioritised. Water may be recycled back to the process upstream of the WWTW or recovered as water of 'fit for purpose' quality.

Recovery of the product is easiest when it is inherently in a different phase to the bulk liquid, which is why gaseous products such as biogas have been strongly favoured (van Haandel & van der Lubbe, 2012; Verster et al., 2014). Figure 2-4 is an initial guideline for wastewater biorefinery bioreactor selection Verster et al., (2014) It demonstrates that the approach used depends on the whether the product is; intracellular, biomass-associated or extracellular.

If downstream processing is considered in the reactor design and operation, then biomass associated products such as biopolymers, that are either stored in intracellular vacuoles or associated with extracellular polymeric substances (EPS) can be recovered through exploiting biomass retention. Through this approach, the burden on DSP is reduced and productivities improved.

If the product is extracellular and not associated with biomass (excreted into the bulk liquid and soluble), separating the biomass from the broth is necessary and involves concentrating the product prior to purification. This could be done through precipitation, distillation, ultra-filtration or chromatography (Doran, 1995; Harrison et al., 2017). It is preferable to avoid using additives to drive this phase separation e.g., chemicals solvents that cause precipitation or flocculation, as this can have

consequences on the quality of the water that is a priority to recover. These additives would also need to be removed as part of the downstream processing.

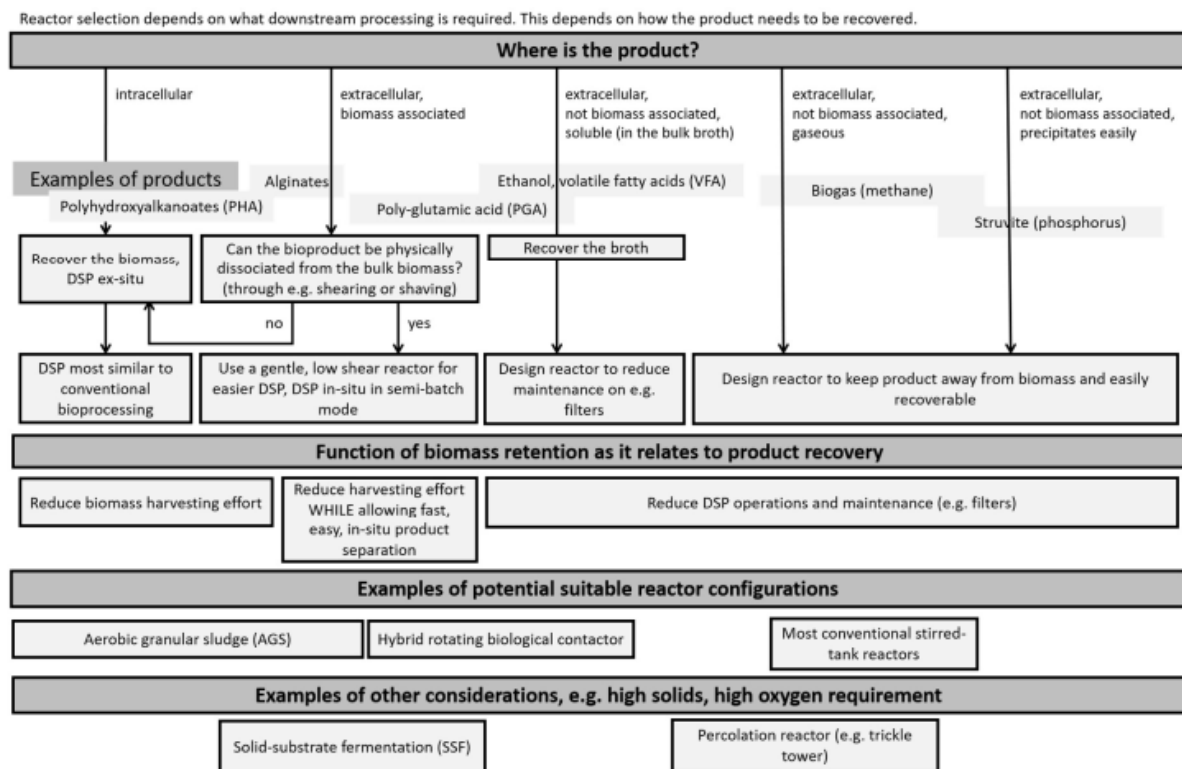


Figure 2-4: Suggested guideline for wastewater biorefinery bioreactor selection (Verster et al., 2014; Harrison et al., 2017; Verster, 2019)

Non-sterile operation and environmental considerations

The ability for a WWBR to operate and produce the commodity products under non-sterile conditions is a key requirement based on the principle of operation; large volumes of wastewater being processed, and reactors being exposed to the surrounding environment. This removes the practicality and possibility for sterilisation and maintaining sterile boundaries.

Operating under non-sterile conditions requires careful thought when selecting the microorganism and product. There are limitations on modifications that can be made to the microbial community, currently favoured for traditional bioproduction. The use of genetically modified organisms may be prohibited due to environmental regulations and social acceptability. Instead, the most robust and resilient microorganisms make up a mixed community which is well adapted to the physicochemical environment in which it exists and can withstand shock loads and hostile environments (Chen, 2013). To ensure product formation, the environment of the desired producers must be enhanced through the reactor design. Further, the mixed community can also become a strength by offering process robustness in the microbial community (Verster, 2019).

The water product of the WWBR must comply with regulatory standards for release into the wider environment or meet the quality requirements if being reused. If the water is for reuse, any additives introduced into the stream should be non-hazardous. The pathogenic loading also needs to meet the regulated levels.

2.1.5 Evaluation criteria for bioreactor design and selection

The challenges discussed in Section 2.1.4 were summarised by Harrison et al. (2017) and converted into Table 2-1 that a selected bioreactor would need to comply with. This table is used as an evaluation tool when reviewing whether a reactor technology is suitable for application in a wastewater biorefinery. Should a bioreactor be unable to fill the requirements in Table 2-1 then it will be unlikely to be able to produce the selected product in a phase and quantity that will ensure the process is economically feasible.

Table 2-1: Wastewater biorefinery bioreactor design requirements (Verster et al., 2014; Harrison et al., 2017).

#	Requirement	Comply?
A - Large Volume		
1	Decouple hydraulic and solid retention times	✓
2	Continuous or semi-continuous (cannot store flows)	✓
3	Think big! Commodity rather than niche	✓
B - Complex, Variable		
4	Influence microbial community, non-sterile	✓
5	Give advantage to product: create ecological niche	✓
C - Environment		
6	Water released into environment eventually	✓
D - Downstream Processing		
7	Product formation in different phase?	✓
8	Can product be recovered?	✓
9	Reactor design conducive to reducing DSP load?	✓

2.1.6 Research motivation on the wastewater biorefinery concept

The WWBR brings together the best aspects of industrial biotechnology and wastewater treatment by integrating and optimising multiple stages, each with the function of ensuring that regulatory-compliant water and valuable bioproducts are produced from waste streams. The multistage approach helps to ensure that the WWBR could be retrofittable within existing WWTW through modification to existing unit operations and that wastewater is fully utilised if possible. The first stage of the WWBR, the bacterial bioreactor aims to remove the largest proportion of carbon and nutrients. This is the most suitable reactor out of the four reactors (see Figure 2-1) in be the significant producer of a value-added product. Bioproduction within this bioreactor from wastewater presents challenges and tensions that need to be overcome through careful process and reactor design. The key aspects that that are necessary for a successful WWBR include:

- Decoupling the solid (biomass) residence time with the hydraulic (wastewater) residence time by retaining and/or recycling biomass.
- High biomass retention enables higher biomass concentrations, resulting in increased volumetric conversion of larger volumes.
- Ecological niche is created through the careful selection of product and chosen reactor. This is applied in a non-sterile environment.
- Ensure that adequate mass transfer and nutrient requirements of the cells are met, without excessively increasing energy requirements.
- Considering the DSP requirements when designing the bioreactor and selecting the biological system.
- Producing clean water, as well as product formation are achieved without competing, through the integration and optimisation of the WWBR as a whole system.

In taking this forward, it is evident that what must be still considered is reactor design that will be used to achieve bioproduction within the bacterial bioreactor of the WWBR. A logical starting point is the review of existing reactor technologies in current WWTW in South Africa, and a review of their criteria fulfilment in Chapter 5.

2.2 The selection of poly- γ -glutamic acid as a commodity product

2.2.1 Potential bacterial products from a WWBR

When selecting a product for bioproduction from a WWBR, it must be able to fulfil two main requirements; it should have a market demand to be considered of value and be suitable to production within the constraints of the WWBR (discussed in Section 2.1.4). These requirements can result in a limitation on which products can be selected.

A study by the European Commission (2009) that was done to promote the implementation of biobased products defined them as non-food products derived from biomass (e.g., plants, algae, trees, marine organisms, household biological waste, animals and food production). They range from high value products in industries such as pharmaceutical, cosmetic or food additives, to high volume products such as biopolymers or chemical feedstocks. An overview of these biobased products and their characteristics was done and is given in Table 2-2:

Table 2-2: Overview of the biobased products and their characteristics/functionalities, excluding food, energy and fuel. (European Commission, 2009)

Product type	Characteristics or functionalities
Chemical and chemical building blocks Various chemicals made from renewable raw materials.	Sustainable chemical production, lower GHG and other emissions in production, lower resource use in terms of energy and water with less waste depending on production process, typically better biodegradability, potentially less toxic.
Biobased plastics, biopolymers and biomaterials e.g., PHA, polyethylene (PE), polylactic acid (PLA) and propanediol based plastics from biotransformation of glucose, sucrose, plant derived carbohydrates or starch.	Sometimes biodegradable and/or compostable, lower GHG emissions, potentially less toxic, materials with new qualities (composite materials, textiles, boards etc).
Renewable construction materials and composite materials from natural fibres e.g., flax, hemp, jute, wood used in building construction and automotive components etc.	Good mechanical properties (impact resistance, acoustic qualities, strongly reduced Weight/lightweight concrete), better waste recycling (easier to recycle or burn than fibreglass).
Surfactants Surfactants with lower surface tension of liquids and are used in soaps, detergents, pharmaceuticals, food additives, etc. and to produce emulsions and foams. Chemical surfactants are produced largely from oils. Next generation biosurfactants can be produced using algae, fungi or bacteria.	Low eco-toxicity offers biodegradability and compostability. Enzyme-based detergents are used in household washing machines and offer environmental advantages (lower temperature, energy savings, more efficient washing, have replaced phosphorus).
Biosolvents Solvents are used in paints, inks, varnishes, adhesives etc.	Biobased solvents do not emit VOCs that are harmful to human health and the ozone layer. Some 23% of VOCs emitted into the air are from petrochemical solvents.
Biolubricants Lubricants made from vegetable oils and their direct derivatives for engines, gearboxes, chains, etc.	Biodegradable, lower toxicity, can be used in sensitive environments, may reduce pollution from non-biodegradable or otherwise environmentally unacceptable lubricants from machines and vehicles.

For the purposes of this research, focus was given to the microbial-based polymers that could be produced from the carbon-rich components of wastewater. A polymer is a chemical compound made from repeating structural units (monomers) that is synthesised in polymerisation or fermentation processes. A biopolymer is a natural polymer produced during the growth cycle of all microorganisms

through complex metabolic processes than can be classed into three groups (Kumar et al., 2007); i) Polynucleotides (RNA and DNA); ii) Polypeptides and proteins that are polymers of amino acids and iii) polysaccharides, linearly bonded polymeric carbohydrate structures

Plastic, by definition, is a blend of one or more polymers and additives. A bioplastic, is a broad and often misleading term which can be defined in a variety of ways (European Bioplastics, 2015):

- Biobased plastic that has biobased raw materials as the source
- Biodegradable plastics that can ultimately biodegrade naturally
- Biocompatible plastics, that are able compatible with humans or animals.

Typically, the understanding of a bioplastic is either biobased or biodegradable, or both. However, not all biodegradable plastics are produced from biobased raw materials (Shen et al., 2009). The definition and clarification around biopolymers and bioplastics can be represented in the following diagram:

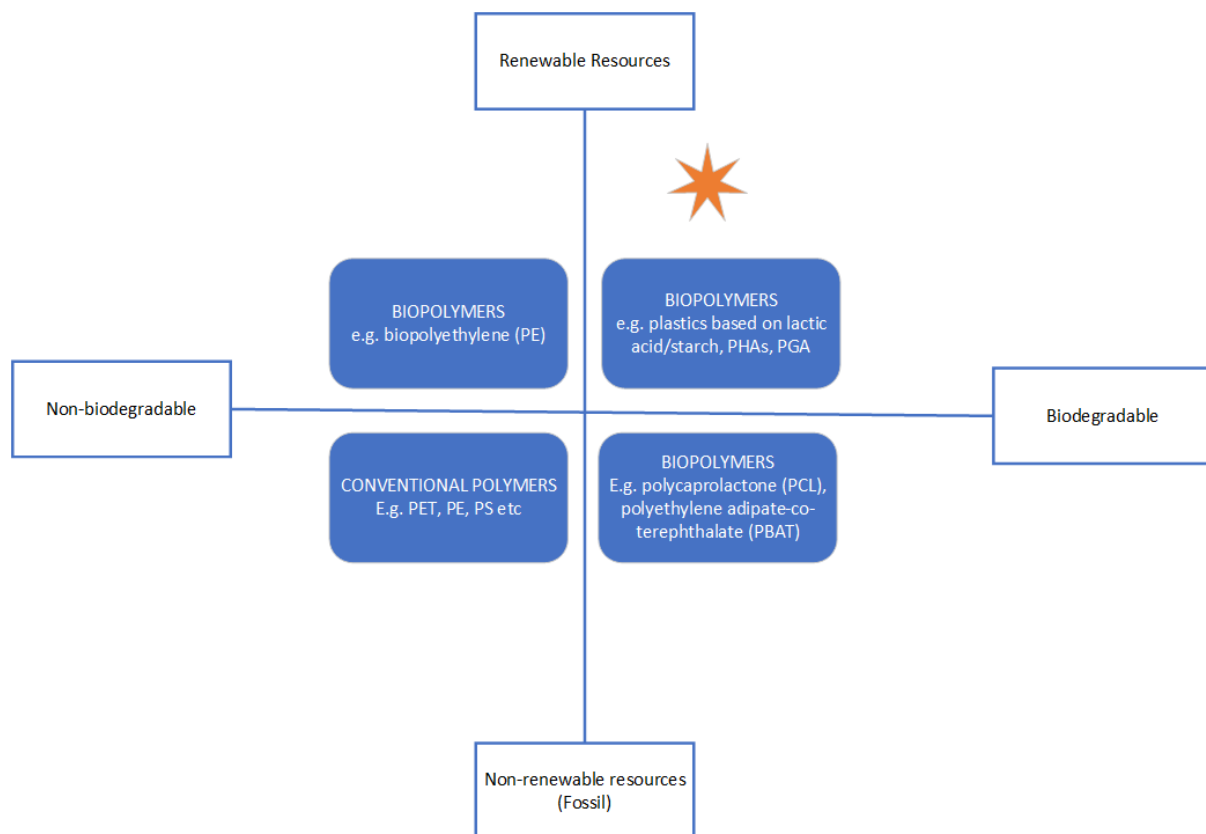


Figure 2-5: Clarification matrix for bioplastics (European Bioplastics, 2015)

The most logical biopolymer class for bioproduction in a WWBR, would be the biobased and biodegradable class of bioplastics (the class of product that falls under the upper right quadrant in Figure 2-5). Products that fall under these categories, are fully degradable by microorganisms, and occur naturally in a variety of organisms. Being able to produce these types of biopolymers from waste streams containing the nutrients they require, would provide an opportunity to close resource and carbon cycles, by exploiting the value of the wastewater components (Harrison et al., 2017). One such biopolymer that falls under this category, is γ -PGA. This will be explored as the bioproduct of choice in this research.

2.2.2 Poly-γ-glutamic acid as a valuable bioproduct

Poly-glutamic acid (PGA) is a highly anionic polymer that can be produced either chemically or bacterially. It is a naturally occurring polyamide consisting of D- and L-glutamic acid isomers polymerised by α-amino and γ-carboxyl amide linkages (Kumar & Pal, 2015). When produced chemically in a well-established process, α-PGA is formed. The polymer was first discovered in 1937 in the capsule of *Bacillus anthracis*; however, due to the microorganism's pathogenic nature, it was unsuitable for the industrial scale production of γ-PGA (Ivanovics & Bruckner, 1937).

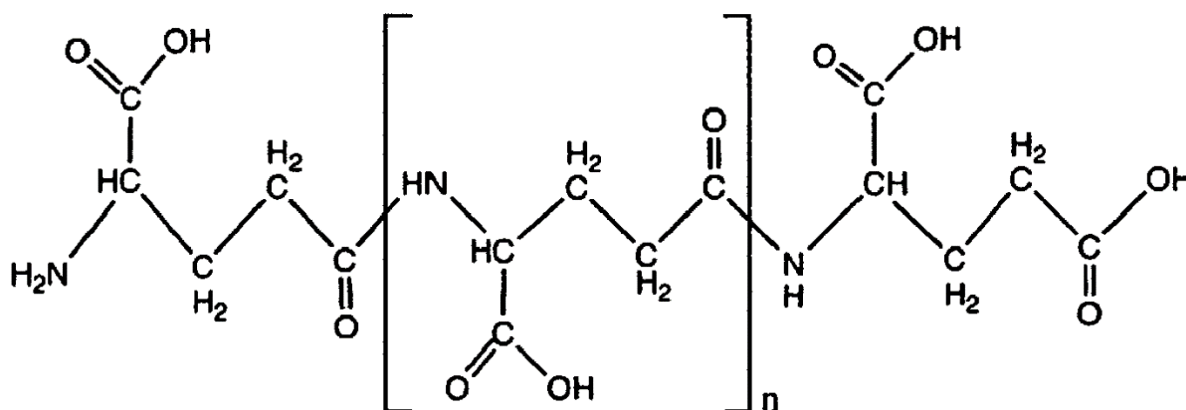


Figure 2-6: The chemical structure of poly-(γ-glutamic acid) (Chen et al., 2013).

This biopolymer is of great interest due to its valuable uses and properties, ranging from biodegradability in food, soluble sutures in medicine, and flocculating properties in water treatment industries. It is non-toxic to both humans and the environment and edible (Shih & Van, 2001; Zhang et al., 2012; Moraes et al., 2013). Its applications include, but are not limited to use as a thickener, humectant, bitterness relieving agent, biodegradable fibre, biopolymer flocculant, heavy metal absorber, sustained-release material, highly absorbable hydrogel and animal feed additive (Shih et al., 2001). Its most widely reported application is its use as a biodegradable bioflocculant. The highly flocculating nature of γ-PGA is particularly valuable in the wastewater treatment space, as it could potentially replace non-biodegradable chemical flocculants such as acrylamide that have possibly damaging health side effects such as being carcinogenic and neurotoxic (Bajaj and Singhal, 2011; Buthelezi et al., 2009; Shih et al., 2001; Wu and Ye, 2007; Yokoi et al., 1995).

The large-scale production is limited by the current cost of its production. It requires high quality substrates of a high concentration in a mono-septic environment. Thus, there is an opportunity to find a low-cost production process that is profitable and industrially logical (Soliman et al., 2005)

γ-PGA can be produced from a range of microorganisms including *Bacillus licheniformis*, *Bacillus anthracis*, *Bacillus megaterium* and *Bacillus subtilis* (Shih & Van, 2001; Potter et al., 2001; Soliman et al., 2005). Most noticeably *B. licheniformis* and *B. subtilis* have shown the highest yields of γ-PGA. γ-PGA is excreted into the bulk culture medium (as well as loosely associated to the biomass) to increase the survival of the strains when exposed to environmental stresses (Soliman et al., 2005). This quality of *Bacillus* is favourable in terms of WWBRs, as it helps in creating the ecological niche environment for the survival of the dominant commodity-product producer.

2.2.3 Production of γ-PGA by species of *Bacillus* in bioreactors

Bioproduction of γ-PGA in bioreactors by various species of *Bacillus* is widely studied in literature. Table 2-3 summaries a few of the studies that have investigated γ-PGA production in different bioreactor types, operational modes (batch, fed-batch and continuous conditions) and conditions. The WWBR bacterial bioreactor will be operated under continuous conditions due to the high volumes of wastewater and not being able to store large flows of incoming wastewater. The studies in Table 2-3 are largely

conventional bioreactors (stirred tank reactors) used to investigate the production of γ -PGA using suspended cell culture conditions, with no biomass retention. Notably, a few less conventional bioreactor configurations (non-CSTR reactors), that have relevance to this research were found and have been included, since the bacterial bioreactor used in WWBR will not be a traditional stirred tank reactor. The results achieved from the various reactor types and knowledge gaps are discussed below.

Jiang et al. (2016) investigated the addition of biomass carriers to a stirred tank reactor (7.5 L Bioflo 115, New Brunswick Scientific) operated under fed-batch conditions with a rich medium (60 g/L glucose, 50 g/L glutamate and 5 g/L $(\text{NH}_4)_2\text{SO}_4$). A high productivity of 1.24 g/L/h and product concentration of 74.2 g/L was achieved; however, a rich medium was used and higher concentrations are expected. In this study, the reactor was classed as a moving bed biofilm reactor (MBBR) however the inclusion of an impeller under aerated conditions is atypical for the MBBR unless under anaerobic conditions (van Haandel & van der Lubbe, 2012). There were no results presented or a demonstration on whether biomass retention was achieved on the carriers e.g., through SEM imaging of the carriers showing biofilm attachment. The biofilm attachment onto carriers is a critical aspect in achieving biomass retention, to optimise substrate utilisation from dilute streams.

Xu et al., (2014) used cell immobilisation in a novel aerobic plant fibrous-bed bioreactor (APFB) under fed-batch conditions to achieve improved γ -PGA productivities and yields compared to the suspended cell batch fermentations. γ -PGA concentrations in the batch phase were 32.9 ± 0.6 g/L with a productivity of 0.456 ± 0.002 g/L/h. The repeated fed-batch fermentations achieved significantly higher γ -PGA concentrations at 71.2 ± 0.83 g/L and 1.25 ± 0.008 g/L/h. The increased performed from batch to fed-batch demonstrated the value in operating in fed-batch, over batch, as well as the impact that cell retention has on increasing productivities. Xu et al. (2014) also noted that during the repeated fed batch fermentations, the lag time was decreased as the retained biomass reseeded when fresh medium was replenished. This finding is significant for WWBR applications

Kumar & Pal (2015) investigated operating a conventional continuous stirred tank reactor (CSTR), with an integrated membrane-based downstream product separation and purification to concentrate the γ -PGA. Biomass was separated from media through microfiltration, and cell recycling was incorporated to increase cell concentration in the bioreactor. The microfiltrate permeate underwent additional ultrafiltration, to concentrate γ -PGA and recycle permeate still containing sucrose, back to the reactor. The pH of the fermenter was monitored, but not controlled through acid/base addition. Dilution rates of 0.025 to 0.4 h^{-1} with cell recycle and γ -PGA concentration steps were incorporated. The maximum productivity (1.2 g/L/h) was associated with the higher dilution rate of 0.4 h^{-1} .

Limited literature was found on the continuous bioproduction of γ -PGA by *Bacillus* species in a conventional CSTR, or other reactor types other than the studies already discussed in this section. For the remainder of the studies reported in Table 2-3, STRs were operated with no biomass retention.

Operation in fed-batch mode (Jiang et al., 2016; Tang et al., 2015; Xu et al., 2014) showed consistently higher productivities (0.81 g/L/h to 1.25 g/L/h) and γ -PGA concentrations (66.4 g/L to 74.2 g/L), except for Kongklom et al. (2017) who reported lower fed-batch concentrations at 27.5 g/L and 39.9 g/L. This is likely due to several factors, including the use of lower carbon concentration (<20 g/L), use of *Bacillus licheniformis* vs *Bacillus subtilis* whereas the other fed-batch studies utilised richer medium such as Yeast Extract and L-glutamic acid/glutamate and higher glucose concentrations

The studies also all operated at temperatures in the range of 32°C to 37°C and at substrate concentrations that are not considered dilute in nature (relative to what would be seen in wastewater treatment). An observation on the temperature in relation to the γ -PGA concentration and γ -PGA Productivity is that lower temperatures out of the studies assessed (32°C), consistently showed higher γ -PGA rates despite the operation mode. Publications investigating lower temperatures ($<32^\circ\text{C}$) were not found at the time of this review.

Table 2-3: Literature examples of γ -PGA production in varying bioreactor types, operation modes and different substrates.

<i>Bacillus</i> Strain	Substrate Nutrients	Operation Mode	Reactor Type	Reactor Conditions (Temp, pH)	γ -PGA concentration (g/L)	γ -PGA Productivity (g/L/h)	μ (h^{-1})	Reference
<i>Bacillus licheniformis</i> NCIM 2324	Glutamic acid Citric acid NH ₄ Cl	Continuous	CSTR	35 °C pH 5.0 - 7.0	36.5 32.4 30.2	0.91 0.97 1.2	0.025 0.03 0.04	Kumar & Pal, (2015)
<i>Bacillus subtilis</i> NX-2	Glucose Glutamate (NH ₄) ₂ SO ₄	Fed-Batch	MBBR	32 °C pH 7.0	74.2	1.24	-	Jiang et al. (2016)
<i>Bacillus subtilis</i> NX-2	Rice straw hydrolysates Glutamate (NH ₄) ₂ SO ₄	Fed-batch	STR	32 °C pH 7.0	73.0	0.81	-	Tang et al. (2015)
<i>Bacillus subtilis</i> NX-2	Glucose Glutamate Yeast Extract	Fed-Batch	APFB	32 °C pH 7.0	71.2	1.25	-	Xu et al. (2014)
<i>Bacillus subtilis</i> NX-2	Glucose Glutamate Yeast Extract	Fed-Batch	APFB	32 °C pH 7.0	66.4	0.98	-	Xu et al. (2014)
<i>B. licheniformis</i> TISTR 1010	Glucose Citric Acid NH ₄ Cl	Fed-Batch	STR	37 °C pH 7.4	39.9	0.93	0.11	Xu et al. (2014)
<i>B. licheniformis</i> TISTR 1010	Glucose Citric Acid NH ₄ Cl	Fed-Batch	STR	37 °C pH 7.4	27.5	0.29	0.06	Kongklom et al. (2015)
<i>B. subtilis</i> ZJU-7	L-Glutamic acid Glucose Tryptone	Batch	STR	37 °C (ND)	54.0	0.84	-	Chen et al. (2010)
<i>B. subtilis</i> NX-2	Glutamate Glucose (NH ₄) ₂ SO ₄	Batch	STR	32 °C pH 7	40.5	0.56	0.26	Zhang et al. (2011)
<i>B. subtilis</i> CMGCC 0833	L-Glutamic acid (NH ₄) ₂ SO ₄ DMSO Glycerol	Batch	STR	32.5 °C pH 7	34.4	0.72	-	Wu et al. (2008)

Bacillus Strain	Substrate Nutrients	Operation Mode	Reactor Type	Reactor Conditions (Temp, pH)	γ -PGA concentration (g/L)	γ -PGA Productivity (g/L/h)	μ (h ⁻¹)	Reference
<i>Bacillus subtilis</i> NX-2	Glucose Glutamate Yeast Extract	Batch	STR	32 °C pH 7.0	34.0	0.72	-	Xu et al. (2014)
<i>B. licheniformis</i> WBL-3	Glycerol Citric Acid L-Glutamic Acid NH ₄ Cl	Batch	STR	37 °C pH 6.5	29.4	0.33	0.08	Du et al. (2005)
<i>B. subtilis</i> RKY3	L-Glutamic acid Glucose Citric acid NH ₄ Cl	Batch	STR	38 °C (ND)	28.4	0.59	0.28	Jung et al. (2005)
<i>B. licheniformis</i> ATCC 9945 A	L-Glutamic Acid Glycerol Citric Acid NH ₄ Cl	Batch	STR	37 °C pH 6.5	23.0	0.48	0.11	Cromwick et al. (1996)
<i>B. subtilis</i> IFO 3335	L-Glutamic acid Citric acid Glycerol (10 g/L) (NH ₄) ₂ SO ₄	Batch	STR	37 °C pH 7	23.0	0.77	0.18	Richard & Margaritis, (2003)
<i>B. licheniformis</i> TISTR 1010	Glucose Citric Acid NH ₄ Cl	Batch	STR	37 °C pH 7.4	3.4	0.11	0.06	Kongklom et al. (2015)

2.2.4 Production of γ -PGA from isolates obtained from South African municipal wastewater

Madonsela (2013) obtained 18 isolates from an activated sludge sample from the Mitchell's Plain wastewater treatment plant in Cape Town, South Africa. Through Polymerase Chain Reaction (PCR) analysis, six isolates were identified as carrying γ -PGA synthesis potential. A reference organism, *Bacillus licheniformis* JCM 2505 was used as a base case comparison throughout the preliminary screening studies. Based on the media optimisation studies conducted by Madonsela (2013), Isolate 1 (identified as *Bacillus subtilis* strain DmB55, by 16s rRNA gene sequencing) was the most promising organism in terms of growth rates, γ -PGA production and ability to adapt to substrates with lower carbon concentrations. Gravimetric extraction of the polymer, cultivated in shake flasks for 48 hours in standard (un-optimised) MME, yielded 22.5 g/L of biopolymer, which contained fractions of carbohydrates and proteins, also precipitated out from ethanol extraction.

Finding the appropriate media that adequately supports cell growth, as well as stimulate γ -PGA production is essential. Appropriate carbon sources for cell growth and γ -PGA production have been found to be glucose, glycerol, ethanol, maltose and starch (Li et al., 2009). Nitrogen sources that were suitable were found to be yeast extract, urea, ammonium chloride and tryptone (Chen et al., 2010; Cheng et al., 1989). Providing metabolic pre-cursors to the TCA cycle of bacteria, increases both cell growth as well as offering maximal γ -PGA yield, and this can be achieved by addition of citric acid L-glutamine, L-glutamic acid and α -ketoglutaric acid (Bajaj & Singhal, 2010). There are some species of bacteria that are L-glutamic acid independent, and do not require its addition to the medium for stimulation of γ -PGA production. A few of these strains are listed in Table 2-3 and include *B. licheniformis* TISTR 1010 which was grown on glucose, citric acid and ammonium chloride (Kongklom et al., 2015); *Bacillus subtilis* NX-2 which grew on glucose as a carbon source and yeast extract as the carbon source (Xu et al., 2014). Ito et al. (2009) grew *Bacillus subtilis* TAM-4 on different sources of sugar (glucose, fructose, maltose etc) and ammonium salts as the nitrogen source and were able to product up to 22.1 g/L of γ -PGA.

Madonsela (2013) performed media optimisation studies in multi-well plates to optimise the concentration of glucose, glycerol and citric acid, in order to assess what isolate achieved best growth by lowering carbon source concentration. The chemicals that were optimised between standard MME and Optimised MME (Table 2-4) were the glucose, glycerol and citric acid and ammonium chloride. The remainder of the salts stayed the same concentration. The results found that the utilisation rates of glycerol were quite low, and thus unnecessary in the medium. In the interest of keeping raw material costs down, glucose was also used in replacement of L-glutamic acid.

Table 2-4: Comparison between standard Medium E and Modified Medium E optimised by Madonsela (2013)

Chemical name	Standard Medium E (ME) concentrations (g/L) Birrer et al. (1994)	Chemical name	Modified Medium E (MME) concentrations (g/L) Optimised by Madonsela (2013)
L-glutamic acid	20	Glucose	20
Glycerol	80	Glycerol	1
Citric Acid	12	Citric Acid	12
Ammonium Chloride	7	Ammonium Chloride	3.48
di-Potassium Hydrogen Phosphate	1.5	di-Potassium Hydrogen Phosphate	3
Magnesium Sulphate	0.5	Magnesium Sulphate	0.5
Manganese Sulphate	0.104	Manganese Sulphate	0.104
Ferric Chloride	0.04	Ferric Chloride	0.04
Calcium Chloride	0.15	Calcium Chloride	0.15

The growth rates and characteristics are outlined in Table 2-5, of the two short-listed isolates that under different culturing modes. An overall biomass yield of 1.36 g_x/g_s was given for Isolate 1 using glucose as the limiting carbon substrate in the batch cultivation Sixfors® bioreactors. γ -PGA was quantified by Madonsela (2013) using the standard Medium E (Table 2-4) in shake flasks, and only for Isolates 1 and

7. Isolate 1 produced a concentration of 22.5 g/L and Isolate 7 was 19.3 g/L. The crude polymer was extracted using ethanol precipitation method.

Table 2-5: Performance of top two isolates and reference organisms cultivated shake flasks for 48 hours, in Modified Medium E by Madonsela (2013).

Organism	Medium	PGA (g/L)	Biomass (g/L)	OD ₆₀₀	$\mu_{\max}$ (h ⁻¹)	Experiment mode
Isolate 1 (<i>Bacillus subtilis</i>)	Optimised MME	No data given	7.85	5.42	0.287	Microtitre plates
Isolate 7 (<i>Serratia</i> sp.)	Optimised MME	No data given	8.74	6.03	0.224	Microtitre plates
Isolate 1 (<i>Bacillus subtilis</i>)	Optimised MME	No data given	8.38	5.78	0.143	Batch cultivation Sixfors® bioreactors

Madonsela (2013) also performed a short cultivation of Isolate 1 in continuous mode in the Sixfors® bioreactors and reported a steady state cell concentration of 25 g/L at a dilution rate of 0.115 h⁻¹, 20 g/L at a dilution rate of 0.124 h⁻¹ and 5.3 g/L at a dilution rate of 0.136 h⁻¹. The results of the media optimisation study by Madonsela (2013) which showed that Isolate 1 and Isolate 7 grew well in lower carbon substrates. Isolate 7 was ruled out as the selected organism due to it being an opportunist pathogen and has been known to cause illness amongst livestock (Madonsela, 2013). Based on the pathogen nature of Isolate 7, the ability of Isolate 1 to grow well on lower carbon substrates and that it produced higher γ -PGA concentrations, it was selected as the organisms for use in this study.

2.2.5 Research motivation on selection of γ -PGA from *Bacillus*

The selection of a bioproduct to demonstrate the feasibility of the WWBR concept, needs to ensure that the core function and value of the WWBR is proven, through the sufficient removal of nutrients and conversion to the desired products, that have potential value (either economically, environmentally and/or socially). The value of γ -PGA as a product is a well-studied and researched biopolymer on richer medias and in ideal reactor conditions. However, the study of bioproduction of γ -PGA on more dilute medias and reactor technologies that differ from traditional STRs has only scarcely been researched at the time of conducting this research. The studies reviewed in 0 showed that through implementing biomass retention and operating modes such as fed-batch or continuous, improvement in productivities and γ -PGA concentrations could be achieved. There was limited knowledge generation or publications available on bioproduction at room temperature conditions (or conditions that are minimally temperature controlled), as well as reactors utilising biofilm retention or development under continuous and dilute conditions. This provides a clear opportunity for this research to contribute in a new way to the knowledge generation around wastewater biorefineries and their real-world demonstration.

By selecting a *Bacillus* species isolated from a South African municipal wastewater plant with proven capabilities to produce γ -PGA, there is a unique opportunity to demonstrate both the feasibility of the WWBR as well as the ability to investigate the production of valuable bioproducts under 'non-ideal' conditions and in a different type of reactor technology and operating mode.

2.3 Research Objectives

Wastewater biorefineries are designed and optimised with the aim of ensuring effective treatment of water to the required standards and converting components removed to valuable bioproducts. This is achieved through careful reactor design and selection to accommodate the dilute and variable feedstocks in a high flow continuous system. The bacterial bioreactor, where the primary bioproduction occurs, needs to ensure that biomass retention is achieved and that bioproduction is achievable under non-sterile and varying conditions. The product should support the niche microbial environment provided by the selected reactor technology. Through identifying the need for reactor design and

selection, and identifying γ -PGA as a suitable bioproduct to demonstrate the bioreactors requirements, the following aims and objects, key questions and hypothesis of this research are presented:

2.3.1 Aims and Objectives

1. Identify the bioreactor configuration/technology that meets the key requirements of a bacterial bioreactor in WWBR application.
2. Assess the performance of Isolate 1 (*Bacillus subtilis*), previously isolated from a WWTP, and provide kinetic results and yields to assist with the process design.
3. Explore the biofilm formation of Isolate 1 on the carrier matrix to facilitate biomass retention in the selected reactor.
4. Explore varying dilution rates and thereby the productivity and performance of the selected reactor
5. Demonstrate the proof of concept that though selecting a reactor technology that meets the WWBR bioreactor requirements, bioproduction is possible.

2.3.2 Key Questions

1. What are the kinetic and yield data for *Bacillus subtilis* (Isolate 1) under room temperature operation in a STR and how do these compare at 37°C?
2. Can a reactor technology that is currently used in wastewater treatment plants be found that adequately fulfils the WWBR bioreactor selection criteria of decoupling solid and hydraulic residence times through biomass retention?
3. Can adequate and active biofilm attachment be demonstrated using Isolate 1?
4. What is the relationship between γ -PGA productivity and substrate utilisation rates at room temperature and non-sterile operating conditions, in a continuously operated lab-scale bioreactor with biomass retention?
5. Can the role of biomass retention be demonstrated at dilution rates $> u_{\max}$ (critical dilution rate) for Isolate 1?

2.3.3 Hypothesis

It is hypothesised that the achieving biomass retention and biofilm development using Isolate 1 will be demonstrated and thereby demonstrate the importance of decoupling solid and hydraulic residence times to achieve bioproduction at high flowrates.

It is hypothesised that bioproduction of γ -PGA in a reactor technology, that meets the requirements for bioreactor selection for use in a WWBR, will achieve increasing productivities when operated at increasing dilution rates (beyond the critical dilution rate) due to the formation of biofilm and biomass retention.

3 Materials and Methods

This chapter outlines the selection of the organism of choice, the materials used and the experimental methods as well as the biological and chemical analyses used across the dissertation. The detailed reactor studies including both the reactor design, configuration and operation are presented in detail in their respective chapters.

The chapter ends with a description of the experimental approach, consisting of three main sections. The results on growth kinetics of *B. subtilis* under sterile, ideal conditions in a STR in batch and fed-batch operation at different temperatures are reported in Chapter 4. The selection of the MBBR as a suitable reactor design for WWBR is detailed in Chapter 5 and the growth of *B. subtilis* under non-sterile, continuous operation in the MBBR at room temperature is reported in Chapter 6.

All the results datasets with data, calculations and graphs for this dissertation can be found at the following links: <https://figshare.com/s/214e568bf25433e9b781>; Public DOI: [10.25375/uct.14294393](https://doi.org/10.25375/uct.14294393)

3.1 Cultivation of the microorganism

3.1.1 *Bacillus subtilis* as the organism of choice

Madonsela (2013) isolated a range of species from a WWTP and demonstrated that Isolate 1 (identified as *Bacillus subtilis* by 16s rRNA gene sequencing) was the most promising organism in terms of growth rates, γ -PGA production and ability to adapt to substrates with lower carbon concentrations (Section 2.2.4). It was decided to proceed with Isolate 1 (*Bacillus subtilis*) as the organism of choice for this study.

3.1.2 Medium Preparation

Two media were used to cultivate Isolate 1 (*Bacillus subtilis*). A complex medium was used during the initial stages of the inoculation train to achieve high biomass concentrations in the inoculum. The culture was inoculated into a defined minimal medium (modified MME) in the latter part of the inoculum train and for the reactor studies.

Complex medium preparation

Tryptone soy broth (TSB) (Sigma-Aldrich, RSA) was used at a concentration of 30 g/L as a pre-inoculum media during the initial steps (Step 1a – Step 2 in Figure 3-1) of the inoculation train. Tryptone soy agar (TSA) plates were made by addition of 15 g/L of bacteriological agar to liquid TSB.

Chemically defined medium – “Modified MME”

The optimised Modified Medium E (MME) used by Madonsela (2013) and, was used at full strength in the inoculation train and half strength in the reactor studies, the latter to investigate the productivity and growth of Isolate 1 at lower substrate concentrations to mimic lower concentration wastewaters as recommended by Madonsela (2013).

All chemicals, except glucose, were combined and dissolved in distilled water, resulting in a final pH of between 2.7 and 3.2. The pH was adjusted to 6.50 ± 0.1 using NaOH before autoclaving. The glucose solution was prepared separately in a Schott and autoclaved separately to the other chemicals to prevent the browning Maillard reaction. It was added aseptically to the remaining media, after autoclaving.

Table 3-1: Composition and concentration of media used in this study and optimised concentrations of MME studied by (Madonsela, 2013).

Chemical name	Chemical Formula	Full Strength Concentration (g/L) (Madonsela 2013)	Half Strength Concentration (g/L) (Used in this study)	Supplier (Used in this study)
Glucose	C ₆ H ₁₂ O ₆	20	10	Sigma Aldrich
Glycerol	C ₃ H ₈ O ₃	1	0.5	Merck
Citric Acid	C ₆ H ₈ O ₇ .H ₂ O	12	6	Merck
Ammonium Chloride	NH ₄ Cl	3.48	1.74	Merck
di-Potassium Hydrogen Phosphate	K ₂ HPO ₄	3	1.5	Merck
Magnesium Sulphate	MgSO ₄ .7H ₂ O	0.5	0.25	Merck
Manganese Sulphate	MnSO ₄ .H ₂ O	0.104	0.052	Associated Chemical Enterprises
Ferric Chloride	FeCl ₃ .6H ₂ O	0.04	0.02	Merck
Calcium Chloride	CaCl ₂ .2H ₂ O	0.15	0.075	Merck

3.1.3 Inoculation train used in cultivations

In the inoculation train (Figure 3-1), a 1500 µL vial of 50% glycerol stock of *Bacillus subtilis* was used to inoculate (750 µL) two 10 mL volumes of sterile Tryptone Soy Broth (30g/L) in duplicate 125 mL Erlenmeyer flasks and incubated at 37°C for 24 hours (Step 1a). A loopful of the culture from each flask, was then streaked onto a TSA plate and cultured for 8-10 hours at 37°C, to prevent overgrowth of mucoidal colonies on the agar plate (Step 1b). A sterile wire loop was used to transfer an individual colony from the agar into two 50 mL volumes of Tryptone Soy Broth in duplicate 250 mL Erlenmeyer flask and cultivated at 37°C and 200 rpm for 24 hours (Step 2). An aliquot of each culture was transferred into 1000 mL Erlenmeyer flasks containing 250 mL modified MME to achieve an initial OD_{600nm} of 0.1 at 600 nm. This pre-inoculum culture was cultivated for 24 hours at 200 rpm and 37°C (Step 3). An aliquot of this culture (calculated using the dilution equation) was transferred to 2000 mL Erlenmeyer flasks containing sterile 400 mL MME to achieve an initial OD_{600nm} of 0.1 (Step 4). This seed culture, after growth for 24 hours at 37°C and 200 rpm was used to inoculate the STRs in Chapter 4.

For the inoculation of the MBBR in Chapter 6, 5000 mL Erlenmeyer flasks containing 2000 mL sterile MME were inoculated from the previous step (Step 4) with a starting OD_{600nm} of 0.1 and cultivated for 24 hours at 37°C and 200 rpm. Each step of the inoculation train was performed in duplicate flasks and checked for purity under the microscope. Gram stains were performed before the final step (Step 4/Step 5), prior to inoculation of the reactor, to ensure the inoculum was a pure culture of gram-positive rods. The MBBR (7 L total volume) was inoculated with 4 L of pure culture to provide a strong inoculum to ensure the dominance of *Bacillus subtilis* in the non-sterile operation.

Figure 3-1: Summary of the inoculation strategy used to cultivate the organism prior to reactor inoculation

Step 1a	Step 1b	Step 2	Step 3 Pre-Inoculum	Step 4 (CSTR) Inoculum	Step 4 (MBBR) Inoculum
 2 x 125 mL flasks 37°C 200 rpm 24 hours	 2 x TSA Plates 37°C Static 8-10 hours	 2 x 250 mL flasks 37°C 200 rpm 24 hours	 2 x 1000 mL flasks 37°C 200 rpm 24 hours	 2 x 2000 mL flasks 37°C 200 rpm 24 hours	 2 x 5000 mL flasks 37°C 200 rpm 24 hours

TSM: 10 mL Inoculum: 1 ml of glycerol stock vial.	Loopful of pure culture Inoculum: from Step 1a streaked onto TSA plates.	TSM: 50 mL per flask. Inoculum: Loopful of pure culture from TSA plates.	MME 1: 200 mL per flask Inoculum: Sufficient to provide starting OD _{600nm} of 0.1	MME 1: 400 mL per flask Inoculum: Sufficient to provide starting OD _{600nm} of 0.1	Full strength MME: 2000 mL per flask Inoculum: Pooled inoculum of 4000 mL into MBBR at start-up. ombined starting inoculum OD _{600nm} of 3
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3.2 Biological analysis methods

3.2.1 Cell Dry Weight (CDW)

Cell dry weight (CDW), or biomass concentration, was measured in triplicate from samples that were taken at discrete sampling times during experiments. Culture was sampled and 2 mL was pipetted into pre-labelled, pre-dried, pre-weighed microfuge tubes and centrifuged at 14000 rpm for 20 minutes (Eppendorf Centrifuge 5418R, Merck, South Africa) The supernatant was pipetted off, filtered with a 0.22 µm cellulose syringe filter and frozen at -20 °C for further analysis (HPLC for substrate utilisation and γ-PGA concentration). The pellet was re-suspended, washed in 1.5 mL of pH 7 sodium phosphate buffer and centrifuged again under the same conditions. The wash buffer was discarded, and the cell pellet and centrifuge tube were dried for 48 hours in an 80°C oven. After allowing to cool to room temperature in a desiccator, the microfuge tubes were weighed using a four decimal place scale (Radwad AS 220.R2, Lasec, South Africa).

3.2.2 Turbidity (optical density)

The turbidity, or optical density (OD), of the cell culture suspension was read using a spectrophotometer (Genesys 10s UV-VIS, Thermo Scientific, South Africa) at a wavelength of 600 nm. Deionised water was used to auto-zero the spectrophotometer, as well as to dilute samples where the OD_{600nm} was over 1.0 absorbance units (AU) to ensure that all readings were between 0.1 and 1.0 absorbance units. The OD_{600nm} values were corrected for the dilution factors. All OD_{600nm} readings of samples were measured in triplicate, with the average and standard deviation being reported. The spectrophotometer was switched on for a minimum of 30 minutes prior to the absorbances being read to ensure the fluorescent bulb was sufficiently warmed up.

3.2.3 Gram staining

Gram staining was performed (Cowan & Steel, 1965) prior to all reactor inoculations to ensure that the inoculum was a pure culture of gram-positive, rod-shaped bacteria. The Burke modification to the method was used (Burke, 1922). A small volume of culture (± 10 µL) was added to a microscope slide and spread thinly and evenly with a sterile wire loop. The culture was then heat-fixed by passing it through a flame in a circular motion to ensure even heating. The culture was stained with crystal violet (10 – 60 seconds), iodine (10 – 60 seconds), decolourising solution until no further colour was observed (± 5 seconds) and counterstained with fuchsin solution (40 – 60 seconds). After each step, the slide was gently rinsed with deionised water. The slides were viewed under 100 times magnification with immersion oil (model no. BX40, Olympus Optical Company Ltd, Japan). The detailed methodology and reagent preparation is outlined in the CeBER Methods Manual (CeBER, 2018).

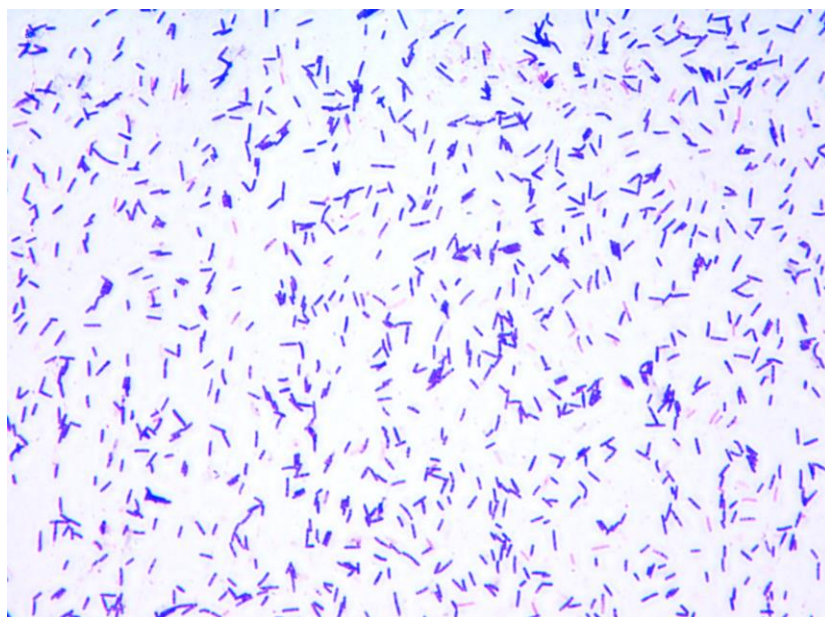


Figure 3-2: Microscope image under 100 x magnification of gram-positive *Bacillus subtilis* in MME.

3.2.4 Scanning Electron Microscope (SEM) Imaging of MBBR Carriers

Standard protocol for the preparation of the samples for SEM was followed (See Appendix 7.4A.1.1). The attachment of the cells on the carriers was fixed by placing them in 2.5 % glutaraldehyde in the fridge at 4 °C for at least 8 hours. They were then rinsed with pH 7 sodium phosphate buffer and then rinsed again with distilled water. Thereafter the dehydration process was carried out, by soaking the carriers in increasing concentrations of ethanol, consisting of 30 %, 50 %, 70 %, 90 %, 95 % and 100 %, for 10 minutes at each concentration. A small section of the carrier was carefully cut out, mounted onto an aluminium stub and covered in carbon glue. The stubs are then sputter-coated with gold palladium alloy. SEM images were captured at varying magnification ranges from 2000 times to 45000 times (FEI Nova Nanosem 230 with field emission gun).

3.2.5 Crude γ -PGA concentration by ethanol precipitation

Crude γ -PGA was precipitated from the thawed supernatant of cultivation samples by pouring it into 3 volumes of cold absolute ethanol into pre-labelled, pre-dried and pre-weighed microfuge tubes. The precipitate was recovered by centrifugation at 14 000 rpm and 4°C (Eppendorf Centrifuge 5418R, Merck, South Africa) and placed with microfuge lids open at 37 °C to allow excess ethanol to evaporate for 24 to 48 hours. The precipitate was dried in an oven at 80°C for 48 hours and cooled in desiccator for a minimum of 1 hour before weighing. This method was used to report crude γ -PGA concentrations in the continuous MBBR experiments.

3.3 Chemical analysis methods

Planktonic cells were removed by centrifugation, immediately after sampling. The supernatant filtered through a 0.22 μ m cellulose syringe filter and stored at -20°C for further testing by HPLC and γ -PGA analysis (crude and UV-Spec assay). The following analyses were performed on the thawed supernatant of samples from all experiments.

3.3.1 HPLC for substrate utilisation

All samples were analysed for substrate utilisation of glucose, glycerol and citric acid using a High-Pressure Liquid Chromatography (HPLC) system (Thermo Scientific ASC3000) with UV-Vis at 210 nm (Thermo Scientific, Spectra System UV1000) and Refractive Index (Thermo Scientific, RefractoMax52) detectors. The latter was used to quantify the concentration of glucose and glycerol and UV-VIS was

used for the quantification of citric acid. Samples were loaded onto a Biorad Aminex[®] HPX-87H ion exclusion column (7.8 mm x 300 mm). The operating parameters are detailed in Table 3-2 below.

Table 3-2: Summary of operating parameters for quantification of glucose, glycerol and citric acid by HPLC.

Operating Parameters	Setpoint
Mobile phase	5 mM H ₂ SO ₄
Column oven temperature	65°C
Sample injection volume	10 µL
RI detector operating temperature	55°C
Mobile phase flowrate	0.3 mL/min
Sample run time	55 min

A stock solution consisting of 10 g/L glucose, 6 g/L citric acid and 0.5 g/L glycerol was made up and diluted to known concentrations. Standard curves for each analyte were generated using Thermo Scientific[™] Chromeleon[™] 7.2 Chromatography Data System (CDS) software. The software was setup to determine the concentrations of the analyte in the samples based on the standard curves automatically generated. An example of the standard curve generated is provided in the corresponding datasets for each relevant chapter accessed here: A standard curve for each analyte was generated prior to each HPLC run. HPLC was only conducted on one sample for each sampling run due to the time constraints on the HPLC equipment, and the necessity to run each vial for 55 min. Detailed methodology regarding the sample, standard preparation and operating procedure are outlined in Appendix A2.1.

3.3.2 UV-Spectrophotometer method for γ -PGA identification and quantification

Due to the potential simplicity, rapidity and availability of equipment and low-cost raw materials, it was decided to investigate and adapt the method described by Zeng et al. (2012) for the identification and quantification of γ -PGA in the experimental work in this dissertation.

γ -PGA assay verification process

In order to use this method, it was required to show that reproducible, stable and accurate linear standard curves could be obtained. Since not all the raw materials and equipment were identical or available, investigations into the validity and applicability of this method with the available resources were required.

Table 3-3: Comparison between critical raw material and equipment used for development of UV spectrum method

List of raw materials & equipment	Zeng et al., (2012)	This study
Poly- γ -glutamic acid standard	γ -PGA sodium salt (monomer, C ₅ H ₆ NO ₃ Na = 151.3 g/mol Mw = 200-500 kDa; 1500-2500 kDa; 4000-6000 kDa) 98% purity Wako Pure Chemical industries (Japan)	γ -PGA, Mw= 10 – 20 kDa 95 % purity Xi'an Realin Biotechnology Co., Ltd (China)
Spectrophotometer	Multiskan GO UV/vis microplate spectrophotometer (Thermo Scientific, USA)	Genesys 10s UV/vis spectrophotometer (Thermo Scientific, RSA)
Microplate	UVStar 96-well microplate (Greiner Bio-One GmbH, Germany)	Not used
Cuvette	Not used	Hellma 1000 µL quartz cuvette (104.002-QS, 10 mm with lid)

As a starting point, a standard solution of 1 g/L γ -PGA was prepared by dissolving in deionised water (pH fluctuation of deionised water was between pH 6.3 - 7.0). The standard solution was then diluted with deionised water to create concentrations between 0.02 g/L and 0.1 g/L. The absorption spectra was recorded between 190 nm and 390 nm for the different concentrations, in triplicate. The absorbance reaches a maximum of 1.0 at a concentration of 0.1 g/L for this system. Figure 3-3 shows the preliminary scans performed in unbuffered deionised water, where the pH fluctuated between 6.3 and 7.0. This is indicated by the shift in maximum absorbance between the different concentrations.

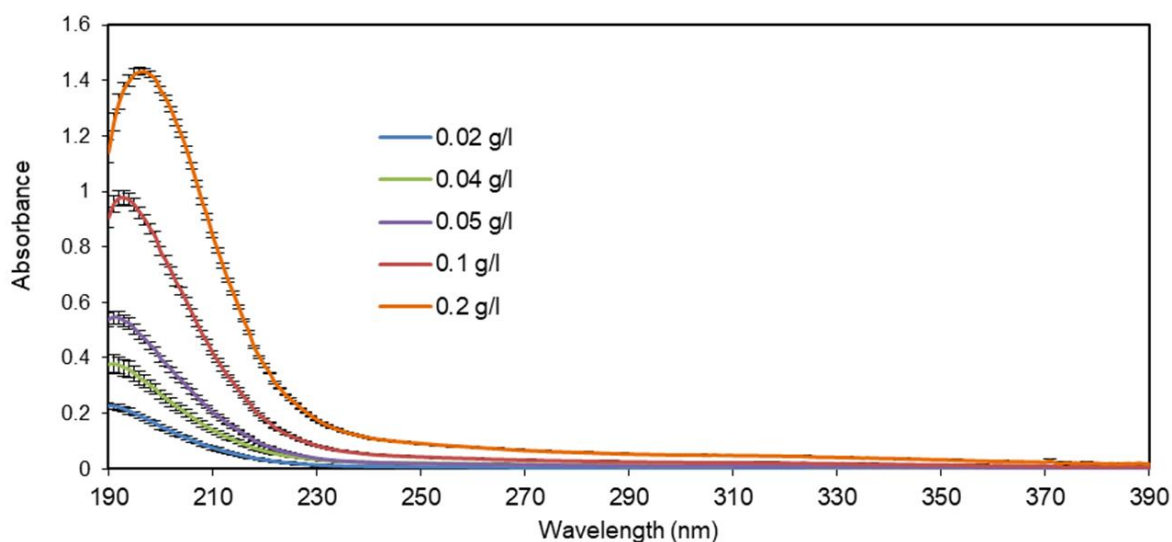


Figure 3-3: Absorption spectrum of PGA in deionised water (pH 6.3 - 7.0).

Since this method is highly pH sensitive, this resulted in it being increasingly difficult to produce a linear calibration curve that was consistent and gave accurate repeatability for sample analyses. Further optimisation of this method was required due to the fluctuation in the pH values of the deionised water.

It was demonstrated by Zeng et al. (2012) that a neutral pH solution (sodium phosphate buffer, pH 7.0) showed the best linearity and R^2 values closest to 1, in comparison to a pH <3 or pH >10, as shown in Table 3-4 and Figure 3-4 below.

Table 3-4: Standard curves at 216 nm generated for γ -PGA in aqueous solutions in pH 3, 7 and 10, where y refers to absorbance and x refers to concentration of standard solution. Adapted from (Zeng et al., 2012).

pH value	Linear fit equation	R^2 value
< 3	$y = 0.0013x + 0.1227$	0.9033
7	$y = 0.0059x + 0.0064$	0.9997
> 10	$y = 0.0041x + 0.0441$	0.9937

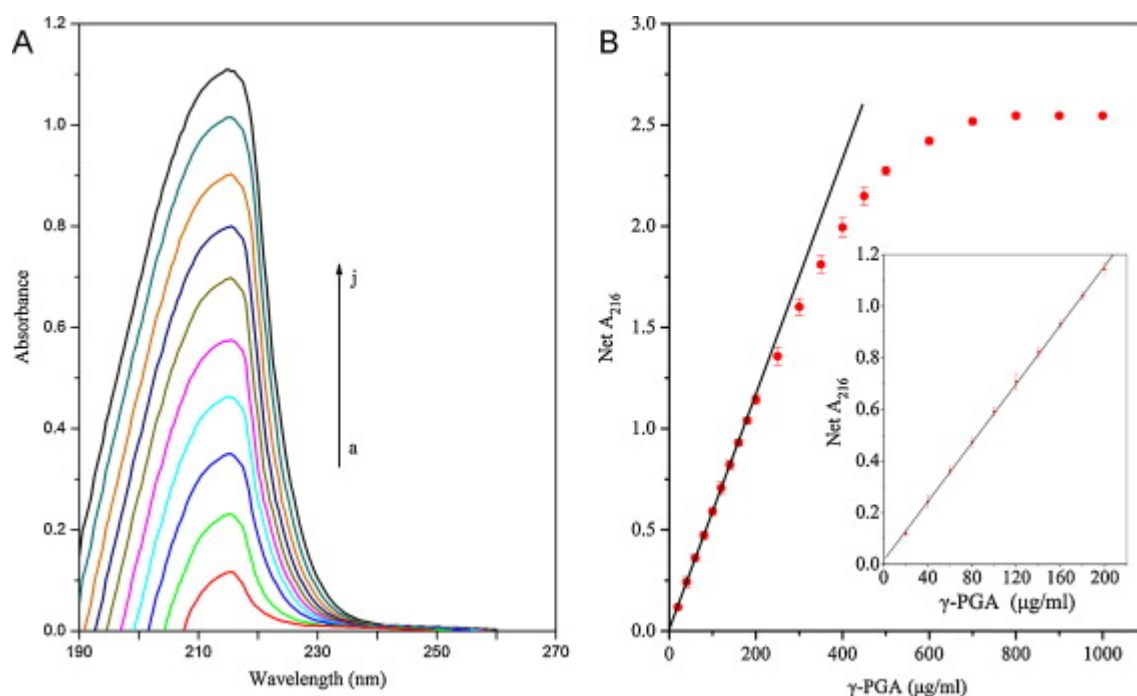


Figure 3-4: (A) UV spectrum absorption and linearity produced by (Zeng et al., 2012) in pH 7 aqueous solution with concentrations of (a) 0.02 g/L, (b) 0.04 g/L, (c) 0.06 g/L, (d) 0.08 g/L, (e) 0.1 g/L, (f) 0.12 g/L, (g) 0.14 g/L, (h) 0.16 g/L, (i) 0.18 g/L, (j) 0.2 g/L. (B) shows linear range up to a concentration of 0.2 g/L.

Based on these results and findings, it was decided to investigate using buffered deionised water as the solution of choice. Sodium phosphate buffer was prepared (see Appendix 7.4A.2.2), and the pH confirmed to be 7.0 ± 0.1 . A stock solution of 1 g/L γ -PGA was prepared in deionised water in a volumetric flask and triplicate serial dilutions made from 0.04 to 0.2 g/L (see Appendix 7.4A.2.2), then filtered through a $0.22 \mu\text{m}$ cellulose syringe filter and scanned in the UV range from 190 nm to 250 nm using a 1.0 mL quartz cuvette. Figure 3-5 shows the full scan of the serial dilutions from a range of 190 to 250 nm, with a maximum peak absorbance at 204 nm (indicated by the dashed vertical line).

The absorbance of each standard dilution at 204 ± 1 nm was plotted against the corresponding concentrations and the curve fitted through zero, to produce a standard curve in Figure 3-5 (B). This was reproduced over different days using freshly prepared buffer and standards to show the reproducibility of the method, by ensuring the slope of the standard curve did not vary significantly.

Further calibration curves were generated to demonstrate inter-day reproducibility using freshly prepared pH 7 buffer solution and standard solution. Figure 3-6 demonstrates three examples of such curves, and the similarity in the slope and R^2 values. Curves 1 to 3 were generated in different months, using the same ultraviolet bulb. Variations in the slope of the curves are hypothesised to be due to the age of the ultraviolet lamp decreasing over time.

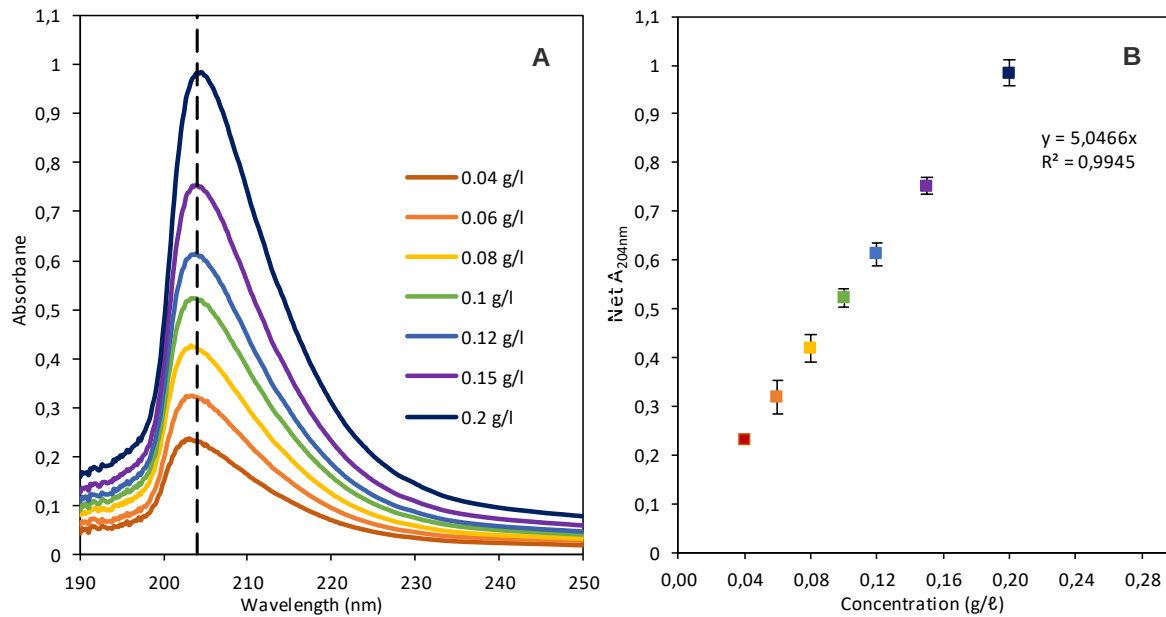


Figure 3-5: (A) UV Spectrum absorption pH 7 phosphate buffer at varying concentrations. The vertical dashed line indicates the maximum absorbance at 204 nm. (B) Linearity of γ -PGA scans from (A) at a maximum absorbance of 204 nm.

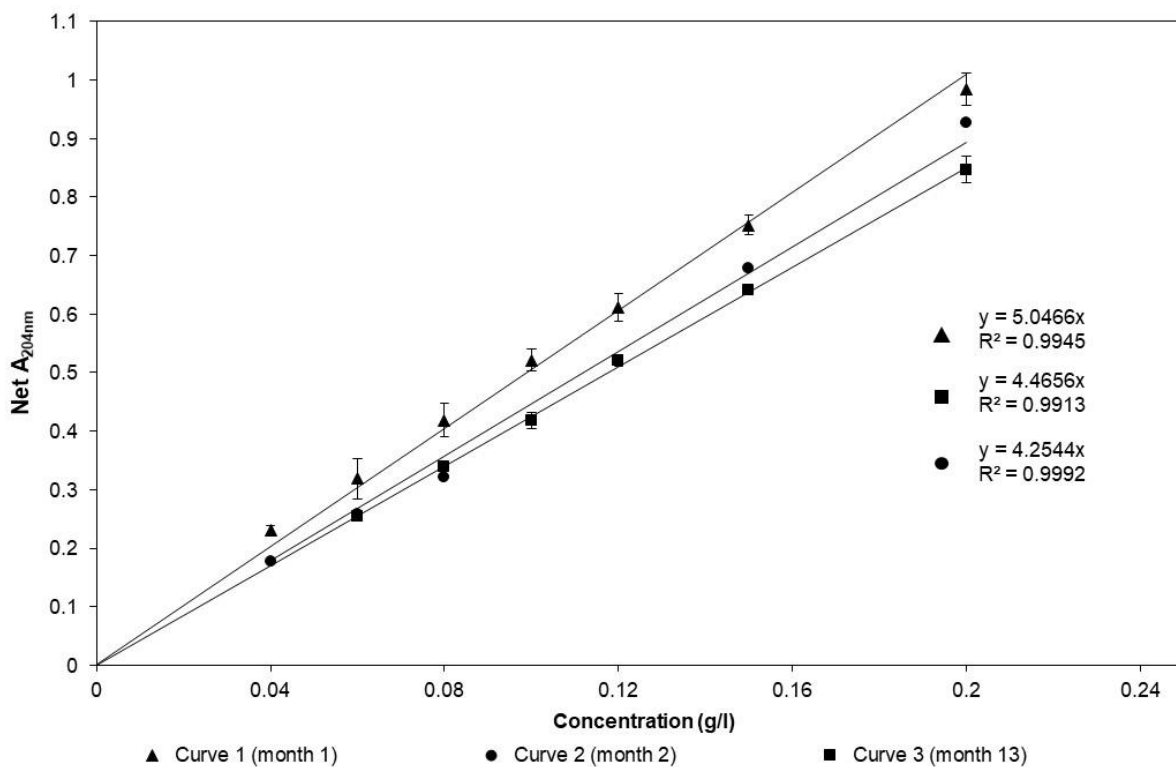


Figure 3-6: Examples of three linear curves generated on different days, in pH 7 aqueous solutions.

Analysis of cultivation samples for γ -PGA using UV-Spec Method

Cultivation samples from the continuous MBBR experiments were analysed for γ -PGA by first centrifuging at 14 000 rpm for 20 min to remove cells. The cell pellet was resuspended and rinsed with 1.5 mL of pH 7 sodium phosphate buffer, and the rinse solution retained to check for remaining γ -PGA. The supernatant was then transferred to a microfuge tube. An aliquot of 1425 μ L of cold absolute ethanol was added to 75 μ L of supernatant, and gently mixed. Samples were centrifuged again at 4°C and 14 000 rpm for 10 min to allow the precipitate to form a pellet. Ethanol was evaporated off by leaving

samples open to atmosphere for up to 48 hours. The precipitate was dissolved in 1.5 mL of pH 7 sodium phosphate buffer and filtered through a 0.22 μm cellulose syringe filter to remove insoluble contaminants. Further dilution of the sample in solution, with pH 7 phosphate buffer was undertaken where necessary to remain within the linear range of the standard curve. This method was used to quantify the presence of γ -PGA in cultivation samples from the continuous MBBR experiments.

3.4 Bioreactor studies

The methodology and operating conditions used for the setup and operation of the STRs (Chapter 4) and MBBR (Chapter 6) experiments, are outlined in the respective chapters.

3.4.1 Mass transfer studies in the MBBR

The static gassing in-out method was used to determine the volumetric mass transfer coefficient (k_La) in the MBBR. These results were used to ascertain the fill percentage of carriers and aeration rate for optimal mass transfer. The oxygen concentration was decreased to zero by sparging with nitrogen. Aeration was then started and the increase in oxygen concentration recorded with a calibrated DO probe (Mettler Toledo InPro 6050/120).

The rate of oxygen transfer (OTR) from air bubbles to the liquid phase can be described by the equation:

$$\frac{dC}{dt} = K_L a (C^* - C) \quad \text{Equation 3-1}$$

where C is the concentration of dissolved oxygen in the liquid phase, t is time, dC/dt is the change in oxygen concentration over time, K_La is the volumetric mass transfer coefficient and C^* is the saturated dissolved oxygen concentration. Integration of Equation 3-1 yields:

$$\ln(C^* - C) = -K_L a t \quad \text{Equation 3-2}$$

Plotting $\ln(C^* - C)$ against time during the gassing in phase yields the straight-line slope of graph B in Figure 3-7, with the slope proving the estimation of the volumetric mass transfer coefficient.

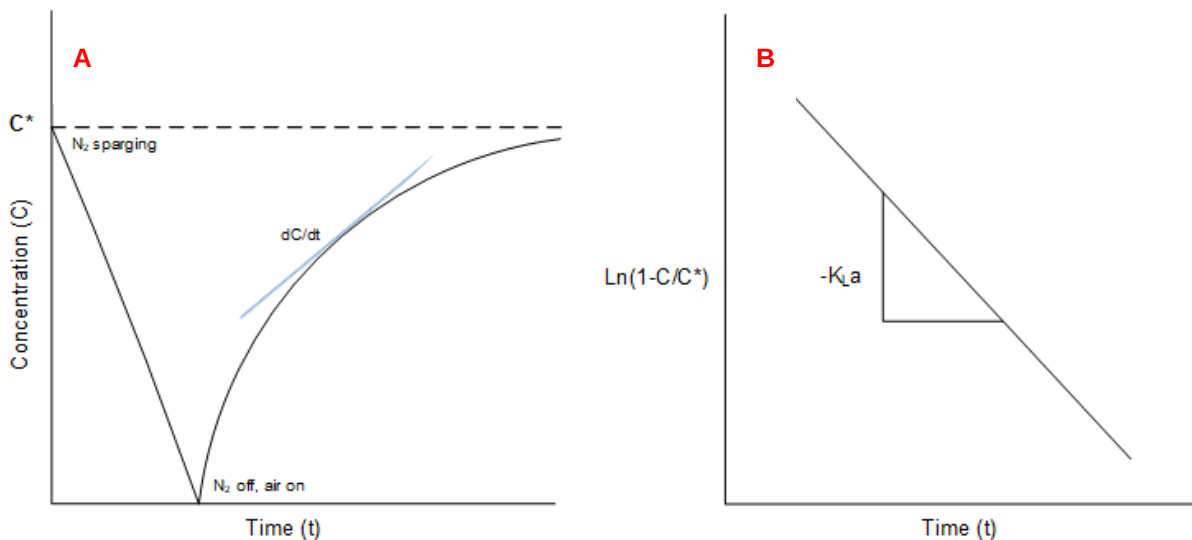


Figure 3-7: Graph A demonstrates the change in dissolved oxygen concentration over time during the gassing out method. Graph B demonstrates the graphical determination of the mass transfer coefficient. Adapted from Doran (1995).

3.4.2 Hydrodynamic studies in the MBBR

To determine the mixing time and patterns of the MBBR, hydrodynamic studies were performed with the optimal carrier fill fraction of 40 %, determined from the mass transfer studies. This was done using the pH indicator phenolphthalein, which is colourless in acidic liquids and pink in alkali liquids. The addition of the indicator allows the visualisation of movement of liquid through the bioreactor to identify dead zones or poorly mixed areas.

The reactor was filled to the working volume of 5.5 litres with dH₂O along with a few drops of phenolphthalein indicator (~2 mL per 2 to 4 litres). 1M NaOH (40 g NaOH pellets in 1 L dH₂O) was added using a pipette dropper until the solution turned bright pink. A solution of 1 M Hydrochloric Acid (96 mL of 32% HCl in 1 L of dH₂O) was pumped into the reactor at various flowrates. The mixing time in the reactor is determined by the time it takes for the solution to turn from pink (alkali pH at 8.2 – 12) to completely clear (pH lower than 8.2). The acid and base addition was repeated for each test in tracer pulse format. Each study was done in duplicate and the mixing time reported as an average of the two.

3.5 Summary of Experimental Approach

The key aim of this thesis was to demonstrate the plausibility of achieving the production of a valuable bioproduct, γ -PGA, from a low concentration feed resembling a wastewater stream while using a reactor technology typical in existing wastewater plants, to achieve both a product of value and treated or partially treated water. The concept was tested on a lab-scale, under non-sterile room temperature conditions. A fast-growing organism, *Bacillus subtilis* (Isolate 1), isolated from a local WWTP and with proven capabilities to produce a biopolymer (γ -PGA) was selected to achieve this aim. This proof of concept is sought as an example of the first stage of a wastewater biorefinery, established to reduce organic loading and some loading of nitrogen at high flowrates, while producing the product of value.

Chapter 4 investigates the base case experiments in a STR using the selected isolate, *B. subtilis*, and optimised MME (with the concentrations of the MME halved) to investigate this organism's ability to handle lower medium concentrations, as demonstrated by (Madonsela, 2013). These experiments were carried out in a 5 L working volume STR in a batch mode at 37 °C initially, and then at room temperature (20-25°C) in batch and fed batch modes. The time-based growth profiles for the batch and fed-batch experiments are presented, and the kinetic data (maximum specific growth rate, overall biomass yield, maximum biomass productivity, overall productivity and overall γ -PGA productivity (fed-batch only) are discussed. The maximum specific growth rates calculated during the batch experiments were used to calculate a range of critical dilution rates for the continuous reactor experiments in Chapter 6.

Chapter 5 builds on the concept of a wastewater biorefinery by outlining the biological reactor requirements for application in a WWBR. A detailed review of existing reactor technologies in South African WWTPs is discussed and the suitability for application in a WWBR is assessed against the requirements. Five of the most suitable reactor technologies were shortlisted and researched in detail. The selection criteria of the reactor technologies were refined into two main categories: design and operational priorities. The five shortlisted reactor technologies were then assessed against the refined criteria, a SWOT analysis performed on the three most suitable technologies and a final reactor technology, the moving bed bioreactor, selected for design, commissioning and 'proof of concept' study.

Chapter 6 investigates the continuous operation of a moving bed biofilm reactor using the same media (half strength MME) and *Bacillus* species (Isolate 1) in Chapter 4, where the RT batch experimental results assisted in the selection of dilution rates used in continuous MBBR reactor. The design and assembly of the reactor was discussed. Hydrodynamics and mass transfer studies were investigated to determine ideal aeration rates and biofilm carrier percentages. The acclimatisation and proof of cell attachment onto the biofilm carriers was proven through SEM imaging. Finally, the inoculation and start-up operation of the MBBR was detailed. The effects of decreasing dilutions rates on productivity, growth rates, substrate utilisation and yields (biomass and crude γ -PGA) were investigated and discussed.

4 Batch and fed-batch cultivation of *B. subtilis* in a traditional STR

The main objective of this chapter was to determine approximate growth kinetics and performance parameters from time-based growth profiles of *B. subtilis* (Isolate 1) under batch conditions (Section 4.5) at different temperatures (37 °C and a lab room temperature range of 19 °C and 23 °C). By investigating growth kinetics of *Bacillus subtilis* under ambient conditions in comparison to mesophilic cell conditions, the potential impact of temperature on growth rates and biomass productivity in the continuous production in the ambient bioreactor for WWBR application can be predicted.

Two fed-batch cultivations (Section 4.6) at room temperature was carried out to further study the growth rates, while feeding with glucose-enriched half strength modified MME feed with the aim of increasing biomass productivity at a constant feed-rate. The results of the calculated maximum specific growth rates during the batch cultivations provide a range of maximum dilution rates (under non-attachment conditions) at which a continuous run could operate, before cell washout occurs.

4.1 Experimental setup and reactor conditions

All cultivations were performed in the 7 L benchtop New Brunswick Bioflo 110 bioreactors (New Brunswick Scientific, USA) at a 5 L working volume. The batch and fed-batch equipment setup are shown in Figure 4-1 and Figure 4-2 respectively. Temperature was controlled using a fitted heating jacket for the 37 °C batch cultivation and cooling coils. The heating jacket was removed for the room temperature batch and fed-batch experiments. The lab temperature range was between 19 °C and 24 °C for room temperature experiments; these mimics fluctuating ambient temperatures in a WWBR application i.e. a reactor exposed to natural elements with limited temperature control. Bioproduction in a WWBR application would occur under fluctuating ambient temperatures.

Half strength modified MME was used for all batch cultivations and is described in Section 3.1.2. The inoculum train described in Section 3.1.3 was followed. The inoculum size was calculated to ensure a starting OD₆₀₀ of 0.10 ± 0.05 to a total working volume of 5 L of culture medium. The parameters for the batch and fed-batch cultivations were kept consistent and are described in Table 4-1.

The reactors were aerated with compressed air at a rate of 0.5 vvm (volume of gas per volume liquid per minute). Dissolved oxygen was controlled by agitation speed (300 – 650 rpm) to maintain DO% at 30% and monitored by means of an internal dissolved oxygen probe (InPro 6100/220/S/N, Mettler Toledo, Ohio, USA). The impeller was fitted with two six-blade Rushton impellers (59 mm in diameter), spaced one impeller width apart. The pH was measured through an internal probe (405-DPAS-SC-K8S120, Mettler Toledo, Ohio, USA) and controlled by automated titration with 5M NaOH. The air was passed through a rotameter and 0.22 µm Millipore Millex® membrane filter (Merck, Modderfontein, RSA) before entering the reactor. Exhaust gases were vented through the condenser, cooled using tap water. The resultant gases were filtered through a depth filter followed by a membrane filter. Antifoam was controlled with the addition of sterile solution of 10% Antifoam 204 (Sigma Aldrich, RSA).

Table 4-1: Summary of cultivation parameters

Working volume	5 L
Agitation	300-650 rpm (cascade control to maintain DO >30 %)
Aeration	0.5 vvm
pH	6.5 ± 0.2
Temperature	37 ± 1 °C (temperature-control); 19 – 24 °C (room temperature)

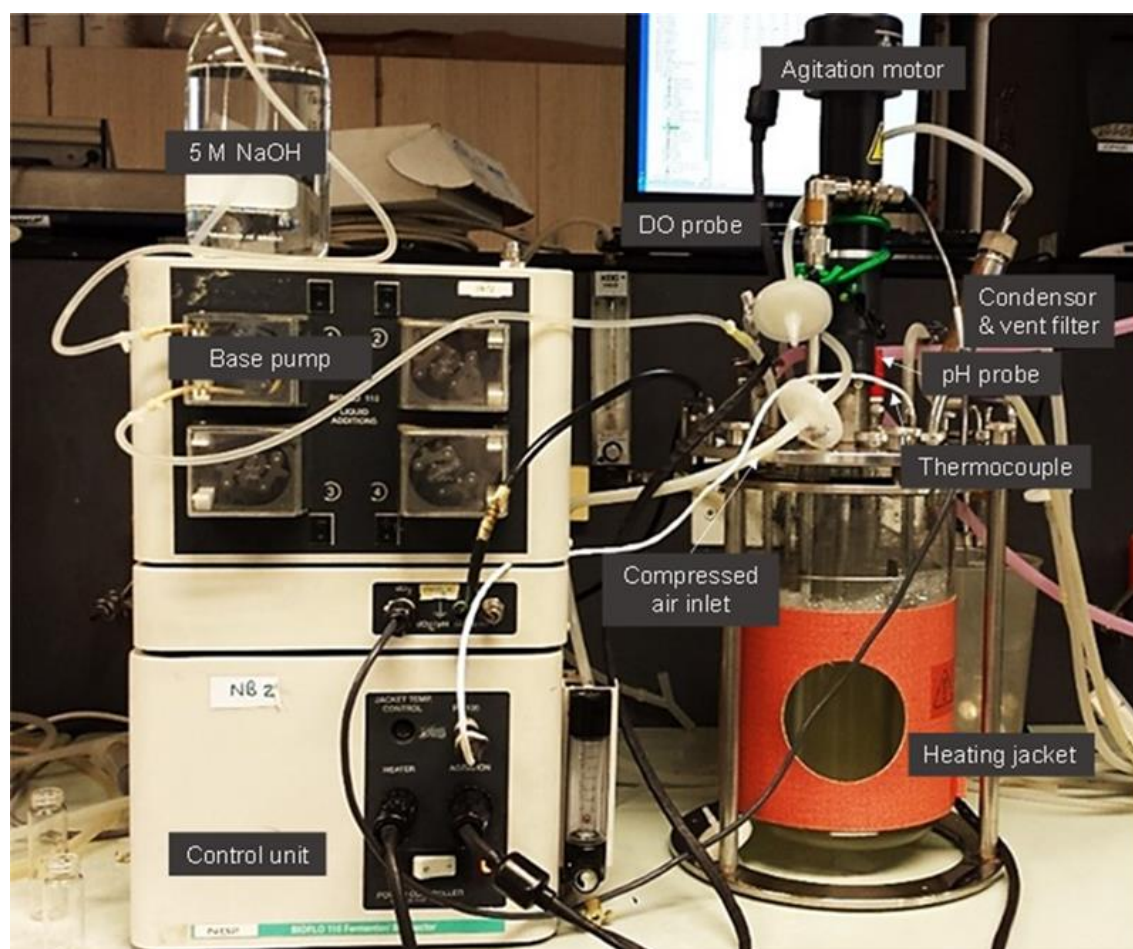


Figure 4-1: Setup of Bioflo 110 New Brunswick bioreactor for batch cultivations



Figure 4-2: Setup of Bioflo 110 New Brunswick bioreactor for fed-batch cultivations

4.2 Fed-batch feeding strategy

The fed-batch cultivation commenced in late exponential growth phase in the batch process by the removal of 2.5 litres of culture through the sample port. A modified, glucose-concentrated MME was pumped into the bioreactor at a controlled rate to match the maximum glucose utilisation rate during the batch face, until a final volume of 5 litres was achieved. The composition of the feed is shown in Table 4-2. The mass of feed and 5M NaOH bottles were recorded independently at each sample point to monitor the change in vessel volume through the addition of reagents.

Table 4-2: Concentration of MME feed used for fed batch cultivations.

Chemical name	Chemical Formula	Feed Concentration (g/L)	Batch Concentration (HS MME) (g/L)
Glucose	C ₆ H ₁₂ O ₆	60.00	10.00
Glycerol	C ₃ H ₈ O ₃	0.3750	0.5000
Citric Acid	C ₆ H ₈ O ₇ .H ₂ O	4.500	6.000
Ammonium Chloride	NH ₄ Cl	5.220	1.740
di-Potassium Hydrogen Phosphate	K ₂ HPO ₄	1.121	1.495
Magnesium Sulphate	MgSO ₄ .7H ₂ O	0.1880	0.2500
Manganese Sulphate	MnSO ₄ .H ₂ O	0.03900	0.05200
Ferric Chloride	FeCl ₃ .6H ₂ O	0.01500	0.02000
Calcium Chloride	CaCl ₂ .2H ₂ O	0.05600	0.07500

The feed-rate was calculated from the rate of glucose addition required to meet the maximum glucose consumption rate during the room temperature batch cultivation, at a volume of 5 litres. The glucose utilisation rate, R_G (g/L/h), of the room temperature batch was calculated as follows:

$$R_G = \frac{S_{G2} - S_{G1}}{t_2 - t_1} \quad \text{Equation 4-1}$$

where amount of residual glucose consumed, S_{Gx} (g/L), is at a maximum. The feed rate, F (mL/h), was then calculated based on the glucose concentration in the feed of 60 g/L ($S_{G,f}$) and where the glucose utilisation rate was at a maximum, R_{Gmax} (g/L/h) and V_R is working batch volume (L):

$$F = \frac{R_{Gmax}}{S_{G,f}} \times V_R \times 1000 \quad \text{Equation 4-2}$$

4.3 Kinetic parameter calculations

The maximum biomass yield coefficients ($Y_{X/S}$) and maximum specific growth rates were determined for the batch and fed-batch bioreactor runs. The calculation formulae are shown in this section, and the results are summarised and discussed in Section 0 and Section 4.6.

Maximum specific growth rates

The maximum specific growth rates, μ_{max} , were calculated based on the Malthus Equation in Equation 4-3

$$\frac{dx}{dt} = \mu x \quad \text{Equation 4-3}$$

Its integrated and linearised form is shown as Equation 4-4:

$$\ln(x) = \mu t + C \quad \text{Equation 4-4}$$

where t is the incubation time, x is the biomass concentration and μ is the specific growth rate. In the exponential growth phase where μ remains constant at approximately μ_{max} , the straight-line slope of $\ln(x)$ as a function of time yields the μ_{max} .

The specific growth rate was calculated between by integrating the simplified Monod equation (Equation 4-3) between same points, x_f and x_i and elapsed cultivation time of the samples, Δt , and demonstrated by Equation 4-5:

$$\mu = \frac{\ln(x_f) - \ln(x_i)}{\Delta t} \quad \text{Equation 4-5}$$

Biomass yield coefficient

Overall biomass yield coefficients $Y_{x/s}$ (g $\times$ /g s), were calculated based on the glucose as limiting substrate:

$$Y_{x/s} = \frac{\Delta X}{\Delta S} \quad \text{Equation 4-6}$$

where X is cell dry weight formed (g) and S is glucose (substrate) consumed (g).

Biomass productivity

The biomass productivity (g/L/h) on a volumetric basis, Q_x , is defined as the volumetric rate of biomass formation. The maximum biomass productivity was approximated by multiplying the maximum specific growth rate with the biomass concentration, where it was at the highest concentration during exponential phase. This is given by given by Equation 4-7:

$$Q_x = \mu_{max} x \quad \text{Equation 4-7}$$

Where μ_{max} is the maximum specific growth rate, and x is the maximum biomass concentration (g/L) during exponential phase.

During the fed-batch phase, volumetric productivity was calculated using the equation used by Borzani (2008) that accounts for the variable volume and its influence on biomass concentrations. This is given by Equation 4-8:

$$Q_x = \frac{x_b V_b - x_a V_a}{V_b \Delta t} \quad \text{Equation 4-8}$$

where X_b and X_a are biomass concentrations (g/L) at t_b and t_a , V_b and V_a are the volumes in the reactor at each corresponding biomass concentration and $\Delta t = t_b - t_a$ is the elapsed time (hours).

Activation Energy

The Arrhenius equation can be used to describe the relationship between temperature and growth rate (Doran, 1995; Shuler & Kargi, 2002):

$$\mu = A e^{\frac{-E_a}{RT}} \quad \text{Equation 4-9}$$

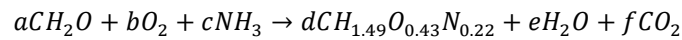
where μ is growth rate in h^{-1} , A is Arrhenius constant, R is universal gas constant ($kJ/mol.K$), T is temperature (Kelvin) and E_a is activation energy for growth (J/mol). Taking the natural logarithm of both sides of Equation 4-9:

$$\ln(\mu) = \ln(A) - \frac{E_a}{RT} \quad \text{Equation 4-10}$$

A plot of $\ln(\mu)$ vs $1/T$ results in a straight-line plot, with slope as $-E_a/R$, from which the activation energy can be calculated. The results of μ_{max} and the average temperature at which μ_{max} was calculated was plotted to calculate the activation energy for the room temperature batch cultivations.

4.4 Elemental balances

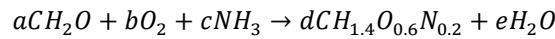
The maximum theoretical biomass and γ -PGA yield coefficients were calculated from growth stoichiometry and elemental balances. With the assumption that the only extracellular products formed were CO_2 and H_2 , the following equation can be written for aerobic cell growth with biomass composition as estimated from a known *Bacillus* species (Duboc et al., 1999), using glucose as a substrate:



The maximum theoretical biomass yield coefficient can be calculated as follows, using a equal to 1:

$$Y_{\frac{x}{s}}^{max} = \frac{d \left(MW \frac{g}{Cmol} \text{biomass} \right)}{a \left(MW \frac{g}{Cmol} \text{glucose} \right)} \quad \text{Equation 4-11}$$

The theoretical maximum yields for product formation can be calculated using the same approach, but with the mass balance equation written to assume that all the carbon source forms only the product, in this instance γ -PGA:



The maximum theoretical yield of γ -PGA can be calculated as follows, using a equal to 1:

$$Y_{\frac{p}{s}}^{max} = \frac{d \left(MW \frac{g}{Cmol} [\text{Glutamic Acid}] \right)}{a \left(MW \frac{g}{Cmol} \text{glucose} \right)} \quad \text{Equation 4-12}$$

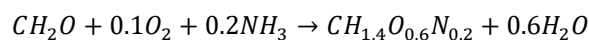
Where the product (Glutamic Acid) is described by the singular glutamic acid monomer (the building block of γ -PGA).

Biomass and product mass balances

The theoretical maximum biomass yields were calculated by performing an elemental balance with the assumption that only biomass is formed, and that the only carbon source used is glucose. The stoichiometric coefficients were calculated by performing carbon, oxygen and nitrogen balances to arrive at the following balanced reaction equation (with biomass composition for a known *Bacillus* species (Duboc, Marison & von Stocar, 1999):



An elemental mass balance was also performed for the production of γ -PGA using glucose as the carbon source and the assumption that γ -PGA was the only carbon containing product:



Based on the balanced equations, the theoretical maximum biomass and product yields were calculated using Equation 4-11 and Equation 4-12 to be 0.29 g_x/g_s and 0.86 g_p/g_s respectively.

4.5 Batch Cultivation Results

4.5.1 Time-based growth profiles

The results of the batch cultivations are shown in Figure 4-5 and Figure 4-6. The 37 °C batch cultivation reached the end of exponential phase after 9 hours, with a maximum OD₆₀₀ of 7.23 ± 0.14 and a corresponding CDW concentration of 3.32 ± 0.25 g/L. The most rapid glucose utilisation occurred towards the end of the exponential phase (high biomass concentration) at 1.43 g/L/h of glucose. After complete glucose consumption, a decrease in the growth was observed indicating that glucose is both the preferred and limiting substrate. Minimal citric acid consumption was observed, with only a slight decrease from 7.5 g/L to 6.5 g/L in the last 2 hours. This finding is consistent with the results for the same isolate presented by Madonsela (2013). The pH was maintained at 6.5 using 5M NaOH. A time-lapse of the 37 °C batch fermentation is shown in Figure 4-3.



Figure 4-3: Time-lapse photographs of 37 °C batch cultivation.

The room temperature batch cultivations were repeated in triplicate. The time-lapse of the batch phase is shown in Figure 4-4 (Fed-batch phase is not shown). The first repeat was initially run as a batch cultivation to determine growth profile at lower temperatures, glucose consumption rates and cultivation time. The second and third repeats consisted of a batch phase (under same conditions as first repeat), followed by a constant feed-rate and variable volume fed-batch phase. The fed-batch phase is discussed in Section 4.6. The room temperature batches all had an acclimation phase of 16 ± 1 hour, before entering exponential growth which continued until 34 ± 1 hours. A maximum OD₆₀₀ of between 5.27 ± 0.45 and 6.23 ± 0.03 and CDW concentration of 2.5 ± 0.15 and 2.73 ± 0.24 g/L respectively, was achieved. Following glucose depletion in room temperature batch repeat 1 (RTB1) at 36.5 hours, growth decreased until the run was terminated at 41 hours. In RTB2 and RTB3, fed-batch phase was started before significant effects of glucose depletion could be observed. The overall average reactor temperature of the room temperature cultivations were 25.4 ± 0.780 °C, 25.7 ± 1.54 °C and 22.2 ± 1.40 °C for the first, second and third repeats respectively, with the largest increase noticed during exponential growth phase due to effects of metabolic heat generation. The kinetic data productivities were discussed in Section 4.5.2 and 4.6.2.



Figure 4-4: Time-lapse photographs of room temperature batch cultivation.

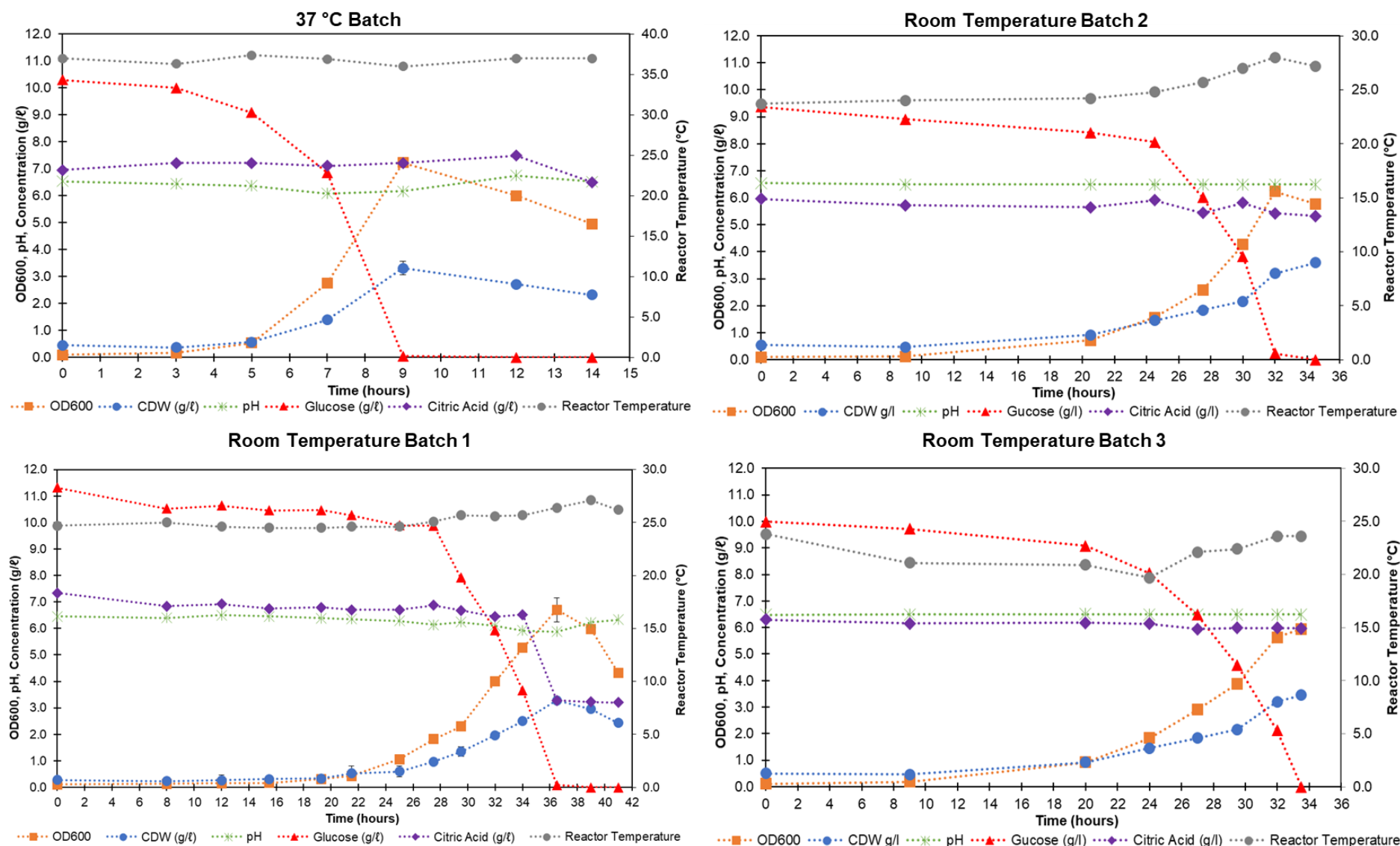


Figure 4-5: Time-based growth profiles of 37 °C and room temperature batch experiments. Top left and bottom left: 37 °C batch and first repeat of Room Temperature Batch. Top right and bottom right: Room temperature batch repeats 2 and 3.

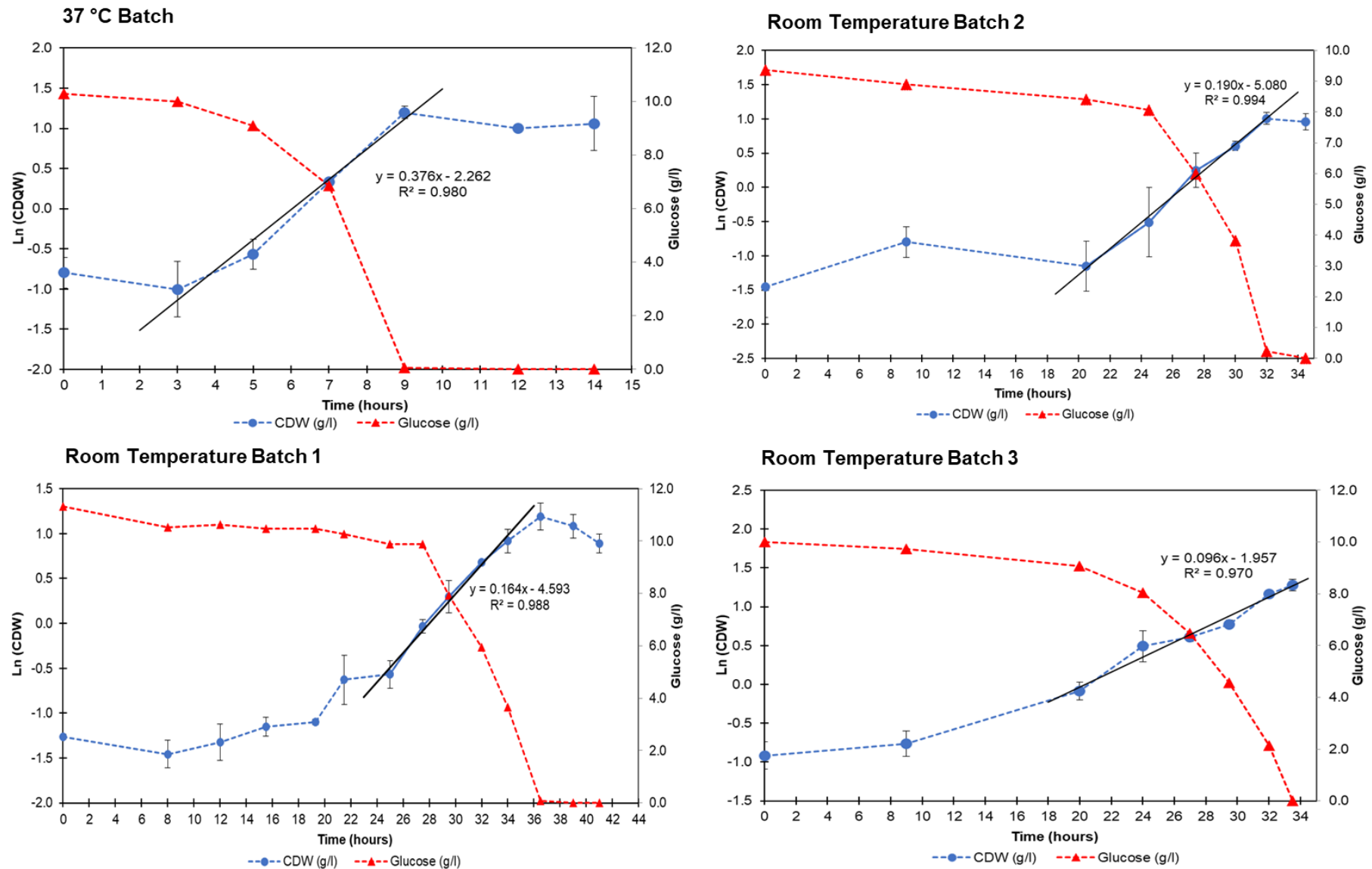


Figure 4-6: Time-based natural log of OD₆₀₀ and CDW for 37 °C batch and Room Temperature batch experiments. Top and bottom left: 37 °C batch and average of room temperature batch runs. Top right, middle and bottom right: Room temperature batch repeats 1, 2 and 3. Repeats 2 and 3 were the batch phase of the fed-batch cultivations discussed in Section 4.6.

4.5.2 Kinetic parameters and performance

Table 4-3: Kinetic data and results from the batch experiments

	37 °C Batch (37B)	Room temperature batch 1 (RTB1)**	Room temperature batch 2 (RTB2)	Room temperature batch 3 (RTB3)	Adjusted values of Room temperature batch 3 (RTB3)
Elapsed Run time (hours)	14.0	34.0 **Total run time 41 hours	34.5	33.5	33.5
Max OD ₆₀₀	7.23 ± 0.01	5.27 ± 0.043	6.23 ± 0.03	5.94 ± 0.08	5.94 ± 0.08
CDW at max OD ₆₀₀	3.32 ± 0.25	2.50 ± 0.13	2.73 ± 0.24	3.60 ± 0.26	2.65 ± 0.036
$\mu_{\max}$ (h ⁻¹)	0.376 (37°C)	0.164 Temp Range: 25.1°C - 26.4 °C Average: 25.7 ± 0.42 °C	0.190 Temp Range: 24.2 °C - 28.0 °C Average: 25.9 ± 0.71 °C	0.096 Temp Range: 21.0 °C - 23.6 °C Average: 22.1 °C ± 1.24 °C	0.096 Temp Range: 21.0 °C - 23.6 °C Average: 22.1 °C ± 1.24 °C
Overall Y _{X/S} (g/g)	0.277	0.293	0.280	0.343	0.292
Overall Biomass Productivity Q _R (g/L/h)	0.174	0.065	0.069	0.096	0.078
Maximum Biomass Productivity Q _R (g/L/h)	1.246	0.410	0.520	0.346	0.255
**The total run time of RTB1 was 41 hours. For the purposes of comparing the room temperature batch run data, the results of the RTB1 presented here are calculated based on 34-hour run data (RTB2 and RTB3 were the batch phase of the cultivation, pre fed-batch phase).					

The 37 °C batch experiment achieved a Y_{X/S} (g/g) of 0.277. A maximum OD₆₀₀ of 7.23 ± 0.01, CDW of 3.32 ± 0.25 g/L was reached, with a $\mu_{\max}$ of 0.376 h⁻¹ maintained across residual glucose concentration of 10 g/L to 0.05 g/L. Maximum biomass productivity, was found to be 1.246 g/L/h. The overall productivity at 0.174 g/L/h was lower owing to the fact that the experiment was taken well into stationary and death phase, and a decline in OD₆₀₀ and CDW were seen following complete substrate utilisation at 9 hours. *Bacillus* species studied in literature in Table 2-3 and the isolates studied by Madonsela (2013), grown on glucose or similar substrates achieved growth rates in the range of 0.06 h⁻¹ to 0.28 h⁻¹ (32 °C to 38 °C). The values calculated in this study are similar and reasonable in comparison. The calculated theoretical biomass yield was found to 0.289 g/g, which shows that the calculated values are within a reasonable and acceptable range, given the assumptions around biomass composition and the elemental mass balance. In reality, there were metabolites forming and consumption of carbon from glycerol and citric acid which were not accounted for in the theoretical yields.

RTB1 and RTB2 achieved the most comparable results across the room temperature repeats, with a Y_{X/S} (g/g) of a value of 0.294 and 0.280 respectively. RTB3, however, had a higher yield at 0.343 g/g. The CDW and OD₆₀₀ at 33.5 h for RTB3 were not in line with the other two repeats and an adjustment based on the average OD₆₀₀/CDW relationship for the first two repeats of where CDW = OD₆₀₀/2.238. Following this adjustment, the maximum productivities of the three runs were very similar with less than a 6% difference across the 3 repeats, and a slightly higher overall productivity in RTB3 due to higher biomass formation.

The average reactor temperature of RTB1 and RTB2 were closest at 25.7 °C and 25.9 °C, with RTB3 at approximately 3.6 to 3.8 °C lower in comparison to RTB1 and RTB2, over the period where maximum growth rate was observed. This lower overall temperature offers an explanation to the lower maximum specific growth rate of 0.100 h⁻¹, versus the $\mu_{\max}$ of 0.151 and 0.190 h⁻¹ for RTB1 and RTB2 respectively.

The glucose concentrations ranges where $\mu_{\max}$ was maintained for the room temperature batch cultivations were 9.9 to 3.7 g/L for RTB1 (25 to 34.5 hours), 8.4 to 0.2 g/L (20.5 to 32 hours) and 9.1 to 0 g/L (20 to 33.5 hours). It should be noted that for RTB1, complete substrate utilisation was observed at 36.5 hours, and the range reported here is up until, 34 hours to ensure comparability across the room temperature runs. There was no change in the $\mu_{\max}$ value and it remains 0.151 h⁻¹.

Effects of temperature on growth rates

The activation energy for this isolate was calculated by linearising the Arrhenius equation (Equation 4-10) and finding the slope of $\ln(\mu_{\max})$ vs $1/T$ (in Kelvin) as shown in The Activation energy across the 3 distinct average temperatures across the maximum specific growth rates was calculated to be 64.48 kJ/mol. Shuler & Kargi (2002) reported that the activation energies of microbial growth are between 42 and 84 kJ/mol, placing the results well within the expected range. They also report that for every increase in temperature by 10°C, the growth rate approximately doubles. This is evident in the results in Table 4-4, with the growth rate at 37 °C at 0.38 h⁻¹, being more double the growth rate of the room temperature batches, for an increase in reactor temperature of 12 °C to 15 °C.

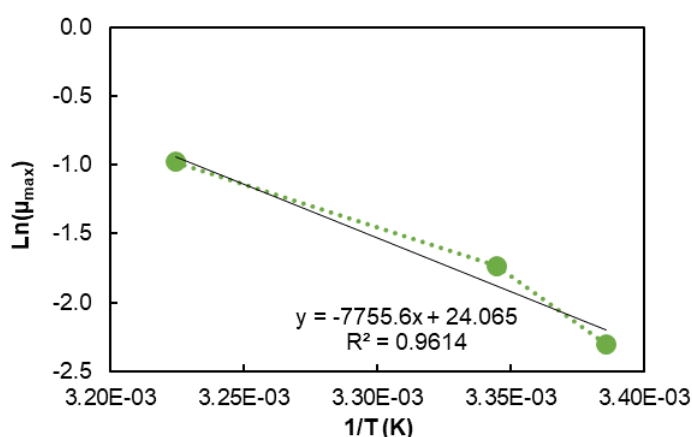


Figure 4-7: Linearised Arrhenius plot of the three distinct temperatures and maximum specific growth rates of the room temperature batch cultivations.

These results provide insight into optimal and realistic operating temperature ranges when a continuous cultivation, with limited temperature control is operated in a wastewater biorefineries application (i.e., a reactor system that is exposed to the environment). The large-scale reactors in a WWBR would see lower heat loss to the environment, due to the larger volume to surface area ratios. This is potentially favourable for large-scale applications of WWBRs, given the exothermic nature of the cultivation. Operating too far from the optimal

temperature of 37 °C for *B. subtilis* would result in slower growth and potentially poorer yields of γ -PGA, given that the product is growth associated (Rademeyer, 2018; Morikawa et al., 2006). A key result of these batch experiments was to determine a range of maximum dilution rates (and retention times) that will be applied in a continuous cultivation. Table 4-4 summaries a range of critical retention times for a suspended culture, at which a continuous fermentation could operate before seeing cell washout. These results provide an initial estimate for the dilution rates, and will be discussed further in Chapter 6, following the selection of a reactor technology in Chapter 5 for continuous runs.

Table 4-4: Average maximum specific growth rates and retention time in batch cultivations.

Batch culture	Average Temp (°C)	$\mu_{\max}$ (h ⁻¹)	Retention time (h)
37 °C	36.8 ± 0.44	0.38	2.63
RTB1	25.7 ± 0.42	0.151	6.6
RTB2	25.9 ± 0.71	0.190	5.3
RTB3	22.1 ± 1.40	0.100	10

4.6 Fed-Batch Cultivations Results

4.6.1 Time-based growth profiles

The results of the time-based growth profiles for both repeats of the fed-batch cultivations are shown in Figure 4-8. Following the batch cultivation discussed in Section 4.5, the 5 L volume was dropped to 2.5 L and the feed media in Table 4-2, pumped in at a fixed rate, to support an increasing productivity and declining growth rate.

The maximum glucose utilisation rate, R_{Gmax} , was calculated in RTB1 to be 1.43 g/L/h (or 7.15 g/h based on 5 L volume) using Equation 4-1, corresponding to an intended feed rate of 59.5 mL/h calculated for a starting fed-batch volume of 2.5 L. The actual feed rate was double, at 119.1 mL/h. This feed rate was applied to both repeats of the fed-batch cultivations (RTFB1 and RTFB2). Considering that glucose is the limiting substrate, it was important to ensure that the glucose consumption rate did not exceed the feed rate, resulting in substrate depletion and significantly decreased growth. In Section 4.5 and Figure 4-5, it was observed that growth was immediately halted when substrate was depleted. Thus, feeding at double the calculated rate was chosen to account for the doubling working volume from 2.5 L to 5 L and the increasing biomass concentration.

Fewer sample points were taken during the initial part of the batch cultivation in RTFB2 and RTFB3, due to the long acclimatisation phase evident from the first room temperature batch (RTB1) of 16 ± 1 hours in Section 4.5.1.

The end of exponential growth in the batch-phase, B1 and B2, was reached by 34.5 and 33.5 hours respectively, compared with 36 h in the room temperature batch run presented in Section 4.4. This was indicated by the negligible concentration of residual glucose and the reduction in OD₆₀₀ and biomass (CDW) concentrations. Citric acid consumption was only evident after glucose depletion at the end of batch exponential growth phase.

The fed-batch cultivations, FB1 and FB2, commenced during exponential batch phase and feeding continued at the constant feed rate of 119 mL/h and 60 g glucose per litre i.e., 7.14 grams glucose per h. FB1 and FB2 reached maximum OD₆₀₀ values of 9.25 ± 0.12 at 54.5 hours and 8.54 at 56 hours, respectively. The corresponding biomass concentrations were 4.90 ± 0.10 g/L and 6.70 g/L CDW, respectively.

The feed rate during FB1 matched the glucose consumption, and there was no glucose limitation reached. This is evident from the glucose added and glucose consumed graphs in Figure 4-8 showing an identical slope. During FB2, the rate at which glucose was fed, exceeded the rate of consumption, with an accumulation of glucose until 47 hours up to 10.1 g/L. Thereafter, there is a decline in residual glucose and an increase in the rate of substrate consumption. This accumulation of glucose did not have an inhibitory effect on the growth or formation of biomass.

One hypothesis for the cause of the accumulation of substrate in FB2, is the temperature of the reactor up to 47 hours. The reactor temperature was an average temperature of 23 °C from the start of the FB phase to 47 hours, after which the amount of biomass formation resulted in sufficient metabolic heat generation to increase the overall reactor temperature to a maximum of 32.7 °C. At the start of this increase in temperature at 47 hours, the residual glucose started decreasing. Once the feed was stopped at 56 hours, the biomass formation plateaued and the temperature decreased.

This was not observed during FB1, where the reactor temperature was on average 27.3 °C; 4.3 °C higher than in FB2. There was a brief period of accumulation at the start of the FB phase, however this is likely due to the feed being started after there was a decline in growth. Based on the first RTB experiment, it was expected that the feed should have been started at approximately 36 hours, but glucose depletion occurred at 32 hours. This was adjusted for in the second FB run, and the feed was started before a decrease in growth was observed.

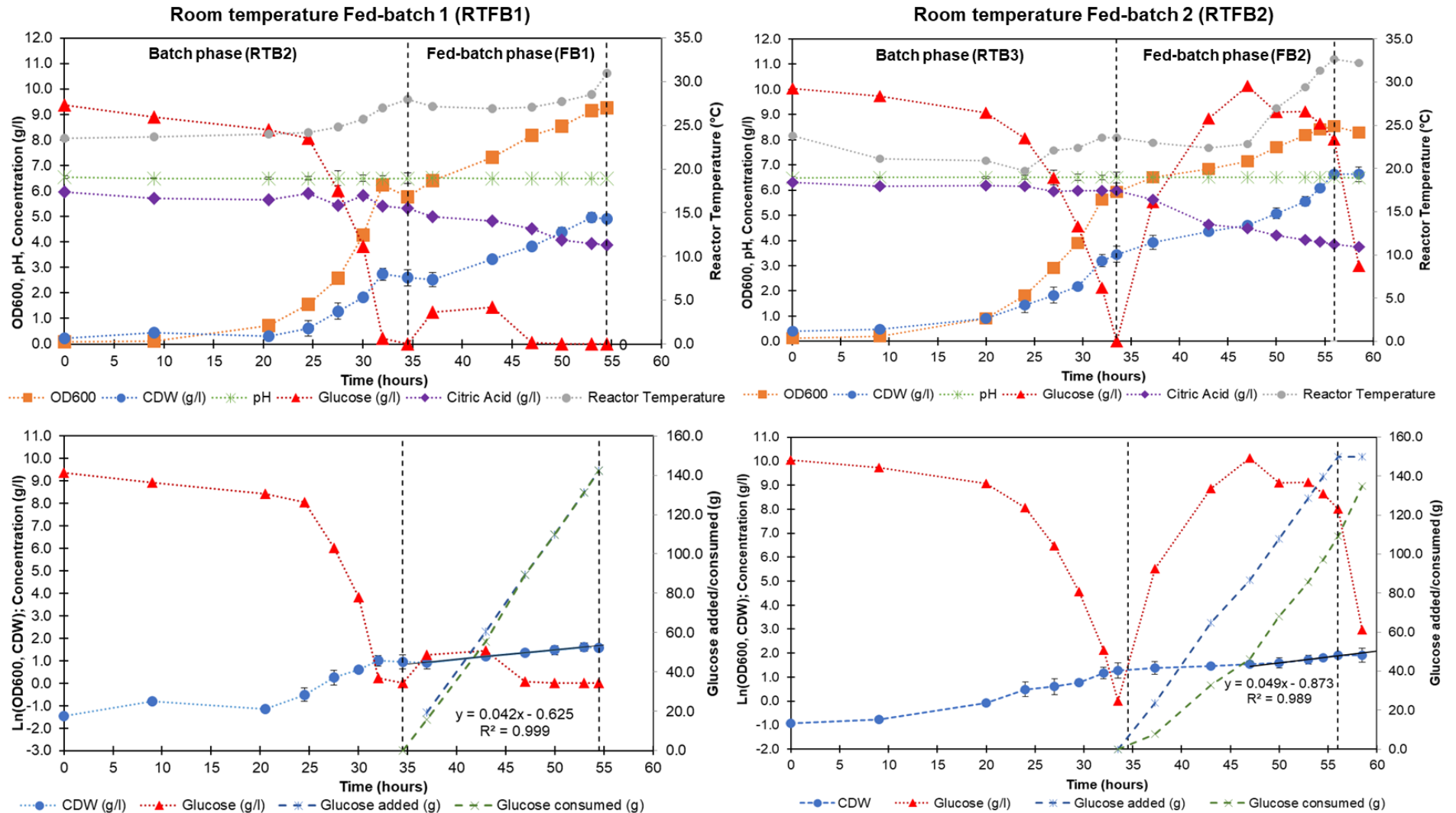


Figure 4-8: Time-based growth profiles of repeat fed-batch cultivations. Top row: Fed-batch cultivation 1 and 2 growth profiles. Bottom row: Fed-batch cultivation 1 and 2 natural logarithmic growth and glucose added/consumed (g). The dashed lines represent the start and end of feeding.

4.6.2 Kinetic parameters and performance

The fed-batch cultivation results are shown in Table 4-5. The main objective from these results it to demonstrate that the productivity can be increased and that overall formation of PGA increased under fed-batch conditions where there was a decreasing growth rate.

Table 4-5: Kinetic parameters of *B. subtilis* (Isolate 1) in batch and fed-batch cultivations.

	Room Temperature Fed-batch repeat 1		Room Temperature Fed-batch repeat 2	
	Batch phase (RTB2)	Fed-batch phase (FB1)	Batch phase (RTB3)	Fed-batch phase (FB2)
Run time in each phase (hours)	34.5	24.5	33.5	25.0
Max OD ₆₀₀	6.23 ± 0.03	9.25 ± 0.12	5.94 ± 0.08	8.54 ± 0.07
CDW at Max OD ₆₀₀	2.73 ± 0.24	4.90 ± 0.10	3.60 ± 0.26 Adjusted: 2.65 ± 0.036	6.70 ± 0.13
γ-PGA g/L (Start of Fed-batch)	N/A	22.2 ± 0.31	N/A	19.11 ± 0.63
γ-PGA g/L (at Max OD ₆₀₀)	N/A	26.4 ± 0.83	N/A	26.44 ± 0.63
μ _{max} (h ⁻¹)	0.190 Temp Range: 24.2 °C - 28.0 °C Average: 25.9 ± 0.71 °C	0.042 Temp Range: 26.9 °C–31.6 °C Average: 27.5 °C	0.100 Temp Range: 21.0 °C - 23.6 °C Average: 22.1 ± 1.4 °C	0.049 Temp Range: 22.8 °C–32.7 °C Average: 28.6 °C
Overall Y _{X/S} (g/g)	0.304	0.168	0.343 Adjusted: 0.292	0.300
γ-PGA yield (g/g)	N/A	0.630	N/A	0.732
Overall Biomass Productivity Q _R (g/L/h)	0.088	0.181	0.096 Adjusted: 0.078	0.197
Maximum Biomass Productivity Q _R (g/L/h)	0.520	0.209	0.346 Adjusted: 0.255	0.287
γ-PGA productivity (g/L/h)	N/A	0.532	N/A	0.801

Results from Table 4-5 illustrate that the constant feed-rate calculated from the room temperature batch cultivation in Section 0 was able to increase overall productivities from 0.088 to 0.181 g/L/h in FB1 and from 0.096 to 0.197 g/L/h in FB2. The declining growth rate was expected due to the linear feed rate and increasing volume, resulting in a dilution effect. Variations in biomass productivity, yields and glucose consumption rates between the room temperature cultivations could be attributed to the variation in starting time of the feed, as well as the temperature differences between the fermentatons, as discussed in Section 4.6.1. Both FB1 and FB2 maintained similar maximum specific growth rates of 0.042 and 0.049 h⁻¹. FB2 had a higher average temperature across the cultivation time that μ_{max} was maintained and a higher yield, productivity and maximum specific growth rate.

The ambient temperatures of the lab were constant at an average of 20.5 ± 0.4 °C during FB1 and 23.3 ± 0.2 °C for FB2, indicating that the rise in temperature in the reactors to a max of 32 °C in FB1 to 32.7 °C in FB2, towards the end of the feeding phase was due to exothermic metabolic activity. The variation in the ambient temperature of the lab was attributed to the fluctuations in the climate control system set to maintain a temperature between 20 and 24 °C.

The theoretical maximum yield of γ-PGA was calculated to be 0.86 g/g in Section 4.4. RTFB1 and RTFB2 had a product yield of 0.532 g/g and 0.801 g/g respectively. These results are in alignment and within an acceptable range compared to the theoretically calculated value of 0.86 g/g.

RTFB1 saw a larger increase in γ -PGA formation from 55.6 g to 135.2 g (19.11 to 26.44 g/L) and RTFB2 from 47.8 g to 135.2 g (22.2 to 25.3 g/L). The higher biomass concentration in FB2 corresponding to a lower OD₆₀₀ in comparison to FB1 could potentially be attributed to the formation of higher levels of γ -PGA, which can be expressed intracellularly, as well as extracellularly (Kongklom et al., 2015). The biomass pellets were washed with phosphate buffer, however if intracellular polymer was formed, this would affect the cell dry weight values. The concentrations of γ -PGA were quite comparable with other reports using species of *B. subtilis*; Bajaj & Singhal (2011) produced 25.4 g/L and Kongklom et al. (2015) achieved 27.5 g/L under fed-batch conditions.

4.7 Conclusions

The main aim of the batch cultivations was to determine the feasibility, impact and reproducibility of growing *B. subtilis* (Isolate 1) under room temperature conditions and determine how the effects of the temperature affect the maximum specific growth rate and reactor performance, with respect to biomass yield and productivity. The room temperature batch runs that had reactor temperatures (RTB1 and RTB2) of 25.7 °C and 25.9 °C respectively, showed similar and reproducible results in Table 4-3. RTB3, while very similar in comparison, had a lower overall temperature of 22.1 °C, thus affecting the maximum specific growth rate and maximum biomass productivity. Following an adjustment to the maximum cell dry weight of RTB3, the overall Y_{x/s}, overall Q_x and maximum OD₆₀₀ achieved showed good reproducibility and similarity. The maximum specific growth rates across the room temperature batch runs showed a linear function to the average temperature of the reactor at which $\mu_{\max}$ was maintained. A linearised Arrhenius equation allowed for the calculation of the activation energy for this organism, that was within expected literature values for bacteria. Operating under ambient conditions in large scale reactors would pose less of a challenge due to the larger surface area to volume ratio, and environmental heat loss being significantly lower than small benchtop bioreactors. Overall, these results demonstrate Isolate 1's ability to achieve similar biomass yields at lower temperatures. The theoretical maximum yield of 0.289 g/g and calculated biomass yields were very close in comparison.

The fed-batch fermentations results were able to extend and increase the biomass productivity with a constant feed-rate and decreasing maximum specific growth rate under room temperature conditions. This was the key aim of the fed-batch phase. Variations between the two runs were as a result of varying temperature in the reactor during the fed-batch phase, and timing for the feed being started. FB2 was on average 1.1 °C warmer than FB1, during the period that maximum growth was observed and as a result, had a slightly higher $\mu_{\max}$. The accumulation of substrate up to 47 hours in FB2 was also due to the lower average temperature until metabolic heat generation drove the temperature up and resulted in substrate utilisation. FB2 saw a higher net increase in γ -PGA formation from the start to end of the fed-batch phase and had an overall product yield of 0.801 g/g. FB1 had a slightly lower product yield of 0.532 g/g however both yields were comparable with the maximum theoretical yield of 0.86 g/g and as well as other literature on similar growth substrate and cultures.

Proceeding into the continuous cultivation studies in Chapter 6, the maximum specific growth rates were calculated from the room temperature batch cultivations and presented in Table 4-4. These results provide a range of retention times, from 5.3 to 10 hours under suspended culture conditions. Wastewater biorefineries operate with short residence times (high dilution rates) and high flowrates (See Section 2.1.4). As a result, in order to exceed the dilution rates calculated under suspended cell culture conditions, biomass retention will be a critical part of the reactor technology selection in Chapter 5.

5 The selection of bioreactors for application in wastewater biorefineries

This chapter expands on the challenges faced from bioproduction in a wastewater biorefinery concept that was discussed in Section 2.1.4. In this chapter, the design and operational requirements and optimisation needed for a bacterial bioreactor in order to address these challenges are considered. Thereafter, the current reactor technologies used in wastewater treatment in South Africa are evaluated against the reactor requirements for bioproduction in the wastewater biorefinery. Reactor technologies are short-listed and a detailed review of the five best technologies is presented. Finally, the selection criteria are refined with a focus on the operation and design requirements and a SWOT analysis in the context of a WWBR is performed to select one reactor technology to be designed and commissioned to demonstrate the early-stage feasibility analysis for PGA production.

5.1 Wastewater biorefinery bioreactor requirements

The dilute nature of wastewater streams, variability in composition, large volumes and difficulty in sterilisation provide unique challenges for the bioreactor design of a wastewater biorefinery to address and overcome, in order to achieve the dual goals of both delivering clean water and a bioproduct of value. Selecting an appropriate reactor technology to address these challenges as well as operating conditions, product separation and water quality requirements is key in the development of a WWBR. Wastewater treatment technologies are well designed to maximise nutrient removal through biomass retention; however, product recovery and accumulation are not factored into their design resulting in limitations in application of waste streams to bioproduction in the WWBR.

For bioproduction from waste streams to be successful, the challenges of large volumes and complex feed streams must be overcome. There should be no adverse environmental impact and the ease product recovery needs to be considered.

The requirements of the bioreactor design to address the challenges of bioproduction were developed by Verster et al. (2014) and Harrison et al. (2017). Table 5-1 provides these requirements; they are further discussed and explored in this chapter in the selection of a suitable reactor technology.

Table 5-1: Wastewater biorefinery bioreactor design requirements (Harrison et al., 2017; Verster et al., 2014)

#	Requirement	Comply?
A - Large Volume Streams		
1	Decouple hydraulic and solid retention times	✓
2	Continuous or semi-continuous (cannot store flows)	✓
3	Think big! Commodity rather than niche	✓
B - Complex Streams of Variable Composition		
4	Influence microbial community, non-sterile	✓
5	Give advantage to product: create ecological niche	✓
C – Meeting Environmental Requirements		
6	Water released into environment eventually	✓
D – Ease of Downstream Processing of Product		
7	Product formation in different phase?	✓
8	Can product be recovered?	✓
9	Reactor design conducive to reducing DSP load?	✓

5.2 Reviewing and assessing bioreactors currently used in WWT in South Africa

Through the WRC projects K5/2000 (Verster et al., 2014) and K5/2380 (Harrison et al., 2017), it has become evident that the implementation of the WWBR concept benefits from adhering to key principles in the selection for each unit operation of the system and that bioreactor selection is a crucial element of this. They provide the framework for bioreactor selection. The key principles are:

- the selection of a product existing in a different phase to the aqueous nutrients to facilitate product recovery
- the selection of a microbial phase favouring retention in the system, to allow the decoupling of the biomass and hydraulic residence times
- application of non-sterile bioproduction systems
- the utilization of a multicomponent system allowing the integrated optimization of the system rather than direct competition between water quality and product formation

A review of bioreactor types in Table 5-2 provides an overview of technologies and bioreactor types used in current WWTP in South Africa, as well as their principle of operation. Their suitability for use in a WWBR, as defined by the selection criteria of Table 5-1, are assessed and the criteria categories that each bioreactor type or technology fulfils are indicated. The bioreactors classified as suitable for WWBRs are reviewed in detail in Section 5.3 to inform final bioreactor selection, detailed in Section 5.4.

The requirements rendering the bioreactor type and technology useful in the WWBR have been numbered in the first column in Table 5-1 i.e., each criterion that the WWBR bioreactor requires or fulfils is indicated by number. The relevance for application of each reactor type in the WWBR, explored in Table 5-2, was based on the number of criteria from Table 5-1 that are fulfilled, with some being classified as “hurdle” or essential criteria. The approach focused on the nature of the bioreactor design and its principle of operation, the ease of downstream processing (category 7 to 9) and their potential for retrofitting for use in the WWBR. Based on the findings of Verster et al. (2014), reviewed in Section 2.1, the highest priority requirements, considered as essential or “hurdle” requirements, were the decoupling of hydraulic and solid residence times (1) and the downstream processing requirements (7 to 9).

The existing reactor technologies used in South African WWTPs that did not fulfil categories 1 and 7 to 9 were excluded from the shortlist as these criteria are considered as essential to restrict the financial investment required based on its principle of operation and reliance on the traditional energy-intensive downstream processing in purifying products from large volume, dilute streams. Purification is more expensive when the product concentration or biomass is low (Doran, 1995). With this in mind, Activated Sludge (AS) and Biological Nutrient Removal (BNR) were not considered further.

Table 5-2: Suitability of bioreactor types or technologies used in WWT in South Africa for application in WWBRs

Bioreactor Type / Technology Used	Principal of Operation	WWBR requirements fulfilled Table 5.1	Suitable WWBR? X: No; ✓: Yes
A Activated Sludge (AS) in a CSTR	This technology makes use of suspended growth bioreactor technology. It consists of flocculated slurry of microorganisms that are used to remove soluble and particulate biodegradable matter from the wastewater. Bioreactors are typically CSTRs in various configurations depending on the conditions desired and or level of treatment (Henze et al., 2008). It is one the most common forms of wastewater treatment technologies used by South African municipalities (DWA, 2008; van der Merwe-Botha & Quilling, 2012)	2, 3, 4, 6 (4/9)	X Does not meet 5 or 7 to 9
B Biological Nutrient Removal (BNR) (in a CSTR in series with recycle)	BNR is similar in operation to the activated sludge systems. These systems are some of the most complicated technologies used for WWT and come in a variety of configurations. BNR processes are divided into different zones where the biological environments are different and allow for removal of nitrogen and/or phosphorus (Henze et al., 2008). BNR usually consists of CSTRs in series with recycles incorporated to achieve the different zones.	2, 3, 4, 6 (4/9)	X Does not meet 5, 7 to 9
C Packed Bed Reactor (PBR)	PBRs fall under the category of submerged attached growth bioreactors. Granules used to create the packed bed are small, typically only a few millimeters in diameter. The particle carriers used can be plastic, rounded sand or fired clay. The packed bed acts as a physical filter for particulates and can be used to oxidise soluble and particulate organic matter and achieve nitrification and denitrification.	1, 3, 4, 5, 6, 7, 8, 9 (8/9)	✓
D Fluidised Bed Biological Reactors (FBBR)	FBBRs are a type of submerged attached growth bioreactor, largely used for the treatment of industrial wastewater. The upward flow of the influent wastewater creates drag forces that suspend the carrier particles upon which the biofilm grows. As the biomass grows, it results in the expansion of the bed height. To prevent the loss of carrier particles and uncontrolled bed expansion, separators are usually included in the process to return carrier particles to the FBBR and remove excess biomass (Henze et al., 2008; van der Merwe-Botha & Quilling, 2012)	1, 3, 4, 5, 6, 7, 9 (7/9)	✓
E Rotating Biological Contactor (RBC)	RBCs are attached growth bioreactors. The microorganisms form biofilms on the disks attached to a shaft and rotate in the liquid (wastewater). The shaft and disks are oriented perpendicularly to the direction of the influent. More than one RBC is typically used, oriented in series to achieve the desired effluent quality. Oxygen transfer is created by the rotation of the partially submerged disks. They are commonly used by WWTPs (Henze et al., 2008)	1, 2, 3, 4, 6, 7, 8, 9 (8/9)	✓
F Trickle Bed Reactor/Trickling Filter (TBR or TF)	The TBR, or trickling filter, is an attached growth biofilm bioreactor in which the substrate is trickled over a fixed carrier. Air is passed counter-current up the bed where diffusion between the wastewater and biofilm occurs. The trickling filter bioreactors used in industrial applications consist of a recycle stream to improve nutrient removal, as well as a liquid-solid separation unit. It was one of the first technologies used to treat wastewater and is well-established and understood (Henze et al., 2008; van der Merwe-Botha & Quilling, 2012; DWA, 2008)	1, 3, 4, 5, 6, 7, 8, 9 (8/9)	✓
G Membrane Bioreactor (MBR)	This technology is a variation of the Activated Sludge process that includes a liquid-solid separation through the use of filtration membranes (flat sheet or tubular). It achieves a high quality of effluent and is increasingly being used in the WWTPs and industry in South Africa (Henze et al., 2008; DWA, 2008)	1, 2, 3, 4, 5, 6, 7, 8 (8/9)	✓
H Moving Bed Bioreactor (MBBR)	The MBBR process is based on attached growth biofilm principles of biological WWT. The core of the process is the biofilm carrier particles. While the biofilm is fixed to the carrier particles, it is thoroughly mixed and retained within a bioreactor. Carrier particle circulation within the bioreactor is provided by the aeration system or by mixers (anaerobic conditions) (Henze et al., 2008; van der Merwe-Botha & Quilling, 2012)	1, 2, 3, 4, 5, 6, 8, 9 (8/9)	✓
I Aerobic Granular Sludge (AGS)	Dense granules of strong biomass structure are formed as densely packed aggregates of microorganisms with a much higher settling rate than conventional sludge. The most desirable attribute is their high biomass retention ability, which allows smaller reactors and HRTs. Sequentially Operated Batch Bioreactors (SBRs) are the only bioreactor type that has successfully been able to cultivate the granules (Adav et al., 2008). The bioreactor is very simple in design and is fed discontinuously, although it can be manipulated to operate under continuous flow conditions. These characteristics of the bioreactor, along with the high settling rates of AGS make it an ideal niche to study the formation of products from wastewater in laboratory settings (Johnson, 2010)	1,3,4,5,6,7,8,9 (8/9)	✓

5.3 Detailed Review of Shortlisted Bioreactors

A detailed review of the bioreactors presented in Section 5.2 led to the selection of 8 reactor technologies that fulfilled the requirements in Table 5-1. For each bioreactor type, its general description, physical characteristics, operating conditions, economic requirements and impact on downstream processing and recovery was considered. The selection of five bioreactors was made based on both current technologies used by South African WWTPs and new technologies showing promise in the large-scale application for wastewater treatment; these also fulfil the requirements for application in the wastewater biorefineries space and are suitable for large flowrates.

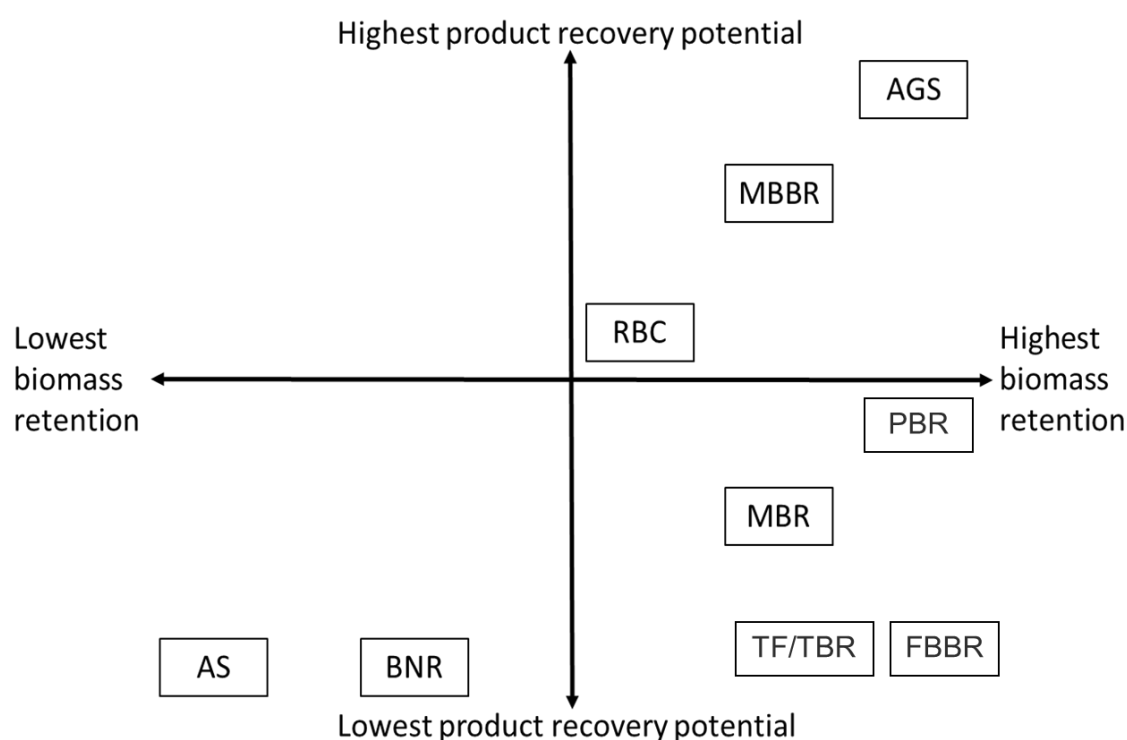


Figure 5-1: Summary chart of the bioreactor technologies in Table 5-2 and their compliance with important criteria for application in WWBRs. AGS: aerated granular sludge; AS: activated sludge; BNR: biological nutrient removal; FBBR: fluidised bed biological reactor; MBBR: moving bed bioreactor; MBR: membrane bioreactor; RBC: rotating bed contactor; TF: trickle filter (trickle bed reactor TBR)

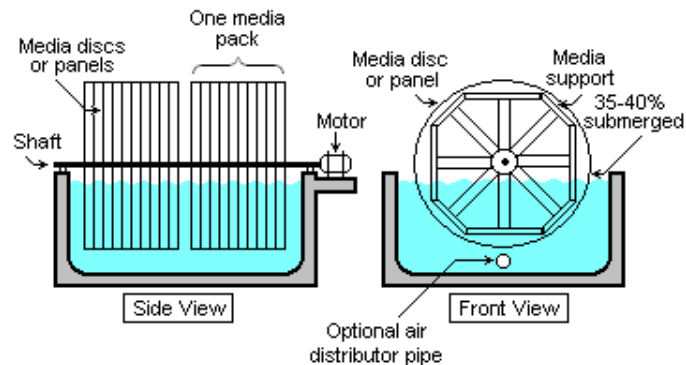
The summary chart in Figure 5-1 illustrates visually the potential of the current wastewater treatment technologies assessed in Section 5-2, to achieve the key goals of high biomass and product recovery potential, critical to overcoming the challenges of bioproduction from dilute waste streams. Out of the ten reactor technologies, the AS, BNR, TF/TBR and FBBR reactors fall short in the biomass and product recovery potentials and thus were not further investigated as potential reactors for application in a WWBR.

Hence, five bioreactors were selected and assessed for applicability through review in Tables 5-3 to 5-7, as follows: the Rotating Biological Contactor (Table 5-3), Trickle Bed Reactor (Table 5-4), Aerated Granular Sludge Reactor (Table 5-5), Membrane Bioreactor (Table 5-6) and Moving Bed Bioreactor (Table 5-7). The packed bed reactor (PBR) has similar principles of operation to the Trickle Bed Reactor/Trickling filter, and detail is provided on TBR only, as it known to be one of oldest and most well understood wastewater treatment technologies. Tables 5-3 to 5-7 summarise the main characteristics of these bioreactors in WWTPs. Where possible, examples of their use in South Africa are provided. The main advantages and disadvantages as well as physical and operational characteristics are also discussed. Associated approaches to use in a wastewater biorefinery have been considered, as well as the effect on downstream processing

Table 5-3: Review of the Rotating Biological Contactor (RBC)

Rotating Biological Contactor

Diagram



[1]

Description

The RBC consists of closely packed circular disks mounted on a horizontal shaft and submerged (typically 40% to 80 %) of the rotating unit as shown above) in the holding tank containing the wastewater. The attached biofilms are exposed to the atmosphere and the wastewater alternatively, allowing aeration and the assimilation of the organic pollutants for nutrient removal. The disks are typically made of high-density polyethylene with UV inhibitors [2]. Depending on the design, temperature, influent concentrations and process parameters such as rotational speed and hydraulic residence times, reduction in organic loads can reach 80 to 90% [7].

When or Why Used in WWTPs

RBCs are a type of static biofilm reactor delivering attached-growth biological treatment. The RBC has been used extensively and is a well-established process, for the pre-treatment of industrial wastewater [2]. They are also used to treat domestic and great water-way, and variable strength wastewater

This type of bioreactor is used in WWTPs with flows below 40 000 m³/day as economies of scale are poor. They are typically used for carbon oxidation and nitrification applications. They have also been used successfully in treating industrial wastewaters containing low to moderate concentrations of hydrogen sulphide [3].

Advantages

[2, 3, 4, 7]

1. Mechanically simple and reliable.
2. Lower energy usage relative to conventional technologies like activated sludge.
3. Motion of shaft causes aeration by exposure of biofilm-covered surfaces to atmosphere.
4. Large-scale operations are successful and well implemented worldwide.
5. Low space requirement
6. Modifications are easy to apply.
7. Biomass can be easily removed (low sludge production)
8. Able to handle lower substrate concentrations (preferable).

Disadvantages

[2, 3, 4, 7]

1. Requires good pre-treatment and primary clarification to avoid solids in the units.
2. Algal growth has been noticed if units are not covered sufficiently.
3. Lack of understanding of biological process may cause system and structural failure.
4. Limited process flexibility. Often more than one unit required to allow cycling and maintenance, taking up valuable land space.

5. High initial investment
6. Skilled technical labour needed to maintain and operate the RBCs.
7. Must be protected against elements such as sunlight, wind and rain. Poor performance at lower temperatures
8. Parts requirement might not be available on the local market

Physical Characteristics

Reactor	The rotating discs are conventionally submerged from 40% to 80%. Typical rotational speed of the shaft is 1 to 2 rpm. Disc diameters are between 0.6 to 3 meters [7]. Oxygen is supplied from the atmosphere into the attached biofilm on the portion of the RBC media exposed to the atmosphere. Oxygen enters the bulk liquid by turbulence created from the motion of the rotating disks. Occasionally, supplemental aeration is required if there is a high oxygen demand.
Arrangement/ Configuration	Typical arrangement is in series with stages dependent on the degree and nature of treatment required. Pre-screening for the removal of solids, debris are required. Typical arrangements are flow parallel to shafts, flow perpendicular to shafts, step feed flow or tapered feed flow parallel to shafts [3]. The bulk processed streams usually require clarification to remove biomass that has sloughed off the disks.

Operation and Maintenance [7, 9]

The start-up phase of this technology typically takes 6 to 12 weeks until sufficient biomass attachment achieves the desired level of nutrient removal. Re-seeding the reactors is not required due to the biofilm formation on the discs (U.S. EPA, 2002)

The treatment and nutrient removal are optimised by adjusting the depth of submergence and rotational speed, as well as supplement the aeration with spargers for the treatment of higher strength influent streams. The systems require monitoring and maintenance by trained and skilled operators. Due to the highly mechanical nature of the technology, the moving parts such as the shaft, bearings and motors required servicing and ensuring they are well lubricated. Excess sludge build-up requires purging and the discs need to be regularly checked for excess biomass accumulation as well as debris build-up (i.e., solids that were not filtered out through the pre-screening stage).

Economic Requirements [6,7]

CAPEX	Medium capital cost for the initial setup costs. Spares and materials may not be available locally and require importing.
OPEX	Medium power consumption due to requirement for continual power supply to the motor. Operationally more costly due to the skilled labour and maintenance-intensive requirements.

Downstream Processing and Product Recovery

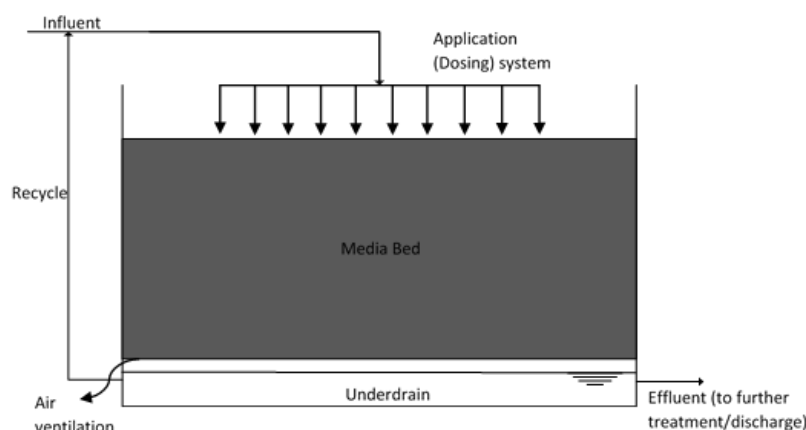
Associated Approaches to Product Recovery	Excess biomass (or 'sludge') that requires settling from the treated wastewater could be used for the retrieval of cell-associated products. Modifications could also be made to the disks to include higher surface area attachments such as meshing. Product recovery can also be achieved through removing disks for biomass harvesting or scraping/cleaning of disks [8].
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Table 5-4: Review of the Trickle Bed Reactor

Trickle Bed Reactor

Diagram



[1]

Description

Trickle Bed Reactors (TBR), are a conventional aerobic biological wastewater treatment technology that is used as a secondary treatment step to effectively reduce the organic matter of wastewaters. They consist of five major components, namely; the carrier bed (also known as filter media), containment structure, wastewater application system, underdrain system and the ventilation system. The biomass grows on the carrier bed surface (or filter media) and the wastewater pre-treated wastewater is 'trickled' over the bed using a rotating sprinkler system. Carrier bed materials vary in size, porosity and shape. They provide a high specific surface area for biofilm attachment. Plastic (PVC or polypropylene) is typically used as the medium material, but rocks, gravel or shredded PVC bottles have been used as well [1,7].

When or Why Used in WWTPs

The TBR, also known as the trickling filter, has been in use for nearly 100 years to treat municipal and industrial wastewaters aerobically. The TBR is a packed bed biofilm reactor in which the wastewater is trickled over a fixed carrier. Air is passed upward through the media, counter-currently. Diffusion between the wastewater and biofilm occurs. The TBRs used in industrial applications include a recycle stream to improve nutrient removal efficiencies. [3, 4, 5]

Advantages

1. Well-established and accepted treatment process.
2. Can be operated at a wide range of organic and hydraulic loading rates.
3. Recycling of the unclarified effluent stream can improve treatment efficiencies and re-seeding of filter media.
4. Lower pressure drops across bed lowers power requirements for ventilation.
5. Able to handle low substrate concentrations.

Disadvantages

1. Primary treatment and removal of solids before the TBR is essential to prevent clogging of biofilm carrier.
2. Clogging of biofilm carrier due to excessive biomass or extracellular polymer growth, or poor pre-treatment of influent (presence of particulates).
3. Channelling and short-circuiting of the wastewater can reduce treatment efficiencies.
4. Susceptible to flies and insect infestations, as well as odour problems.
5. Recycling increases pumping duty and operating costs.
6. A continual aeration and pumping requirement add to operating costs.
7. Prone to ice accumulation in winter season.

Physical Characteristics

	WEF (2010) reports that the depth of typical trickle bed reactors varies from 0.91 to 6.10 m if separation of fine solid particles (roughing filtration) is required [7]. For carbon oxidation, nitrification, and pure nitrification, the bed depth is typically <12 m.
Reactor	The filter media used for bacterial attachment should have a high surface area to volume ratio for maximum attachment and pore sizes large enough to prevent clogging, and allow for free movement of air and pre-treated wastewater flow. Materials such as crushed rock should have a surface area of between 45 to 60 m²/m³ and PVC or plastic packing typically have a surface area of 90 to 150 m²/m³ [8]. The underdrain system allows for the sloughed biomass to be removed to a settling tank where the solids are separated from the treated wastewater.
Arrangement/ Configuration	TBRs are typically arranged in series, with primary clarifiers before the first stage, and secondary clarifiers after the final stage. Occasionally intermediate clarifiers are between stages, to remove the biological sludge and solids generated as a result of the water treatment. They are often part of large wastewater treatment plants following activated sludge reactors.

Operation and Maintenance [8]

To achieve sufficient effluent treatment, a balance between the hydraulic loading rates with the rate of biomass sloughing and re-growth needs to be maintained. The hydraulic loading rates are determined based on the type of filter media used, ambient temperatures, the characteristics of the influent wastewater and treatment level required from the effluent. Recycling is frequently used to ensure higher treatment efficiencies and that the filter bed remains sufficiently wet during periods of lower influent flow.

Operators are required to ensure that the pumps remain operational and that the biomass formation is balanced to prevent clogging and short-circuiting. The biofilm needs to be periodically flushed with higher hydraulic loading rates to ensure that the biofilm remains aerobic, and prevent insect infestations.

Economic Requirements [7]

CAPEX	Medium capital cost; depending on the types of filter materials and pumps
OPEX	Low operational cost, with the main expensing being the electrical consumption from the pumping requirements.

Downstream Processing and Product Recovery

Associated Approaches to Product Recovery	If the product were biomass associated, the removal of the biomass from the media bed would be required. This would require a trade-off between treatment efficiencies and being more reliant on recycle streams for improved efficiencies. If the product is loosely associated to the biomass and extracellular, low shear forces will separate it from the biomass. If the product is in the bulk liquid, the clarified and treated effluent will be similar to traditional bioprocessing.
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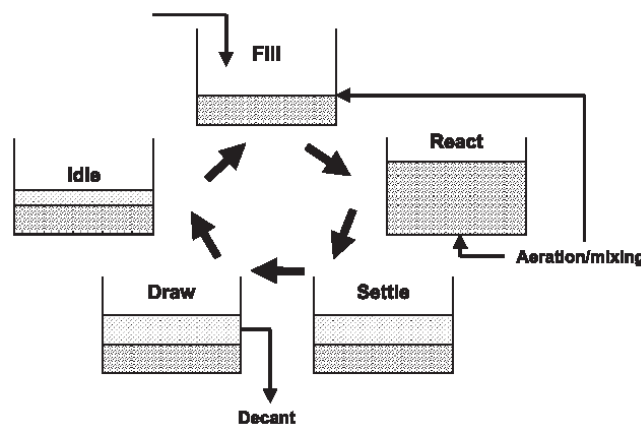
Table 5-5: Review of the Aerobic Granular Sludge Reactor in a Sequencing Batch Reactor

Aerobic Granular Sludge in Sequencing Batch Reactor

Diagram



[1]



[2]

Description

Dense granules, larger than 0.2 mm in diameter are formed as aggregates of microorganisms that are densely packed with a much higher settling rate than the conventional sludge flocs in biological wastewater treatment technologies such as activated sludge [3]. Their unique characteristics include the desirable attribute of high biomass retention ability, which allows the use of smaller reactors with shorter hydraulic residence times. Thus far, Sequentially Operated Batch Reactors are the only reactor configuration that has been used to cultivate the granules successfully [3]. The reactor is very simple in design and is fed discontinuously and cyclically, although it can be manipulated to operate under continuous flow conditions. Nereda® is a wastewater treatment technology that was invented at the Delft University of Technology in the Netherlands, in a private-public partnership. It uses an optimised SBR cycle with continual influent feed and effluent removal, simultaneous removal of organic, nitrogen and phosphorous compounds through an aerated phase, and finally, a fast-settling phase where the biomass granules are separated from the treated effluent. [5, 6, 8, 9]

When or Why Used in WWTPs

A site visit was conducted to the Wemmershoek WWTP in Franschhoek, where two large scale AGS plants using Nereda® technology have been successfully implemented to treat a combination of domestic and municipal wastewater*. This treatment technology has shown great promise in replacing, or for use in conjunction with, Activated Sludge systems in WWTPs to achieve desirable treatment objectives. The granules formed have significantly faster settleability than traditional flocs, whilst still achieving high levels of wastewater treatment for the removal of organics, phosphorous and nitrogen. [9, 12] (Arrojo et al., 2004; Robertson et al., 2015)

Advantages

1. Strong, dense microbial aggregates are formed.
2. High biomass retention and settleability

[3, 4, 7, 9]	3. Able to withstand high flow rates and organic loading rates 4. Uniform and spherical in shape 5. SBRs can be used as a continuous process 6. No separate settling tank required, reducing plant footprint. 7. Granules form biopolymers which make this technology inherently suitable for WWBR applications for cell-associated products. 8. Odour is controlled more effectively by having minimal open areas
Disadvantages [3, 4, 7, 9]	1. If incorrect HRT is employed, washout of slow settling granules will occur. 2. Modifications and improvements are required for streams with low concentration. 3. Pre-screening and filtration is required to remove suspended solids. 4. Whilst it is considered 'easy to operate', it is a new technology so is not without its challenges. Plant operators need to be trained in managing unexpected problem (Naicker et al., 2012)

Physical Characteristics

Reactor	Reactor depths and sizes vary depending on the specific wastewater flowrates and treatment efficiencies required. Verster et al. (2014) reported that the height to diameter ratio (H/D) should be high enough to allow for selectivity of granules based on settling velocities and granule size. The reactors in Gansbaai, which are a demonstration plant for the Nereda® technology, are 18 m in diameter and 7 m in depth [4]. Nereda® bioreactors are designed to process an average daily flowrate of 8000 m³/day, with up to peak flowrates of 36000 m³/day [10]
	Dissolved oxygen is an important variable and it has been noticed that granules have formed at DO concentrations ranging from 0.7 to > 2 mg/L in an SBR. Submerged aeration is used in the Gansbaai AGS process in the form of flat panel diffusers [4]. Fine bubble aeration is used in the Wemmershoek reactors.
Arrangement/ Configuration	Liu & Tay (2004) reported that aerobic granules were formed in column-type up-flow reactors. Wemmershoek WWTP uses two 2.5 ML/day reactors in parallel, with each reactor operating at a different stage (feeding, aeration, settling) at any time. Bruin et al. [4] states that the process configuration is flexible, depending on the desired process conditions

Operation and Maintenance [9]

The hydraulic retention time should be short enough to waste the slow settling sludge but long enough to achieve the treatment objectives and retain faster settling granules. Liu and Tay [6] found that a short cycle time of four to six hours stimulates microbial activity and production of cell polysaccharides, which in turn favours the formation of granules. The Wemmershoek WWTP operates at a retention time of 4 hours. The 5-step sequence of events that occur in a SBR produce effluent that is suitable for environmental discharge [7]. Laboratory studies in an aerobic granular SBRs have shown 90% removal of organic matter and up to 55% ammonia removal [8].

Results from the Gansbaai Nereda® plant indicated a 93.8% removal in COD, 99% removal in ammonia and 83.5% removal in phosphates [4]. Verbal communication with the plant manager at Wemmershoek, also confirmed that those reactors treat the wastewater to environmental and regulatory standards.

The process is fully automated and controlled with a centralised computer, and monitoring of the system pH, temperature and oxygen concentrations during the aeration. Skilled operators are required to ensure that the various stages of the SBR operated as expected and that mechanical equipment is well-maintained.

Economic Requirements

CAPEX	Initial CAPEX investment of this technology could be quite high, however significant savings result from the reduction in plant size and overall footprint for the same treatment efficiencies. Nereda® plants sizes are up to a factor of 4 times smaller than conventional plants [11].
OPEX	Bruin, Gademan & Gaydon (2008) reported that the Nereda® technology installed in Gansbaai reported significant reductions in CAPEX and OPEX. The Nereda® technology system also provides an energy reduction of approximate 35% compared with conventional wastewater treatment plants as demonstrated at a WWTP in Epe, The Netherlands. [9, 13] Operators of the plant would need to be skilled and trained in the operation of the technology, however the process is highly automated, allowing for ease of operation and more remote or unmanned control.

Downstream Processing and Product Recovery

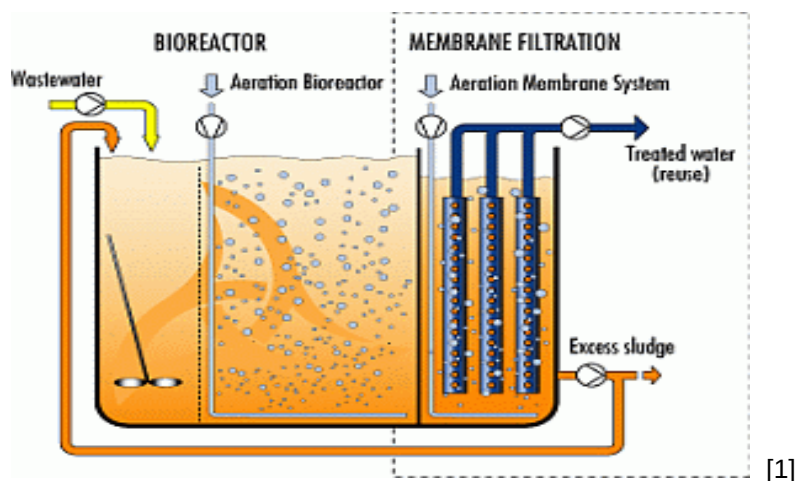
Associated Approaches to Product Recovery	Due to the fast-settling rate of the granules formed, the biomass and biomass-associated product are in solid phase, making it easier to separate a proportion of the granules through discharge valve once sufficient granules and product have accumulated. These granules can be disrupted to release the product into a more concentrated liquid form and the product fraction brought into the solid phase and removed for further downstream treatment. The granules also inherently form biopolymers and extracellular polysaccharide, which can be harvested from the excess sludge [4, 5, 9].
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Table 5-6: Review of the Membrane Bioreactor

Membrane Bioreactor

Diagram



Description

Membrane bioreactors combine micro- or ultrafiltration, with pore sizes ranging from 0.01 to 0.4 μm , within a suspended growth reactor. They can be classified into three generic types: for separation of solids (biomass) from the liquid phase; for bubble-less aeration within the bioreactor and for the extraction of pollutants from industrial or municipal wastewaters. The membranes are used for the separation of biomass and treated effluent and are used for the extraction of pollutants that are usually difficult to treat using traditional biological wastewater treatment processes [2]. The technology allows for much higher biomass concentrations than traditional WWTP technologies such as Activated Sludge, which also allows for smaller bioreactors and plant footprints. [6]

When or Why Used in WWTPs

This technology is increasingly being used to treat wastewater as a key component of water reclamation and reuse systems e.g., reusing it for utilities such as cooling water. It is a variation of the activated sludge process in which a membrane system is used for liquid-biomass separation and is often used as a replacement for secondary (or sedimentation) steps in wastewater treatment plants. These reactors are typically used to achieve a high-quality effluent [2]. MBR technology has been successfully applied to a range of commercial applications and high-strength industrial wastewater streams including food wastes, tannery effluents, textile and dye effluents, landfill leachates with treatment efficiencies ranging from 90 to 99.8%.

Advantage

[5, 8, 7, 6]

1. Combined nutrient removal and solids in one unit.
2. Production of high-quality effluent which can be suitable for immediate reuse e.g., process make-up water, cooling water etc.
3. Retrofitting existing wastewater treatment plants to expand capacity.
4. Reduction of land footprint due to smaller bioreactor sizes.
5. High loading rate capability and short retention times.
6. Capable of treating high strength industrial effluents and wastewaters

Disadvantages

[5, 8, 7, 6]

1. High investment and capital cost.
2. High energy requirements.
3. Frequent membrane fouling from organics, chemical and biological substances.
4. Frequent membrane cleaning and replacement required.
5. Chemicals required for membrane cleaning.

6. Requires skilled operators. (Tchobanoglous et al., 2003; Kennedy & Young, 2007)
7. Does not handle high amounts of non-biodegradable settleable solids. and pre-treatment is required.

Physical Characteristics

Reactor	Reactor depths and sizes vary depending on the specific wastewater flowrates, treatment efficiencies required and influent characteristics. For MBRs that are used in larger scale facilities dealing with a high throughput of wastewater, an additional unit is often included in the design as a redundancy. This allows for flexibility and additional operational capacity, as well as preventing maintenance and cleaning downtime [8].
Arrangement/ Configuration	There are two types of membrane configurations for the MBR: submerged membranes where they are placed inside the bioreactor, or external membranes, where they are external to the bioreactor. The latter requires the bioreactor contents to be filtered under pressure through the membranes and usually has a recycle loop incorporated for biomass recycle. The submerged membrane configuration is carried out in the aerated bioreactor. The submerged configuration can be more economical in terms of energy consumption due to lower transmembrane pressure requirements and no recycle pumping requirements. [5, 7, 8] Aeration in bioreactors can be a combination of fine bubble diffusers to improve mass transfer and a higher oxygen demand from increase biomass retention, as well as coarse bubbles in submerged membrane configurations to aid in keeping membranes free from biofilm buildup and prevent fouling [3].

Operation and Maintenance [5, 6, 7]

The membranes require regular cleaning to prolong their life and delay permanent fouling and reduced flux capabilities. Ensuring that the wastewater has been sufficiently pre-filtered for large solids and debris is also critical in membrane performance. Cleaning can either be performed chemically or physically. Regular, and less intensive cleaning (both chemically and physically) done on a monthly basis prolongs membrane lifetime. Chemical cleaning involves addition of sodium hypochlorite or sodium hydroxide into the bioreactor and membranes are backflushed. Physical cleaning can take place through strategically placed aeration close to membranes, through a high recycle flowrate and backwashing to prevent build up on the membranes. In addition to the requirements of regular cleaning, the pumps require regular maintenance.

Economic Requirements [5]

CAPEX	High capital and investment costs as a result of the initial reactor technology setup, as well as the cost of membrane replacements.
OPEX	High Operating and energy costs due to the high maintenance of the membranes and pumping requirements. Skilled operators are needed to ensure the reactors are maintained well and that membrane permeability is prolonged with regular cleaning

Downstream Processing and Product Recovery

Associated Approaches to Product Recovery	Products in the bulk liquid would need to be assessed to determine if it passes through the membranes with the treated effluent, or is retained with the biomass by the membranes. Its impact on or by membrane fouling also requires consideration. Biomass-associated products would require the
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removal of biomass from the reactor for DSP. The technology might be best suited for a product associated in the bulk liquid that can be extracted and separation from the biomass and broth by exploiting the microfiltration or ultrafiltration.

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Table 5-7: Review of Moving Bed Biofilm Reactor

Moving bed Bioreactor	
Diagram	
Description	The Moving Bed Biofilm Reactor (MBBR) was developed in the 1980s and 1990s, by Professor Ødegaard in Norway. The core of the technology centres on the biofilm carriers, which occupy up to 70% of reactor volume. The specially designed carriers are made of polyethylene or polypropylene and have a density close to water (0.95 g/cm^3). Crucially, they have a high specific surface area of approximately $500\text{ m}^2/\text{m}^3$ or higher. Biofilm carriers circulate and move freely in the reactor, made possible by the aeration system or by mixers (anaerobic conditions), their neutral buoyancy coarse bubble aeration (or mechanical mixing) and only partial filling of the reactor. Biomass and carriers are retained in the bioreactor using screens on the effluent port. Unlike traditional technologies such as Activated Sludge, a biomass retention on the carriers ensures recycle is not required, and only minimal biomass is required to be separated from the effluent. [6,7,8].
When or Why Used in WWTPs	AnoxKaldnes™ commercialised the MBBR technology and are currently the dominant MBBR supplier globally, among other suppliers such as Aqwise, Brightwater, Siemens and Eimco. The technology has been extensively used for organic and nitrogen removal, in industrial and municipal wastewater treatment plants. Existing wastewater treatment plants using Activated Sludge have also successfully been retrofitted with the inclusion of the biofilm carriers [7]. This technology helps to promote a specialised active biofilm that results in higher efficiencies and a more compact reactor. It is a continuous flow process, independent of the solid separation step due to the retention of active biomass within the reactor [3]. MBBRs have also shown robustness in terms of sustained treatment efficiencies at lower or higher temperature conditions. Full scale MBBR plants have been successfully applied in countries with inherently cold climates (Norway and Sweden) as well as India and Arab peninsula for the treatment of municipal sewerage. [6, 7, 8]
Advantages	[3] 1. Uses conventional wastewater treatment equipment due to versatility of the technology (successful retrofitting). 2. A variety of liquid-solids separation approaches can be used 3. Able to process fluctuating hydraulic and organic loads. 4. Potentially easy adaptations/modifications to remove biomass 5. Efficient nutrient removal to environmental specifications. 6. Self-sustaining technology, requiring minimal maintenance. 7. Low power consumption. 8. Significantly smaller plant footprint. 9. Robust technology able to handle fluctuating pH, temperatures and toxic events and variations in influent wastewater. 10. Short hydraulic retention times.

Disadvantages

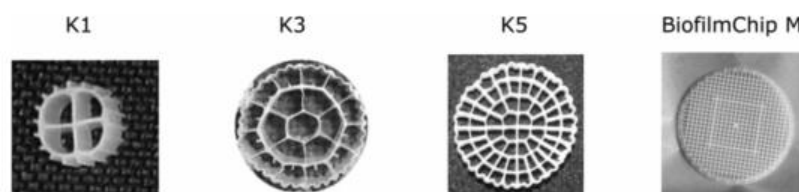
[3]

1. Excess biological phosphorous removal not easily accomplished as cycling biomass through anaerobic and aerobic zones is necessary for biomass to develop.
2. Requires a separate liquid-solid separation step for removal of some biomass from effluent.
3. Volumetric loadings higher than suspended growth systems but lower than other attached growth systems
4. Pre-filtering sieves of the wastewater stream prone to clogging and require removal prior to reactor.
5. Incorrect design and carrier fill % can result in non-uniform motion of carriers under high hydraulic flowrates, resulting in poor performance.

Physical Characteristics

Reactor

Due to the simplicity of this technology, the size and shape of the reactor is dependent on the treatment plant where it is being implemented and sizing depends on the volume and extent of treatment required. The key factor to the success of the MBBR treatment efficiency is in the design of the biofilm carriers and ensuring that adequate specific surface area is provided for the attachment of viable and active biomass attachment. AnoxKaldnes™ carriers have been designed with specific surface areas ranging from 500 m²/m³ to 1200 m²/m³ for some of their most popular carriers (e.g. K1, K3, K5 and BiofilmChip) as shown in the image below from van Haandel & van der Lubbe (2012).



The aeration system should provide an even distribution of air over the reactor, with sufficient power to keep the carriers in suspension, as well as providing sufficient oxygen mass transfer. For this reason, coarse to medium bubble aeration is preferred [6]. Optimising the oxygen transfer rate within the MBBR can be done by adjusting the filling ratios of the carriers, and aeration rates. Studies by Jing et al. (2009) and Barwal & Chaudhary (2015) found that volumetric mass transfer coefficient (K_La) reached its peak value at filling rates of 40%, due to the carriers being able to move freely and slowly, and proved favourable for the dispersion and breakage of bubbles into the liquid phase. Both studies also found that increasing aeration intensity improved K_La , but high aeration isn't always favourable for reactor operation from an economic or operability perspective.

Arrangement/
Configuration

Multiple continuous reactors can be placed in series to achieve the treatment objectives e.g., organic nutrient removal, nitrification and denitrification, with each reactor designated to a specific treatment. MBBR technology can also be used a pre-treatment to remove a large proportion of organic nutrient, before an activated sludge system [3, 6]. The placement of the aerator should ensure that sufficient mixing close to the effluent outlet sieves is provided to prevent any accumulation or 'dead zones' where there is minimal movement [4, 6].

Operation and Maintenance

The MBBR systems have low maintenance demands and are considered easy to operate relative to RBC or MBR technology [6]. The main area of maintenance is around the air blowers, pre-screening

of influent wastewater for debris, liquid level equalisation and controlling separation of biomass in the effluent through a sedimentation (e.g., gravity settling) or a filtration step. Biofilm formation on the carriers is self-maintaining and carriers need replacement every 10 to 15 years [7, 8].

Economic Requirements

CAPEX	MBBRs have high potential to be retrofitted into existing WWTPs such as Activated Sludge, which is one of the most common technologies in South Africa. The initial capital expenditure would be for the biofilm carriers. These, however, can last in excess of 15 years before requiring replacement.
OPEX	The demand on utilities and maintenance is moderate relative to technologies such as the RBC, MBR and trickling filters. Main electro-mechanical operational costs involved are around maintaining pumps, air blowers and monitoring equipment.

Downstream Processing and Product Recovery

Associated Approaches to Product Recovery	In this situation, a solid-liquid separation step would be required regardless of whether the bioproduct is in the bulk liquid or biomass associated, merely due to the nature of operation of this bioreactor. Products which are extracellular but biomass-associated would be preferential to prevent removal of biofilm for product harvesting. The product could be periodically recovered by physically dissociating from the biomass through high shear flow.
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5.4 Final Selection of Bioreactors for WWBR

5.4.1 Refinement of the key criteria for selection

In order to select suitable bioreactors from Section 5.3, the criteria discussed in Section 2.1.4 were prioritised and reorganised. Table 5-8 was constructed to represent this prioritisation by critically analysing each of the selection criteria and identifying whether they satisfy the two key requirements, based on the definition of a WWBR:

- Produce a product in a different phase that is easily removed and separated from the substrate and biomass to decrease the load on the downstream processing through the inherent bioreactor design
- Decouple hydraulic and solid residence times

Table 5-8: Bioreactor Design Requirements in order of priority

	#	Challenge that WWBR presents	Requirement
Design Priority	1	Wastewater as a feedstock: large volumes, dilute concentrations	Decouples hydraulic and solid retention times
	2	Wastewater as a feedstock: variable and continuous influent stream	Continuous or semi-continuous (can't store flows)
	3	Dilute medium: cost of downstream processing for product recovery	Product formation in different phase
	4	Complex and variable feedstock: biomass retention is required and multiple constituent's complicate product recovery.	Bioreactor design facilitates the recovery of the product
Operational Priority	5	Wastewater remediation: need to use the entire wastewater flow for bioproduction	Think big! Commodity rather than niche
	6	Complexity and volume of feedstock: energy for sterilisation unfeasible, robust microbiology needed	Facilitates selection and retention of desired microbial community Non-sterile operation
	7	Complex and variable feedstock: cannot maintain a monoculture	Gives advantage through product; creates ecological niche
	8	Wastewater remediation: non-negotiable production of ecologically compliant effluent	Water resulting is compliant for environmental release

The first four requirements have been labelled as 'Design Priority'. Requirement number 4 is a combination of two requirements in Table 5-1 #8 "Can product be recovered?" and #9 "Bioreactor design conducive to reducing DSP load?" since the recovery of the product is a function of how the biofilm grows and attaches in the bioreactor and whether the bioreactor design facilitates this attachment process. This, in turn, affects how the product is removed and whether additional process units are required to separate product from the bulk liquid. If a bioreactor is unable to fulfil **all** four of the design priorities, then it is unlikely that it will be able to produce the desired bioproduct in a quantity and phase that makes the process economically feasible.

The other four categories have been labelled as "Operational Priority". This set of criteria refers to factors that are independent of the design and pertain to important operational factors of a WWBR that need to be met to ensure its success. Many existing reactor technologies in wastewater treatment plants could fill the operational priority requirements, since the process flows and unit operations of both a WWBR and WWTP are founded on very similar principles. However, should a bioreactor technology fail to comply with the "design priority" criteria, despite fulfilling the "operational priority" criteria, it remains unsuitable for the use in wastewater biorefinery applications.

The desired goal for these wastewater biorefineries is to incorporate the bioreactors into existing wastewater treatment plants. Thus, it is critical that they are able to handle continuous or semi-continuous flows that have seasonal variations in flowrates and composition. WWTPs cannot 'shut down' owing to the continuous flow of wastewater generated by industry and the population.

5.4.2 Criteria fulfilment of the selected bioreactors

In order to adequately justify the selection of one reactor technology for further study, a summary table was compiled for each of the five bioreactors outlined in Section 5.3 to show the degree to which the bioreactors fulfil the criteria outlined in Table 5-8.

The following scale is used to show the extent to which the bioreactors satisfy the requirements in Table 5-9. The number of criteria that completely comply were then totalled to arrive

Completely complies	Mostly Complies	Marginally Complies	Does not comply
+++	++	±	X

Table 5-9: Composite table showing the degree to which the five bioreactor categories fulfil the selection criteria

	Criteria	AGS in an SBR	Rotating Biological Contactor	Membrane Bioreactor	Moving Bed Biofilm Reactor	Trickle Bed Reactor
Most Important	1 Decouples hydraulic and solid retention times	+++	+++	+++	+++	+++
	2 Continuous or semi-continuous (cannot store flows)	++	+++	++	+++	++
	3 Product formation in different phase	+++	+++	++	++	+++
	4 Reactor design facilitates the recovery of the product	+++	++	±	+++	X
Least Important	5 Think big! Commodity rather than niche	++	+++	++	+++	+++
	6 Influences microbial community, non-sterile	+++	++	++	+++	++
	7 Gives advantage to product: creates ecological niche	+++	++	++	+++	++
	8 Water released into environment eventually	+++	++	+++	+++	++
# of criteria that comply completely		6	4	2	7	3

From Table 5-9, it is evident that the reactor types that fulfil the critical requirements are, in order of best compliance: Moving Bed Biofilm Reactor (MBBR), Aerobic Granular Sludge in a Sequencing Batch Reactor (AGS-SBR) and the Rotating Biological Contactor (RBC). This selection is further justified in the SWOT analysis in Section 5.4.3.

The Trickle Bed Reactor did not fulfil criterion number 4, due to the process down time that would be required to remove the packed material, separate the biomass and product and start-up the treatment procedure. This would require the storing of wastewater flows, or having multiple bioreactors, and the re-establishment of equilibrium in the system. Clogging and short-circuiting is a known problem in TBRs used in regular wastewater treatment, resulting in channelling and poor treatment efficiencies (Antonie, 1976). Clogging may be aggravated with product formation, especially if the product is extracellular.

The Membrane Bioreactor also fell short in category number 4. While it is a continuous system, the membranes require replacement and maintenance to prevent clogging. They have high CAPEX and OPEX and require skilled plant technicians. In the context of South Africa's existing WWTPs, this presents an additional obstacle, with current WWTP operations and infrastructure already under pressure (Henze et al., 2001; Henze et al., 2008).

5.4.3 SWOT analysis of the three reactors selected for use in WWBRs.

For the purpose of this comparison, the SWOT Analysis has been based on the ability of each of the bioreactors outlined to fulfil the key criteria that were outlined in Section 5.4.1. The following questions were asked when performing the SWOT analysis on these bioreactors:

Strengths: What characteristics of the bioreactor technology allow it to fulfil the requirements and render it suitable for applications in wastewater biorefineries?

Weaknesses: What are the major drawbacks about this technology, concerning process operation and treatment objectives?

Opportunities: Is there potential for retrofitting and adaption to South Africa's current wastewater treatment plants and infrastructure?

Threats: Does the bioreactor technology have risks associated with its operation, and implementation?

Table 5-10: SWOT analysis of selected bioreactors

Rotating Biological Contactor (RBC)	Moving Bed Biofilm Reactor (MBBR)	Aerobic Granular Sludge in a Sequencing Batch Reactor
Strengths		
Mechanically simple and reliable process. Large scale applications used successfully worldwide. Inherent aeration by nature of shaft rotation. Recycle loops are not required due to continued microbial growth and thus water treatment. Able to handle lower substrate concentrations. Creates a microbial niche – organisms present in the wastewater naturally adhere to the disks. Rotating disks agitated the mixed liquor keeping sloughed biomass in suspension and well mixed at each stage of treatment. Not affected by shock variations in hydraulic and organic loading.	Versatile technology allowing creative solutions. Active biomass is retained in the reactor continuously. The suspended carriers promote the formation of active biomass, resulting in higher efficiencies and process stability. Continuous flow process. Multiple stages can be achieved through arrangement in series, without the need of pumping (similar to AS process). Density of carriers close to that of water, thus minimizing mixing energy required to keep them in suspension. Does not require skilled operators. Simple coarse aeration grid designed on base of reactor eliminates the need for diffuser replacements and maintenance. Robust technology that is able to withstand temperature extremes.	Hydraulic Load variations are readily handled by for AGS systems. High biomass retention. SBRs can be used as a continuous process. Does not require additional clarifiers downstream. Obtains high treatment efficiencies at low oxygen saturation concentrations. Granules have a fast-settling rate. Recycles and mixers are not required saving on energy costs and maintenance. Land footprint of AGS is significantly decreased compared to other technologies such as Activated Sludge.
Weaknesses		
Requires primary clarification and pre-treatment as it does not handle particulate matter well. If insufficient wetting of the biomass occurs, it leaves the disks vulnerable to nuisance organisms such as algae and worms. Development of uneven biofilm growth. Susceptible to environmental conditions such as extreme heat or cold.	Efficient screening and grit removal is required to prevent build-up of inert and debris-type material. Foaming is known to occasionally form at start up. Antifoam added into the process can cause decreased diffusion to the biofilm, be detrimental on the product DSP.	AGS that is formed by slow growing bacteria is more stable than when fast growing bacteria are present. Competency of operators running a relatively new technology at a large scale. Requires pre-treatment to remove solids.
Opportunities		
Modifications to the reactor design could be incorporated to continuously remove surface biomass for product harvesting.	Great potential for modifications to the design to facilitate easy liquid-solid separations. Versatility makes MBBRs suitable for retrofit installation into existing tanks. In	Successful large-scale application of AGS in a SBR in Gansbaai and Wemmershoek have been implemented and showed excellent treatment efficiencies. This

	South Africa, the predominant technology is Activated Sludge. MBBRs could be retrofitted into these existing tanks (large scope for WWTP to be upgraded for lower CAPEX.)	shows great promise for this technology on a large scale
Threats		
Temperatures below 12 °C in colder seasons will result in decreased efficiency. Enclosures are often needed around the RBCs to minimize effects of sunlight, nuisance organisms and temperature fluctuations. These enclosures require odour control and often heat control to avoid condensation and corrosion of the units. Process failures due to inadequate designs of the shaft system	Too much sloughing in the reactor could cause the biomass to flow out with the media and prevent water treatment and product formation.	Process is unstable as washout can easily occur Effects of a biopolymer forming microorganism on a granular formation are not well understood.
References		
(Antonie, 1976; Tchobanoglous et al., 2003; Adav et al., 2008; WEF, 2010; Henze et al., 2008)	(Henze et al., 2001; Borghei & Hosseini, 2004; WEF, 2010; Henze et al., 2008; Cervantes et al., 2006; Odegaard et al., 1994; Ahmadi et al., 2011)	(Henze et al., 2001; de Kreuk et al., 2005; Bruin et al., 2008; Verster et al., 2014)

The SWOT analysis showed the MBBR poses low risk in terms of weaknesses and threats. The challenges mentioned under those categories could be resolved with relative ease and minimal expense. To explain this further, sloughing in the bioreactor was described as a risk. The majority of mixing within a MBBR is caused through the aeration rate and profile. This threat could be minimised by ensuring more gentle aeration and an appropriate carrier fill percentage. However, a threat described for RBCs where temperature shocks can decrease efficiency, and the process being more sensitive to environmental elements is not as simple to overcome and solving this could require increased costs (e.g., a degree of temperature control). Based on the findings of the SWOT analysis, two reactor technologies stand-out as the most suitable for consideration in this study; the MBBR and AGS. The final deciding factor was based on the ease of construction and likelihood of a lab-scale reactor being successful. The MBBR is a well-studied and robust reactor technology, which high potential for retrofitting into South Africa's current wastewater treatment plants. Coupled with this, was the simple reactor design, ease of construction and operability that made the selection of the MBBR the first choice.

5.5 Conclusions

An investigation into wastewater treatment technologies that are frequently used, in relation to their suitability for application in a WWBR context, were evaluated against the design and operability criteria outlined in Table 5-1. Fixed biofilm reactor technologies are the most suitable, and five of the most suitable technologies identified were the Rotating Biological Contactor, Trickling Filter/Trickle Bed Reactor, Membrane Bioreactor, Aerobic Granular Sludge Reactor and Moving Bed Biofilm Reactor.

After an in-depth review (Table 5-3 to Table 5-7) and short-listing of the five most suitable technologies and looking into the operational and design aspects, it was evident from the SWOT analysis that the most promising reactor was the MBBR. It satisfactorily fulfilled the crucial requirements and have been demonstrated at large scale in a South African context a large scale MBBR at the Chevron Refinery in Cape Town. The MBBR has also has been successfully retrofitted into existing WWTPs around the world (van Haandel & van der Lubbe, 2012). With Activated Sludge as the predominant technology used in South Africa, upgrading existing infrastructure would require minimal CAPEX investments and modifications. This is favourable in an economic climate where maintenance and repair of current infrastructure is already behind and failing to provide the full potential it could.

6 Production of γ -PGA with cell retention in a Moving Bed Biofilm Reactor

In this chapter, a proof-of-concept study is presented on the production of γ -PGA in the continuous MBBR. Following its design, construction, commissioning and characterisation, a continuous MBBR operated at room temperature conditions, with γ -PGA production is explored using the same micro-organism and cultivation media as described in Chapter 4 for the base case STR studies. The design and construction of the reactor is presented. The results of studies to demonstrate the proof of microbial attachment to the carriers are discussed. The experimental setup and operational details for the continuous reactor experiments is presented. Thereafter, the results of the continuous reactor studies at varying retention times are presented and discussed with respect to the productivity, substrate utilisation, organic loading rates and yields during steady state.

6.1 MBBR design and commissioning

6.1.1 Basis of design

One of the major advantages of the MBBR technology, described in Table 5-7, is the flexibility and robustness with respect to design factors such as geometry, material of construction, reactor volumes and influent treatment. This flexibility allows easier retrofitting into existing wastewater treatment infrastructure (Odegaard, 2006; Odegaard et al., 1994; van Haandel & van der Lubbe, 2012). A site visit was conducted to the large-scale MBBR at the Chevron Oil Refinery in Milnerton, Cape Town where a rectangular MBBR is used for the treatment of process wastewater containing volatile organic compounds. They have successfully reduced foul odours which previously had caused frustration to the neighbouring communities. Based on this site visit and recommendations from Chevron engineers, as well as research into industrial configurations of MBBRs (Webb, 2008; van Haandel & van der Lubbe, 2012), a rectangular MBBR design was chosen for construction of the lab-scale MBBR (Webb, 2008). The total volume of the MBBR for this study was designed at 7 litres, for consistency with the batch and fed-batch cultivations in Chapter 4.

The selection of the biofilm carrier type strongly influences the performance and success of the MBBR. For the design of this reactor, the AnoxKaldnes™ technology was selected as a well-known and proven technology; these carriers were available through a local Veolia branch. AnoxKaldnes™ provide a wide range of carrier types with specific surface areas ranging from 200 m²/m³ to 1200 m²/m³ and diameters from 9 mm to 64 mm. For the purposes of this study, two carriers were purchased: the BiofilmChip P and K3. Following an initial test where the carriers were aerated in the reactor with water to investigate their mobility, the K3 carrier had far better motion due to the narrower diameter (even with them being considered bare/unacclimatised). Free motion within the MBBR is critical for mixing and mass transfer. Hence, the K3 carrier was selected; it provided a specific surface area of 500 m²/m³, with a depth of 10 mm and diameter of 25 mm.

The filling percentage of the carriers in the reactor affects the mixing patterns. Reactors with a lower fill (<70%) percentage tend to have better movement and this relationship with the carriers results in improved mass transfer (Barwal & Chaudhary, 2015; van Haandel & van der Lubbe, 2012). This parameter was investigated in mass transfer studies in Section 6.2 to determine the optimal filling percentage and aeration rate.

Aeration is also a very important design factor in the performance of the MBBR. An even distribution of air over the surface area of the reactor is required. The aeration provides turbulence to drive movement of carriers in the liquid (van Haandel & van der Lubbe, 2012). This in turns results in bubble breakage and improved mass transfer from the liquid phase into the thin biofilm layer. To ensure the even distribution, coarse-bubble aeration was supplied to the MBBR. A 'grill'-shaped sparger (Figure 6-1)

was designed with uniformly pitched holes. This was aerated from both ends to prevent pressure gradient across the sparger.

6.1.2 Materials and Construction

A rectangular shaped MBBR was constructed with clear Plexiglas (acrylic) sheets with internal dimensions to allow for a final total volume of 7 L. The reactor specifications are given in Table 6-1. The final construction and assembly of the sparger in the MBBR is shown in Figure 6-2. The finished MBBR construction and assembly is shown in Figure 6-3 and Figure 6-4.

Table 6-1: Reactor Specifications for lab-scale MBBR

Specification	Description
Total Volume	7 L
Working Volume	5.0 ± 0.5 L
Reactor body	Constructed using sheets of clear Plexiglas (acrylic sheets). Lid and base: 10 mm clear Plexiglas Body: 8mm clear Plexiglas
Dimensions	310 (h) x 150 (w) x 150 (l) mm Dimensions listed are internal dimensions, not inclusive of the Plexiglas thickness.
Influent	The inlet port was positioned above the height of the liquid, in the reactor. The influent was pumped into the reactor continuously through this port.
Effluent	The effluent port was located at the desired working volume liquid level. It was constructed as a dip tube, located in the lid of the reactor using a compression fitting. Effluent was pumped continuously from the reactor at the same flowrate as the influent by using the same pump head as the influent.
Aeration	A 'grill' shaped sparger (shown Figure 6-1 and Figure 6-2) was constructed from $\frac{1}{4}$ " stainless steel piping with a bend radius of 31 mm. This was placed horizontally in the base of the reactor. 1 mm holes with a 10 mm spacing were drilled along the length of the piping (75 holes total). Air was sparged from both ends of the sparger to prevent pressure drop across the piping.
Biofilm carriers	K3 AnoxKaldnes™ Dimensions: 10 mm (l) x 25 mm (OD) Surface area: $500\text{m}^2/\text{m}^3$ Number per m^3 : 116 000 Composition: high-density polyethylene Density: $0.95\text{ kg}/\text{dm}^3$ (adjustable, depending on cell attachment)

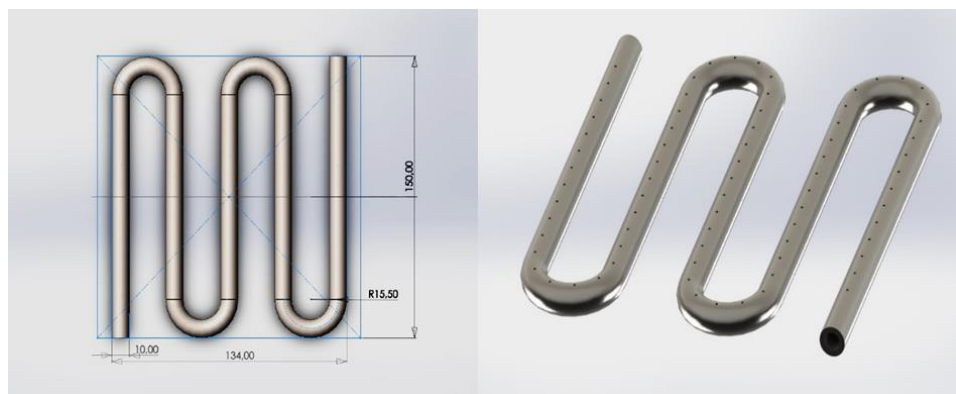


Figure 6-1: Aerial view of top side of the grid sparger situated in the base of the MBBR (left) and rendered image of the grid sparger design from under-side perspective showing the drilled hole (right).

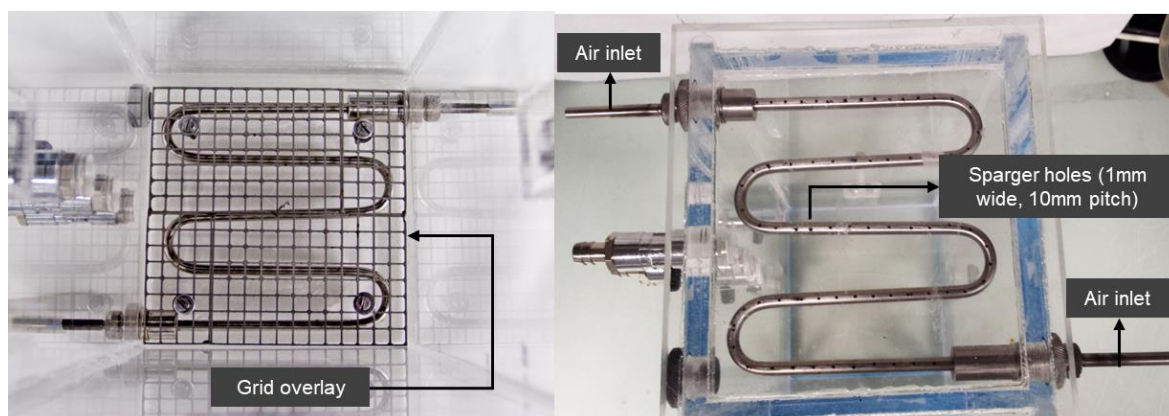


Figure 6-2: Sparger from aerial view inside the MBBR with grid overlay (left) and image of the underside of the MBBR showing the air inlet ports and sparger holes (right).

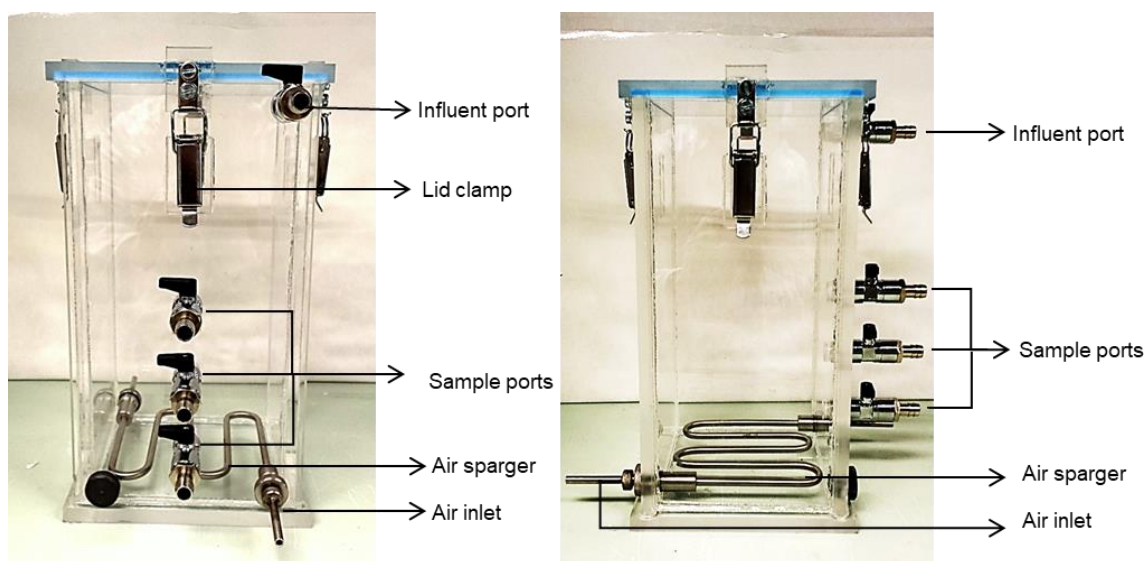


Figure 6-3: Front view and side view of MBBR

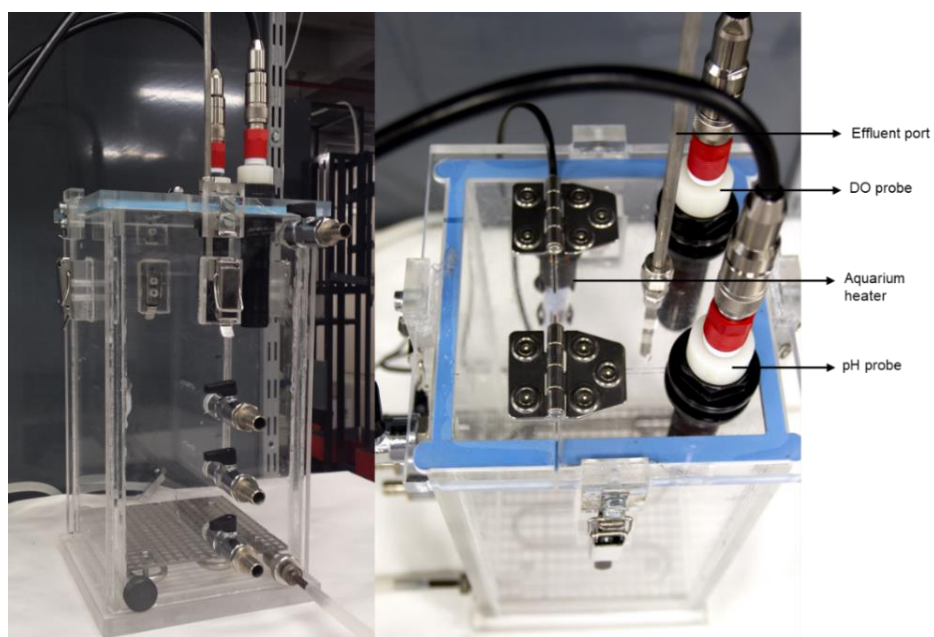


Figure 6-4: The assembled MBBR showing a side and top view with probe and effluent ports in place on the headplate.

6.2 Effect of carrier design on oxygen mass transfer

6.2.1 Introduction

In most bioreactor systems, the mass transfer coefficient is influenced by conditions such as agitation rate, aeration rate, impellor number and position and temperature. In the case of the MBBR both carrier design and carrier fill percentage (number of carriers as a fraction of reactor volume) also impact the mass transfer achieved and are key to optimising the reactor's performance. The carrier fill percentage influences the break-up of the coarse bubbles which in turn influences fluid motion and the motion of the carriers in the liquid media, together with oxygen transfer to the suspended biomass and biofilm.

To determine the oxygen mass transfer delivered in the MBBR and to understand the influence of aeration rate and carrier fill fraction on the mass transfer in the reactor, experiments using liquid media and water were carried out using the static gassing-out method under varying aeration rates and carrier fill fractions. The main objective was to determine the key operating parameters of the MBBR that could influence the nutrient removal: the carrier fill % and aeration rate.

6.2.2 Methodology

The gassing in-out method was used to estimate the volumetric K_{La} and is described in Section 3.4.1.

The tests were performed using 5 L of half strength MME and water, at four different aeration rates (2, 3, 4 and 5 L/min, equivalent to 0.4, 0.6, 0.8 and 1.0 vvm) with carrier fill percentages of 0%, 10%, 20%, 30%, 40% and 50% by volume. At each aeration rate and carrier fill % combination, tests were run in triplicate and the volumetric mass transfer coefficient calculated from the average of the three measurements (total of 144 tests). The number of carriers that occupy this volume percentage is calculated from the specification data for the AnoxKaldnes® K3 carriers, which is estimated at 116 000 carriers/m³, multiplied by the fill fraction. The carriers used were free from active biomass, but had been acclimatised to ensure neutral buoyancy within the liquid.

6.2.3 Results and Discussion

The results presented in Table 6-2, Figure 6-5 and Figure 6-6 show the experimentally determined values of K_{La} with varying carrier fill percentages and aerations rates. The gradient in colour in Table 6-2 (red to green) represents the conditions at which the K_{La} was lowest (red) to highest (green).

Table 6-2: Summary of the volumetric mass transfer coefficient K_{La} (h⁻¹) at varying aeration and carrier fill percentages.

	Water	Media (0%)	20% Fill	30% Fill	40% Fill	50% Fill
2 L/min	10.31 ± 0.19	9.80 ± 0.35	10.44 ± 0.17	11.21 ± 0.07	11.16 ± 0.24	11.30 ± 0.34
3 L/min	14.40 ± 0.36	13.93 ± 0.11	14.76 ± 0.27	16.20 ± 0.51	17.28 ± 0.27	16.70 ± 0.13
4 L/min	18.96 ± 0.22	19.52 ± 0.24	20.16 ± 0.59	21.26 ± 0.30	23.76 ± 0.99	21.31 ± 0.41
5 L/min	25.98 ± 0.58	24.30 ± 0.40	24.12 ± 0.68	27.59 ± 0.44	35.64 ± 0.12	29.45 ± 0.58

These data demonstrate a clear and noticeable trend that with increasing aeration rate, K_{La} increases. This is an expected result given that the higher aeration rates provide more bubbles to drive more oxygen into the liquid phase (Stanbury et al., 1995). Another significant trend is evident between the fill percentage and K_{La} . This is illustrated in Figure 6-5.

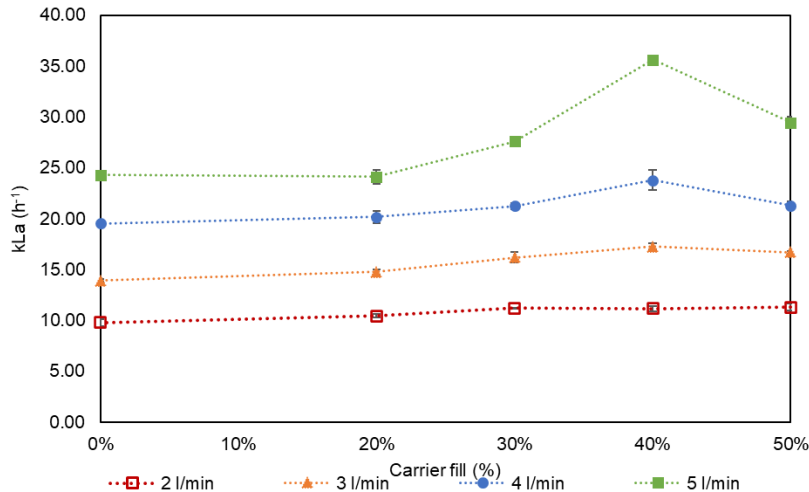


Figure 6-5: Correlation between k_La and aerator rate at different fill percentages, for this MBBR system using media. 0% fill is a comparison between media and water with no carriers.

The k_La improved with an increase in carrier fill percentage from 20% to 40%, thereafter, it decreased. Studies by Jing et al. (2009) and Barwal & Chaudhary (2015) both reported the same trends in mass transfer coefficient and carrier fill %. The cause of this phenomenon is explained by the motion of the carriers within the liquid phase, and the role they play in bubble dispersion and gas hold-up times within in the liquid. Higher fill percentages result in the reduced free motion of the carriers. As the carriers move through the air bubbles, the bubbles are broken down into smaller bubbles causing greater bubble dispersion. There is also temporary attachment of bubbles to the surface of the carriers, increasing the gas-hold up time within the liquid and thus improving oxygen transfer both to the liquid phase, as well as the attached biofilm layer.

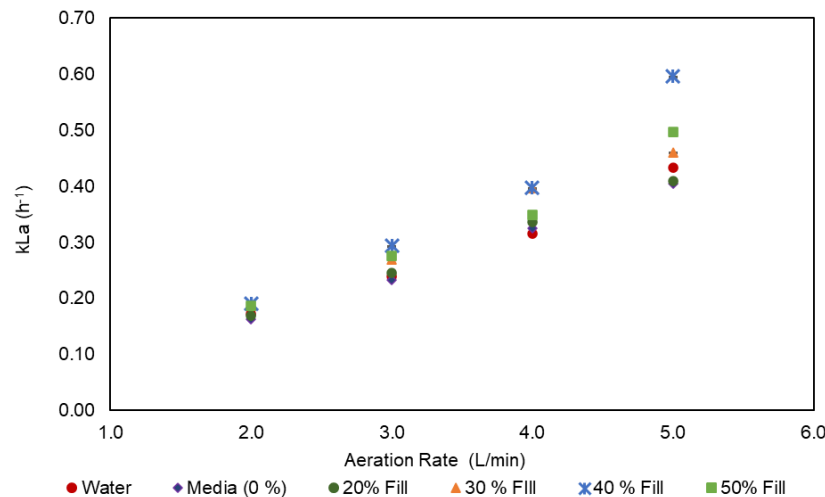


Figure 6-6: Trend of k_La with variation in fill fraction and aerator intensity for this moving bed reactor system

Figure 6-6 demonstrates the relationship between aeration rate and k_La , at varying carrier fill percentages, with the higher aeration rates yielding the highest mass transfer and specifically at 40% carrier fill %. However, at larger scales, high aeration rates are not always desirable for the reactor operation due to the increased operating costs, as well as surface splashing as noted by Jing et al. (2009). Further high aeration rates can result in excessive foaming in biologically active systems.

For this reactor system, the optimal operating range was found at a fill rate of 40 % and an aeration rate between 3 and 5 L/min. It was observed that aeration rates higher than ≥ 5 L/min, resulted in surface splashing at the lid of the reactor. This could result in excessive foaming, increasing the likelihood of contamination and higher risk of biological spills.

6.3 Hydrodynamic Studies

6.3.1 Introduction

The mixing time of a bioreactor is defined as the amount of time liquid takes for the contents to achieve a degree of homogeneity after injection of a tracer (Doran, 1995; Ascanio, 2015). A hydrodynamic study was performed to assess the mixing times and profiles in the MBBR and determine any potential dead-zones in the bioreactor that the mixing time and hydraulic residence times were compared to ensure that the continuous reactor feed was well mixed within a time-frame significantly shorter than the hydraulic residence time.

6.3.2 Methodology

The methodology to determine the mixing times is described in Section 3.4.2.

6.3.3 Results and Discussion

The batch STR cultivations at room temperature described in Section 4.5.2 demonstrated that retention times between 5.3 and 10 hours provide a range of potential critical dilution rates at which cell washout could occur should dilution rates exceed this. At the time of investigating the mixing studies, 6 hours was deemed a reasonable initial retention time, doubling twice thereafter to investigate two other retention times. A retention time of longer than 24 hours was not investigated, due to the high possibility of substrate limitation in the continuous fermentations. The mixing time in this study is the time taken for the reactor to change from pink to completely transparent.

Similar mixing patterns were observed for each of the residence times. The feed entered the reactor through the port situated just above the liquid level and effluent was removed at the liquid level. Colour change within the reactor was gradual and even in distribution, signifying that the aeration and motion of the carriers provided a well-mixed environment with no visible concentration gradients or dead zones.

Table 6-3 summaries the results obtained at the various residence times investigated. A carrier fill percentage of 40% was used, as this was determined to be optimal for the system in the mass transfer studies reported in Section 6.2. Based on the results of the mass transfer studies, it was evident that operating at a range of 3 – 5 L/min would be optimal. Therefore, the aeration rate for the hydrodynamic study was set at 3 L/min to determine what the lowest mixing time would be within that range.

The results demonstrated that the mixing times were all significantly shorter than the residence times. Faster liquid flowrates resulted in a decrease in the mixing time, which is expected given that each unit of liquid had a shorter retention time within the reactor under continuous. There was no visual evidence of short circuiting, hence it is reasonable to conclude that the system is well-mixed under the conditions investigated.

Figure 6-7 to Figure 6-9 show a time lapse of the mixing profiles at each of the liquid flowrates tested. The direction of flow is indicated by the arrows on the first image in each figure.

Table 6-3: Results of the hydrodynamics studies for the MBBR at varying liquid flowrates showing the mixing times with a carrier fill percentage of 40% and aeration rate of 3 L/min.

Retention Time (hours)	Liquid flowrate (mL/min)	Mixing time (min)
24	3.817	40.5 $\pm$ 5.2
12	7.633	24.5 $\pm$ 3.8
6	15.283	12.5 $\pm$ 2.7



Figure 6-7: Time lapse showing the mixing profile in the MBBR at a 24 hour retention time and aeration rate of 3 L/min.



Figure 6-8: Time lapse showing the mixing profile in the MBBR at a 12 hour retention time and aeration rate of 3 L/min.



Figure 6-9: Time lapse showing the mixing profile in the MBBR at a 6 hour retention time and aeration rate of 3 L/min

6.4 Continuous cultivation of MBBR with enhanced *B. subtilis* culture

6.4.1 Introduction

The success of the MBBR is directly linked to the establishment of microbial biofilms on the surface of the MBBR carriers (Biswas et al., 2014). The biofilm formation on the carriers is a critical factor in ensuring that solid and hydraulic residence times are decoupled, thereby allowing high flowrates and minimal cell washout without compromising reactor performance. The ideal biofilm in the MBBR process is typically thin (usually less than 100 μm) and evenly distributed. Thinner biofilms allow for efficient mass transfer between the bulk liquid and biofilm (van Haandel & van der Lubbe, 2012; Morgan-Sagastume, 2018). Attachment is not typically seen on the external surface due to the motion of the carriers and effects of 'bumping'.

Prior to the commencement of continual experiments, attachment studies were conducted to confirm biofilm attachment with Isolate 1 on the Annox Kaldnes® K3 carriers. The methodology and results from these studies are presented and discussed. Following the attachment studies, the experimental equipment setup and plan for the continuous cultivation is detailed. Thereafter the inoculation and start-up of the continuous cultivation is described, with the key results being presented and discussed in Section 6.5.

6.4.2 Biofilm attachment studies

Biofilm attachment and the pattern of development on MBBR carriers has not been widely studied (Biswas et al., 2014). The literature available is typically for application of large-scale MBBRs for use in industrial or municipal wastewater. From the information available, attachment on bare carriers takes 3 to 6 weeks.

Experimental approach

The main goal of the attachment studies was to ensure that Isolate 1 formed biologically active biofilm on the Annox Kaldnes™ K3 carriers that were to be used in the continuous cultivation. To start the acclimatisation, a 5 L round-bottomed flask was fitted with a fine bubble air curtain (commonly used in fish tanks) to provide uniform medium-bubble aeration across the lower cross-sectional area of the flask. The flask was kept at room temperature and filled with 3 L half strength MME. An inoculum of Isolate 1 was used to achieve a starting OD_{600} of 0.1. The bare carriers were chemically sterilised in 2% (vol/vol) hydrogen peroxide solution for approximately 12 hours and rinsed with sterile deionised water, before placing them into the sterile flask with suspended culture. The inoculum was transferred aseptically under laminar flow hood from the shake flasks into the sterile vessel containing the carriers. The experimental setup is shown in Figure 6-10 and Figure 6-11.

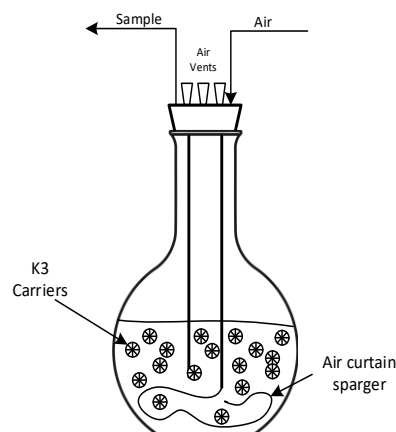


Figure 6-10: Schematic of setup used for attachment studies

The medium was replenished daily (every 18 to 24 hours) with 1.5 L half strength MME and the pH was manually adjusted between 6.5 and 7.0 with 1M H_2SO_4 during the day, as required. Culture (1.5 L) was removed from the flask through the sample port, by creating positive pressure (blocking vents filled with sterilised cotton wool) within the vessel through the aeration. Fresh medium was added under laminar fume hood. The biofilm development was monitored weekly through visual inspection of the carriers. Noticeable biofilm was observed after approximately two weeks by the change in motion (improved suspension as a result of biofilm attachment increasing the density of the carriers) and colour change. After acclimatising for four weeks, images were taken using Scanning Electron Microscope (SEM) to inspect the surface of the carriers for attachment of rod-shaped *Bacillus subtilis*. These results are shown Figure 6-13 and Figure 6-14.

Attachment studies results

Figure 6-11 shows the progression of growth and attachment. Image A demonstrates the aeration and carriers prior to inoculation. The carriers were still bare, and there was minimal suspension due to the density being slightly lighter than water (0.95 g/m^3) without microbial attachment. Image B shows the carriers starting to become more neutrally buoyant after a few days in the suspended culture. After four weeks, extensive biofilm development was evident and the carriers showed good suspension (Image C).

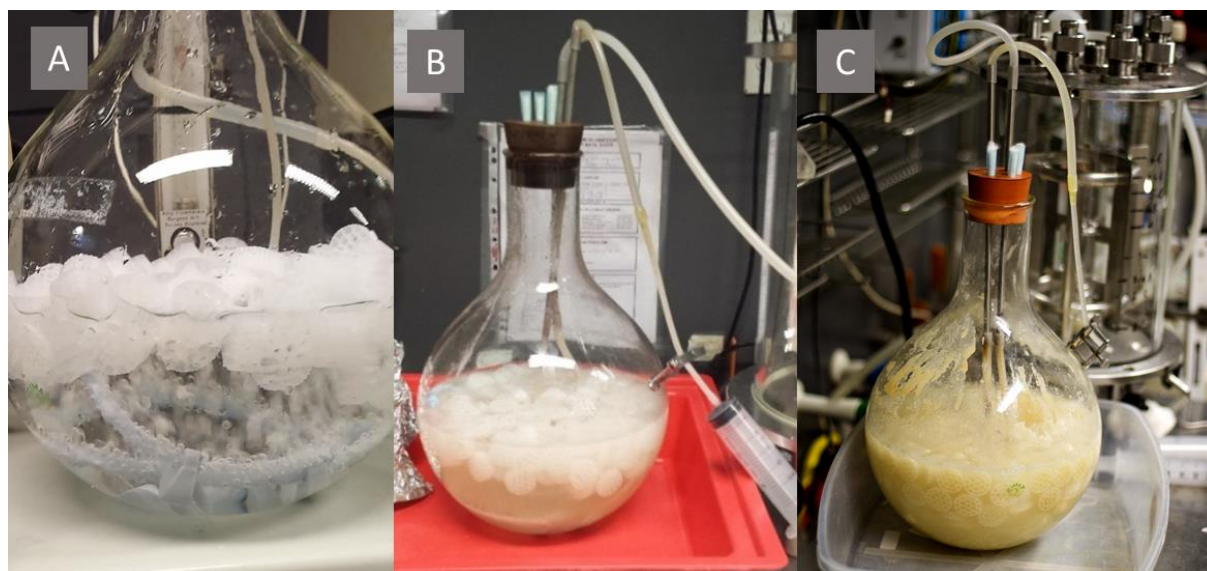


Figure 6-11: (A) Air curtain aeration providing even distribution of air. (B): Carriers after 5 days of acclimation. (C): Carriers after four weeks of acclimation showing dense culture, biofilm development on walls of the flask and carriers.

In Figure 6-12, the change in appearance of the carriers after four weeks was strongly evident. A thin and largely even biofilm developed across the majority of the carriers. These results are a confirmation that *Bacillus subtilis*, and specifically the isolated wild-type *Bacillus subtilis* such as Isolate 1 used in this study, are able to form robust biofilms consisting of EPS as well as γ -PGA (Morikawa et al., 2006).

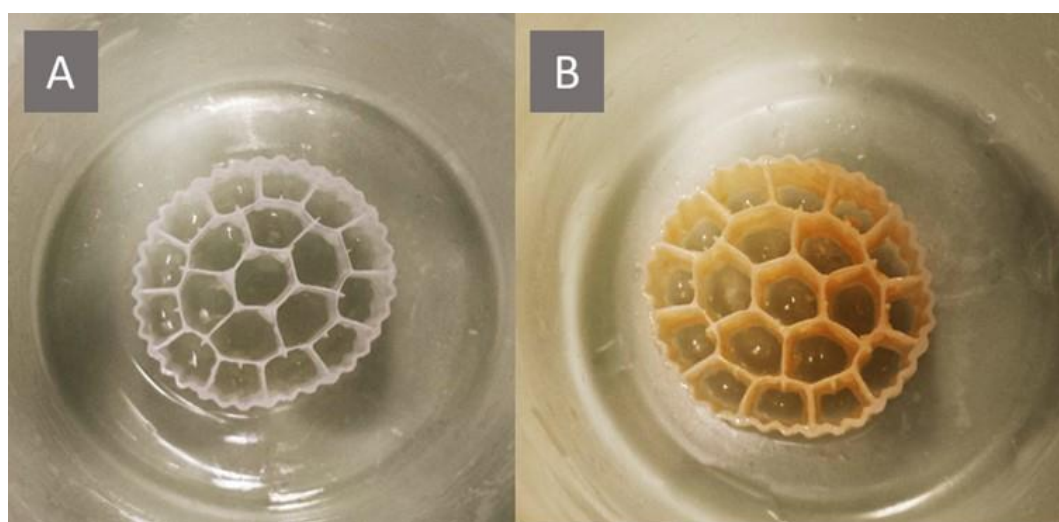


Figure 6-12: (A) Control: carrier with no attachment (B) Acclimatised carrier: after acclimatisation lasting 4 weeks

SEM imaging confirmed the formation of densely packed rod-shaped cells on the carrier surface in Figure 6-13 and Figure 6-14. There was visual evidence of connective structures (EPS, γ -PGA) between the cells, similar to those found in biofilm studies by Morikawa et al. (2006), who investigated biofilm formation by a *Bacillus subtilis* strain that produces γ -GPA. These structures are highlighted in the micrographs and show the structures between the cells.

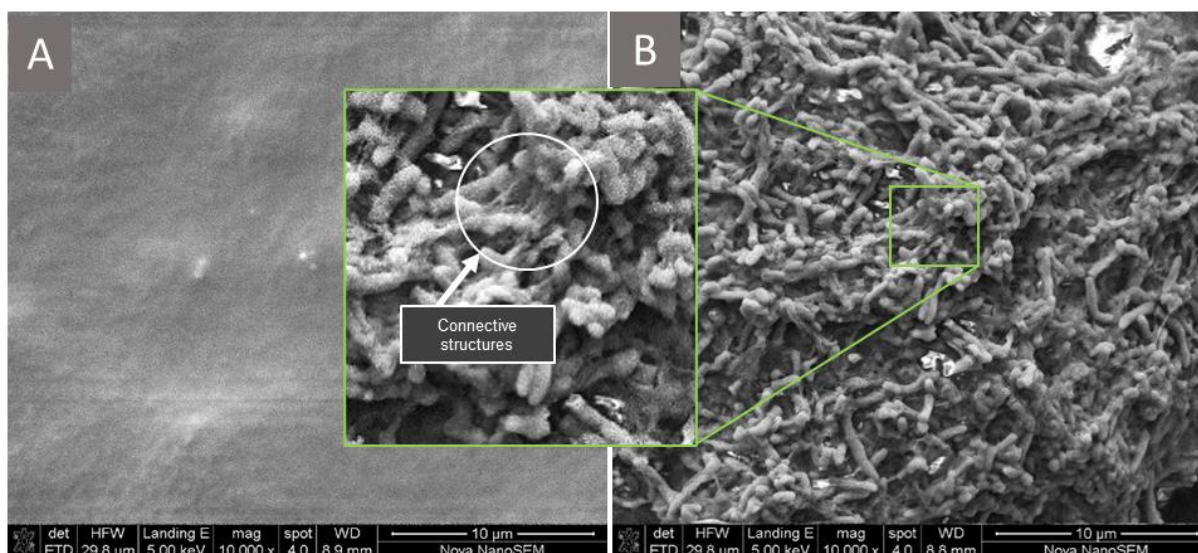


Figure 6-13: SEM images, magnification 10 000 x: (A) control carrier with no attachment on the carrier surface (B) carrier surface with bacterial attachment of rod-shaped bacteria.

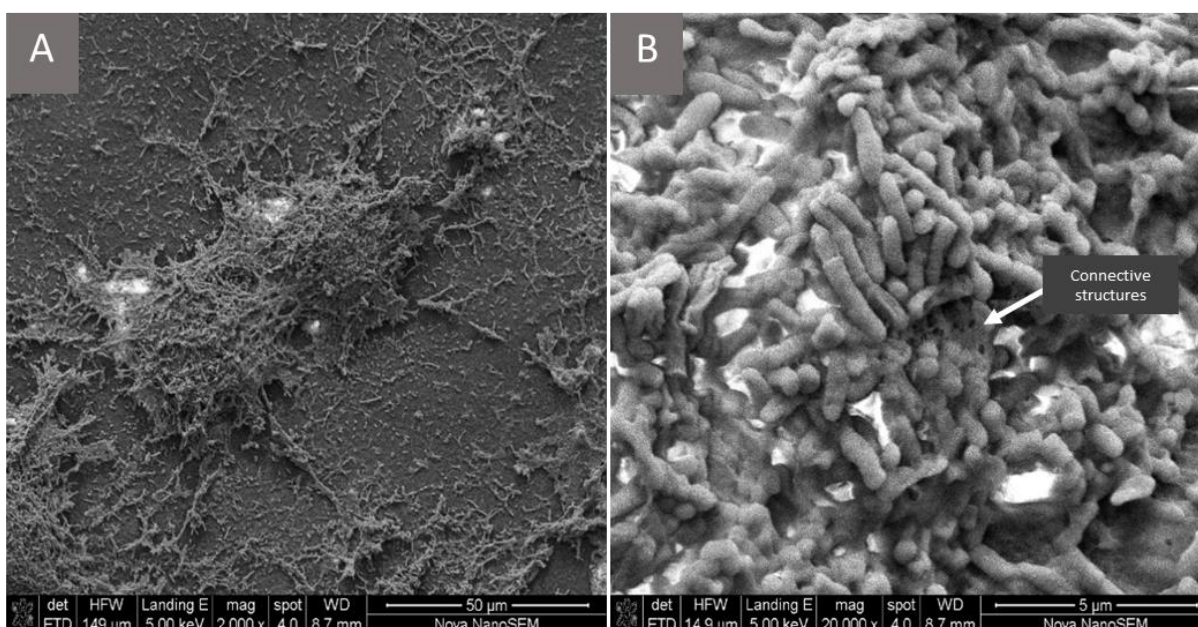


Figure 6-14: SEM images: (A) bacterial attachment, magnification 2 000 x (B) bacterial attachment showing packed rod-shaped bacteria, magnification 20 000x, showing individual rod-shaped bacterial cells.

It was also observed that the biofilm on the carriers was firmly attached and difficult to remove. During sample processing to prepare the carriers for SEM imaging, where the carriers needed to undergo various stages of dehydration in an increasing ethanol to water ratio, almost no biofilm detached from the carriers.

The findings from this attachment study indicated that during the start-up of the MBBR, a period of 2 to 4 weeks is required to achieve biofilm attachment. The surface roughness of the carrier aids in improving the rate of biofilm development. Therefore, the carriers used in this study were also used for the MBBR start-up.

6.4.3 Experimental setup

The MBBR was set up to facilitate continuous cultivation as shown in Figure 6-15. A photograph of the setup is shown in Figure 6-16.

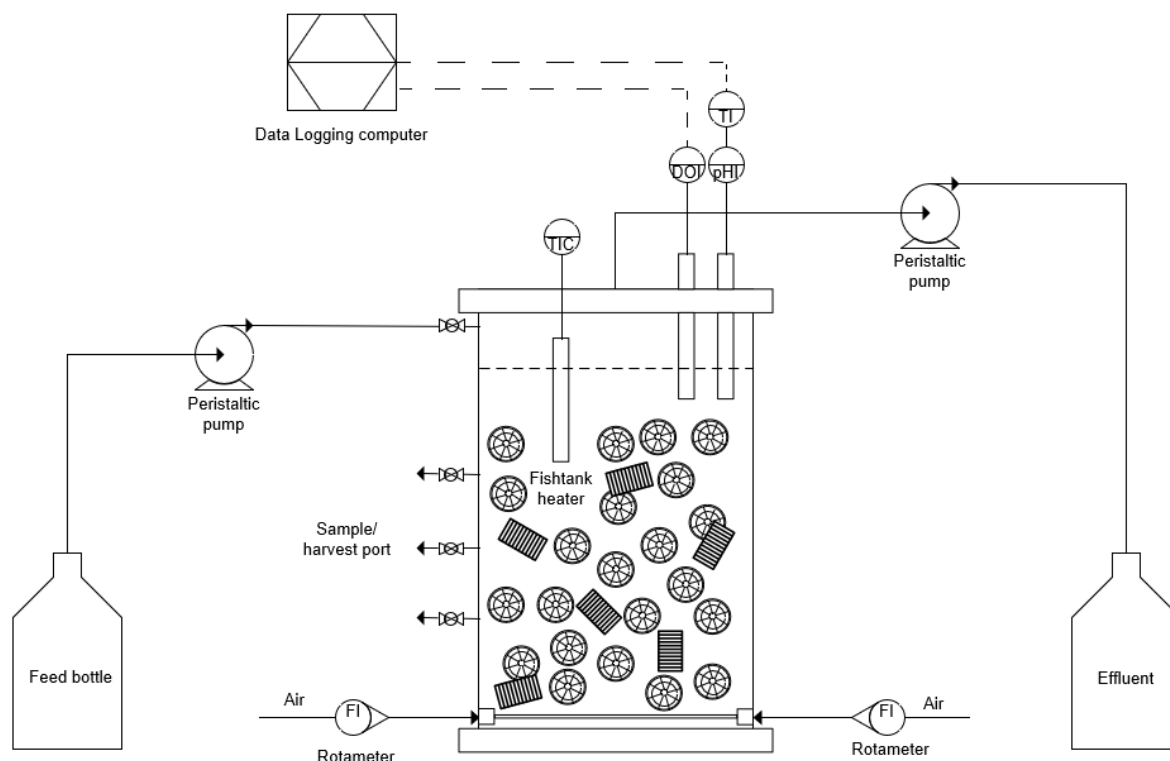


Figure 6-15: Schematic of the MBBR setup

Half strength MME was pumped from a feed bottle in through the influent port, located above the liquid level. The culture was pumped out of the effluent port, positioned at the working volume level, at the same flowrate as the feed using a four-headed pump (two pump heads for influent and effluent) to ensure constant volume control. The influent and effluent double-pump head was calibrated for a range of flowrates across the range pump settings. Calibration curves for the influent and effluent pump heads are detailed in Appendix C.1.

The system pH was monitored using a pH probe (Mettler Toledo InPro® 3250/120/Pt100, Thermo Scientific, RSA). Automated control of pH was not used; however, the feed pH was set to 7.5 ± 0.5 to maintain the pH within 6.5 ± 0.5 . This approach maintained the pH close to 6.5.

Dissolved oxygen was monitored with a DO probe (Mettler Toledo InPro® 6050/120, Thermo Scientific, RSA). Aeration was set at a fixed rate of 4 L/min due to excessive foaming and surface splashing at higher aeration rate. While an aeration rate of 5 L/min, showed the best volumetric gas-liquid mass transfer, it resulted in excessive surface splashing and foaming, despite antifoam 204 being included in the feed. Thus, to lower the risks of contamination and to health and safety, aeration was set to 4 L/min.

The temperature was set to 25 ± 2 °C, controlled by an aquarium (fish tank) heater (SICCE Scuba 25, Sam's Aquarium, RSA) fitted into the lid of the MBBR. Liquid samples were taken from the sample ports, and carrier samples were removed via the lid flap on the bioreactor

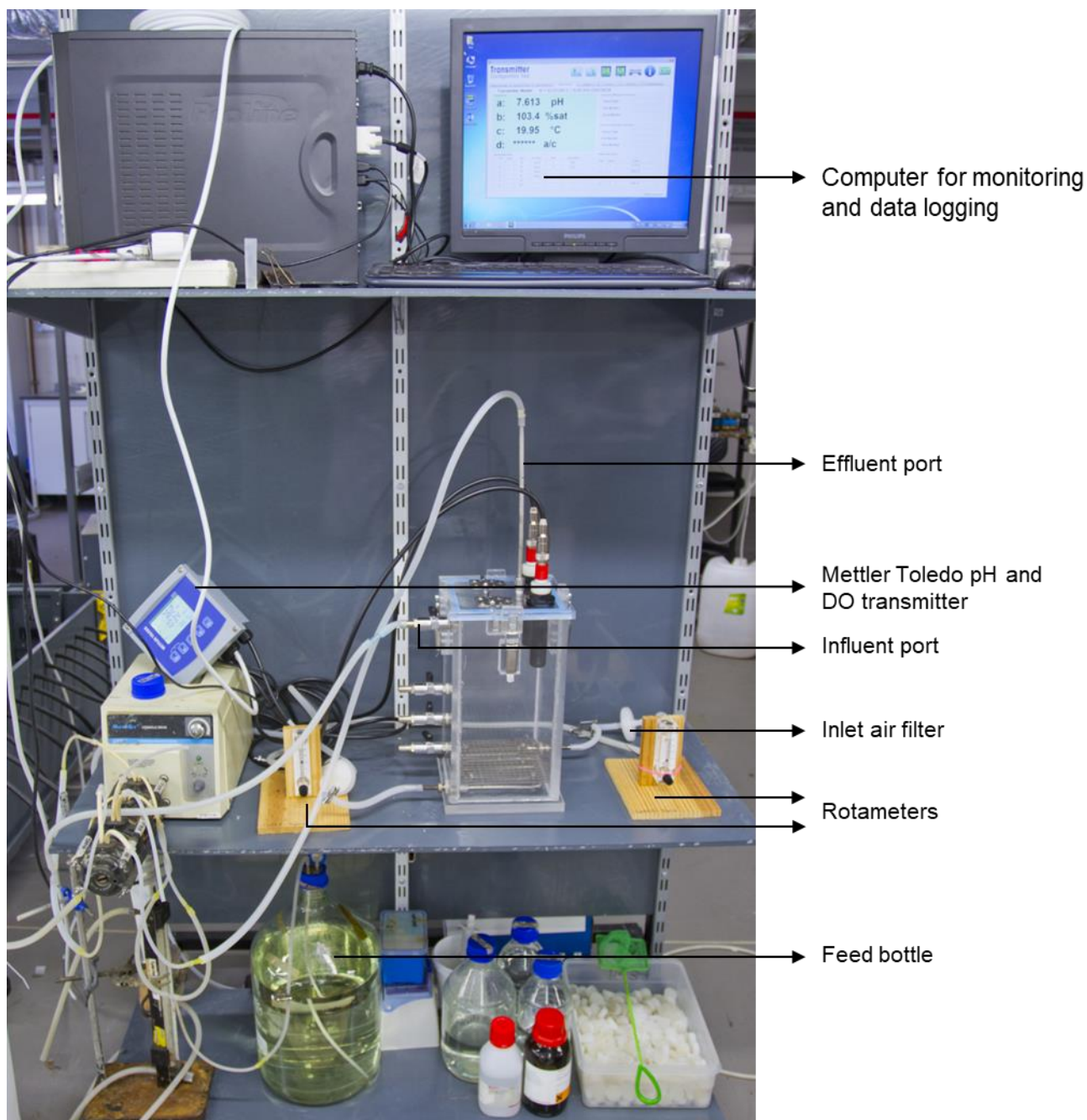


Figure 6-16: Continuous MBBR setup in the Water Quality Lab at UCT

The reactor was chemically sterilised with a solution of hydrogen peroxide (2% vol/vol) and rinsed with sterile water prior to inoculation. The feed and effluent lines were autoclaved at 121 °C for 30 minutes. The carriers used during the carrier acclimatising studies were used, as well as additional bare carriers to ensure a fill percentage of 40% for a working volume of 5.5 L. The bare carriers were chemically sterilised and rinsed with sterile water before start-up. The system as whole could not undergo heat sterilisation due to the Perspex material of construction, however it was operated hygienically. This approach is representative of a real-world WWBR application in which a bioreactor would be run under non-sterile conditions.

The MBBR was inoculated with a 4 L culture of Isolate 1 at OD₆₀₀ of 3 (Figure 6-17) to ensure an enriched and high-density starter culture. This was transferred to the MBBR and the remaining volume made-up with 1.5 L sterile half strength MME. The effluent port was adjusted to ensure that a constant

volume of 5.5 L was maintained. Waste collected from the effluent port was directed into a 20 L drum containing biocide.

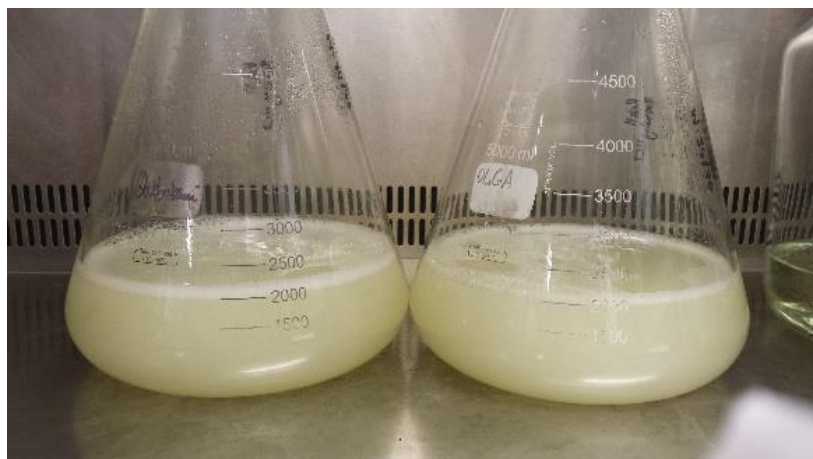


Figure 6-17: Erlenmeyer shake flasks with *B. subtilis* culture used to inoculate the MBBR

Prior to the bioreactor start-up procedure (Figure 6-18), the DO probes were calibrated in sterile media, and polarised for 6 hours. The pH probe was calibrated using pH 4 to 7 calibration standards. The sterile media was drained from the reactor, and the chemically-sterilised carriers added into the bioreactor, along with the inoculum and fresh medium.

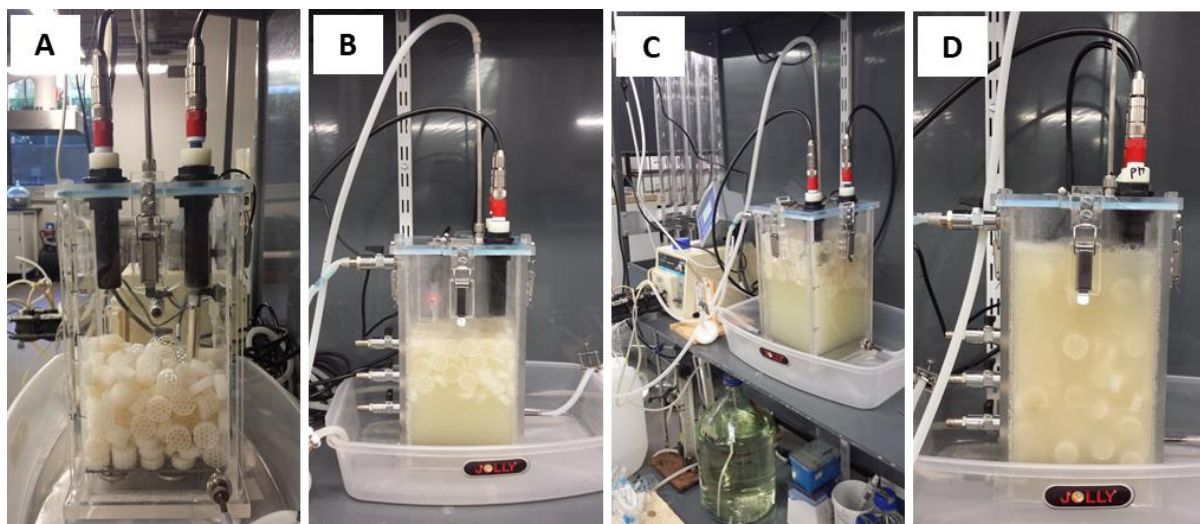


Figure 6-18: Series of images showing the start-up procedure of the continuous experiments. From left to right: **A** – acclimatised carriers placed into washed and cleaned MBBR at 40% fill ratio. **B**: *B. subtilis* inoculum from shake flasks used to inoculate the reactor. **C** – sterile MME used to make up remaining volume in reactor to working volume of 5.5 L. **D** – Aeration and feed started and commencement of the continuous experiment.

6.4.4 Experimental plan

The continuous cultivations were run at a range of retention times with the key aim of testing the hypothesis that a biofilm-based technology would decouple the hydraulic and biomass residence times; that is, when retention times corresponded to dilution rates above the critical point which approximates the maximum specific growth rate of the species, washout of the retained cells would not occur and the system could still achieve nutrient removal (either total or partially).

The approach to the retention times was done in a stepwise manner, starting with the longest retention time (24 h) and decreasing it until it reached the lowest retention time (2 h). The longest retention time was run first, to ensure that adequate biofilm attachment was established on the carriers. This was important to ensure decoupling of solid and hydraulic residence times and preventing cell washout. Each retention time was maintained until a minimum of 4 steady state retention times were achieved,

thereafter it was decreased to the next retention time. The retention times and corresponding flowrates that were investigated are listed in Table 6-4. It

Table 6-4: Retention times and flowrates investigated in the continuous MBBR cultivation

Retention Time (h)	Dilution Rate (h^{-1})	Flowrate (mL/h)
24	0.042	229
22	0.048	250
15	0.067	367
10**	0.100	550
6.6**	0.152	833
4	0.250	1375
2	0.500	2750
**Dilution rates equal to the maximum specific growth rates of the room temperature batch cultivations (Table 4-4).		

A starting retention time of 24 h was selected to ensure that biomass attachment and acclimation to the continuous conditions was achieved. The medium in the attachment studies (Section 6.4.2) was refreshed once daily and achieved successful biofilm attachment with an approximate 'retention time' of 24 hours.

Samples were taken daily from the bulk fermentation broth and analysed for offline pH, OD_{600} , CDW, HPLC analysis for glucose and citric acid and crude γ -PGA concentrations. SEM analysis was performed at 24 h after 2 weeks of continuous operation, as confirmation of biofilm attached before gradually increasing the feed flowrate. The final two retention times of 4h and 2h were selected to be lower than the estimated critical dilution rates, in order to investigate the effects of a high hydraulic load on the systems and test whether the system maintained nutrient removal and productivity under conditions of the removal of planktonic cells. Any microbial activity (consumption of nutrients, maintaining optical densities or cell dry weight) that is observed under very high flowrates (lower than critical dilution rate), can be attributed to activity from retained biomass or active biofilm attachment.

6.5 Continuous cultivation results and discussion

6.5.1 Data processing

The raw data collected at each retention time was analysed for periods where steady state was estimated to have been achieved. The steady state period was defined as the period over which there was the least degree of deviation ($\sim <20\%$ in either OD_{600} or CDW, or 3 to 5 retention times).

Steady-state continuous operation

Under continuous steady-state conditions, the inlet and outlet flowrate are equal, thus maintaining the liquid level. The residence (or retention) time is the inverse of the dilution rate and is defined by Equation 6-1:

$$\tau = \frac{1}{D} = \frac{V_R}{F} \quad \text{Equation 6-1}$$

where τ is retention time (h), D is dilution rate (h^{-1}), V_R is reactor volume (L) and F is feed flowrate (L/h).

Organic loading rate of glucose and citric acid

The total organic loading rate (OLR), glucose (OLR_G) and citric acid (OLR_{CA}) loading rate was expressed in molC/L/h and shown in Equation 6-2:

$$\text{OLR} = \frac{F \times S_f}{V_R} = DS_f \quad \text{Equation 6-2}$$

where F is the flowrate of feed (L/h), S_f is the concentration of glucose ($S_{G,i}$) or citric acid ($S_{CA,i}$) in molC/L and V_R is the reactor working volume. The units of molC were converted from a mass basis (g/L/h) to mole carbon basis (molC/L/h) using Equation 6-3:

$$\text{molC} = g \times \frac{\#C \text{ atoms}}{MW} \quad \text{Equation 6-3}$$

where the MW (g/mol) is for glucose or citric acid, and #C atoms refer to number of carbon atoms in either glucose or citric acid. This approach was taken due to the consumption of both organic carbon sources in the feed.

Biomass and γ -PGA productivity

The volumetric rate of biomass production in the continuous reactor, Q_x , is represented by Equation 6-4, under steady state

$$Q_x = \frac{F_x}{V_R} = D x \quad \text{Equation 6-4}$$

where D is the dilution rate (h^{-1}), x is biomass concentration (g/L) and V_R is the reactor volume.

Similarly, the volumetric rate of γ -PGA formation, Q_P , is:

$$Q_P = \frac{F_P}{V_R} = D P \quad \text{Equation 6-5}$$

where D is the dilution rate (h^{-1}), P is γ -PGA concentration (g/L or mol[PGA]_n/L) and V_R is the reactor volume (L).

Due to the varying chain length of the polymer and the fact that a total mass balance around the MBBR system was not possible, calculations of yields and productivity for γ -PGA were based on a single monomer of γ -PGA i.e. glutamic acid monomer. The monomer of L-glutamic acid has a molecular weight of 129 g/mol and chemical structure of $\text{C}_5\text{H}_7\text{O}_3\text{N}$. For consistency, the organic load was also reported in terms of moles of total organic carbon (glucose and citric acid). The concentration of γ -PGA on a mass basis was converted to a mole basis by dividing the mass concentration by the molecular weight of one glutamic acid monomer.

Biomass and γ -PGA yield coefficients

The yield coefficients were calculated for biomass and γ -PGA on a mass and molar basis. The biomass yield for each steady state retention time was calculated as follows:

$$Y_{\frac{X}{S}} = \frac{\bar{X}}{S_f - S_R} \quad \text{Equation 6-6}$$

where $\bar{X}$ is the average steady-state cell dry weight (g), S_f is the substrate fed (g) and S_R is the average residual glucose (g).

Similarly, the yield of γ -PGA was calculated on both on a mass (g/g) and mole basis (mol[PGA]_n/molC) as follows:

$$Y_{\frac{P}{S}} = \frac{\bar{P}}{S_f - S_R} \quad \text{Equation 6-7}$$

where $\bar{P}$ is the average crude γ -PGA produced (g or mol[PGA]_n), S_f is the substrate fed (molC) and S_R is the residual substrate (molC).

6.5.2 Steady state results

The MBBR results at each retention time are detailed in Figure 6-20. These were averaged over the steady state time period where it was estimated that the system had reach an equilibrium. These estimations became increasing difficult as the retention time approached the critical dilution (nearing cell washout in the system) and exceed the estimated critical dilution rate, due to the disruption of steady state parameters and rate of feeding. This is visually evident in the results at 4h and 2h, in Table 6-5, Figure 6-19 and Figure 6-20 when the planktonic biomass concentration decreased and residual substrate (glucose and citric acid) increased, indicating that washout of planktonic cells is occurring.

Table 6-5: Summary table of steady state analytical results, at each retention time

Retention Time (h)	OD ₆₀₀	CDW (g/L)	Residual glucose S _G (g/L)	Residual citric acid S _{CA} (g/L)	Crude γ -PGA P (g/L)
24	5.37 $\pm$ 0.795	3.76 $\pm$ 0.727	0.00 $\pm$ 0.00	0.03 $\pm$ 0.12	13.11 $\pm$ 0.97
21	4.82 $\pm$ 0.268	3.09 $\pm$ 0.415	0.04 $\pm$ 0.11	0.16 $\pm$ 0.44	13.42 $\pm$ 0.47
15	4.71 $\pm$ 0.399	2.89 $\pm$ 0.243	0.00 $\pm$ 0.00	0.01 $\pm$ 0.03	11.59 $\pm$ 0.65
10	4.42 $\pm$ 0.319	2.97 $\pm$ 0.353	0.00 $\pm$ 0.00	0.50 $\pm$ 0.84	9.38 $\pm$ 0.24
6.6	3.93 $\pm$ 0.332	3.02 $\pm$ 0.164	0.00 $\pm$ 0.00	0.04 $\pm$ 0.007	11.33 $\pm$ 0.47
4	2.72 $\pm$ 0.273	2.08 $\pm$ 0.113	0.13 $\pm$ 0.23	0.47 $\pm$ 0.62	8.98 $\pm$ 0.75
2	1.58 $\pm$ 0.162	1.68 $\pm$ 0.302	5.79 $\pm$ 0.91	4.11 $\pm$ 0.76	15.04 $\pm$ 2.59

Based on the estimated $\mu_{\max}$ values calculated in Section 4.5.2, the critical retention times of 10 and 6.6 were the range at which it was estimated that planktonic cell washout would occur. The system showed relative stability in terms of growth and γ -PGA production between 21h and 10h which is demonstrated by the flat graph profiles in Figure 6-19 . Planktonic cell concentrations started to decrease when the retention time was lowered from 6.6h to 4h. This is evident in the decrease in OD600 from 3.93 $\pm$ 0.33 to 2.72 $\pm$ 0.16 as well as the CDW from 3.02 $\pm$ 0.16 g/L to 2.08 $\pm$ 0.11 g/L, along with an increase in the residual glucose and citric acid concentrations. The operation of the MBBR at a low retention time of 2h was intentionally selected to assess the system behaviour under very high flowrates in which the microbial activity is chiefly attributed to attached biomass and biofilm development.

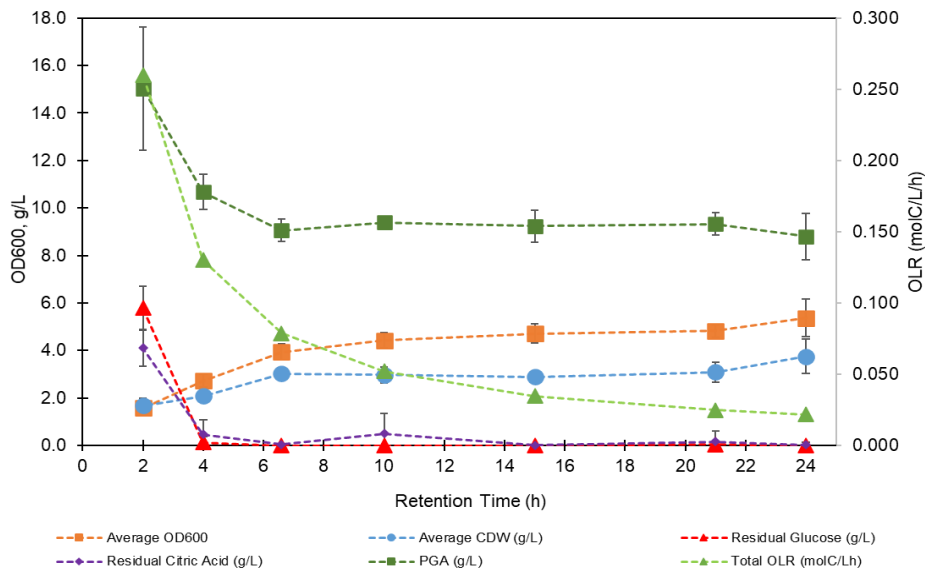


Figure 6-19: Time-based growth profiles and substrate utilisation, of average steady states at each retention time.

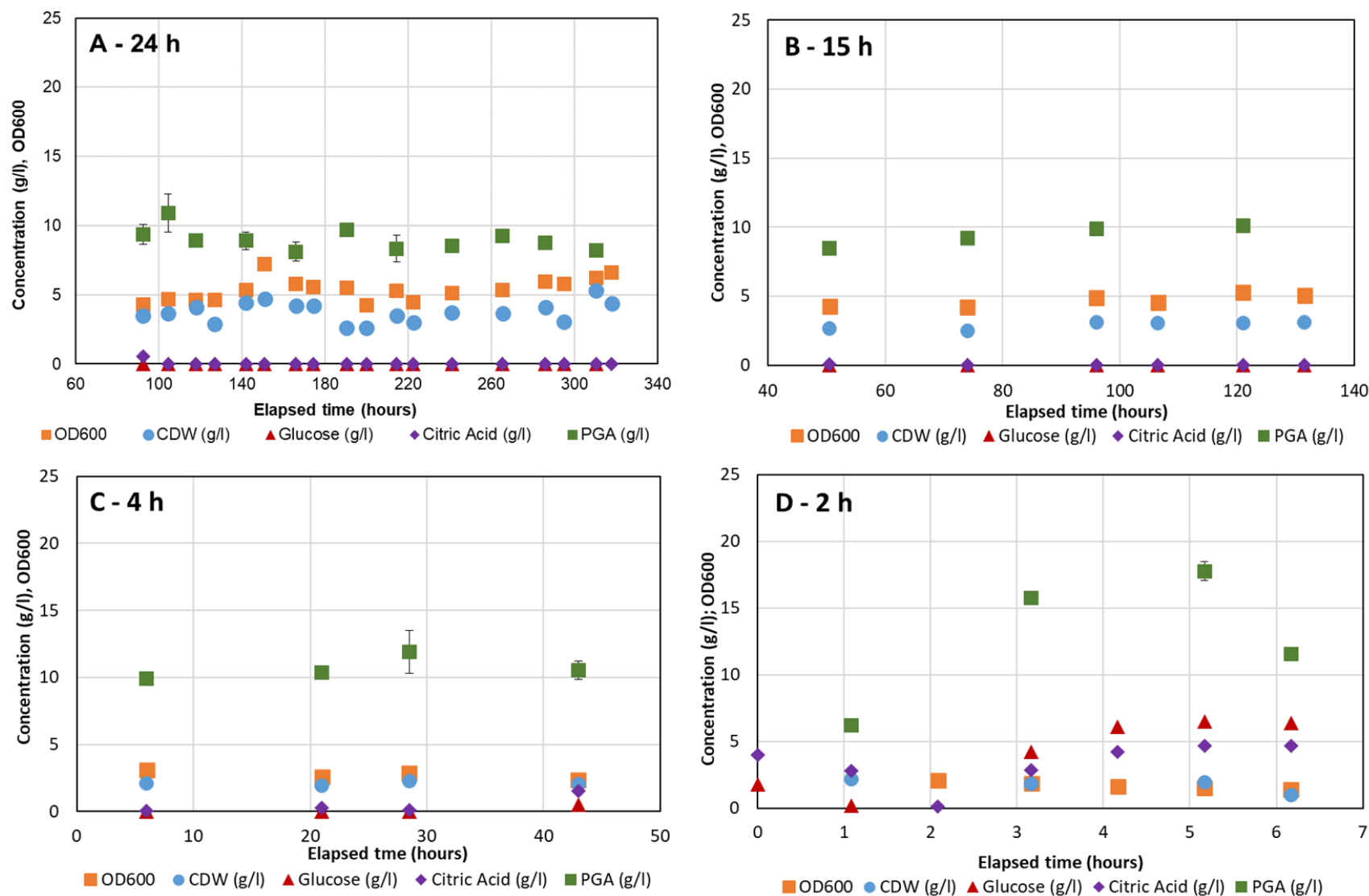


Figure 6-20: Steady state data at four different retention times: A: 24 hours, B: 15 hours, C: 4 hours and D: 2 hours, showing the change in OD600, CDW, substrate concentration and PGA concentration in bulk liquid, for the duration that each retention time was operated

The biofilm development on the carriers was captured through SEM imaging to monitor the level of attachment of the rod-shaped cells. The system was operated under non-sterile conditions, so monitoring the type of cells forming attachment was a way to ensure that two of the conditions for a WWBR bioreactor were met; that is, creating a niche environment providing an advantage for product formation and influencing the microbial community under non-sterile conditions. The first retention time of 24 hours was sampled for SEM imaging after 10 days of runtime and is shown in Figure 6-21. The cell attachment is evident with clear visual of EPS structures and attachment to the carriers.

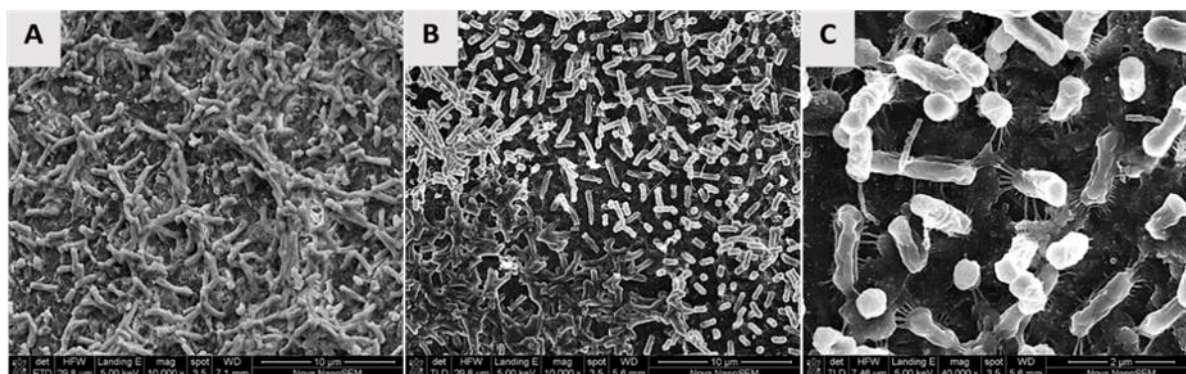


Figure 6-21: SEM images of a section of carrier at 24 h retention time. From Left to Right: A,B are at 10 000 x magnification. C is at 40 000 x magnification

There was a noticeable increase in cell density on the carriers at 10 h retention time in Figure 6-22. The biofilm was also thicker and deeper, with the carrier surface no longer visible, demonstrating the successful establishment of *Bacillus subtilis* biofilm.

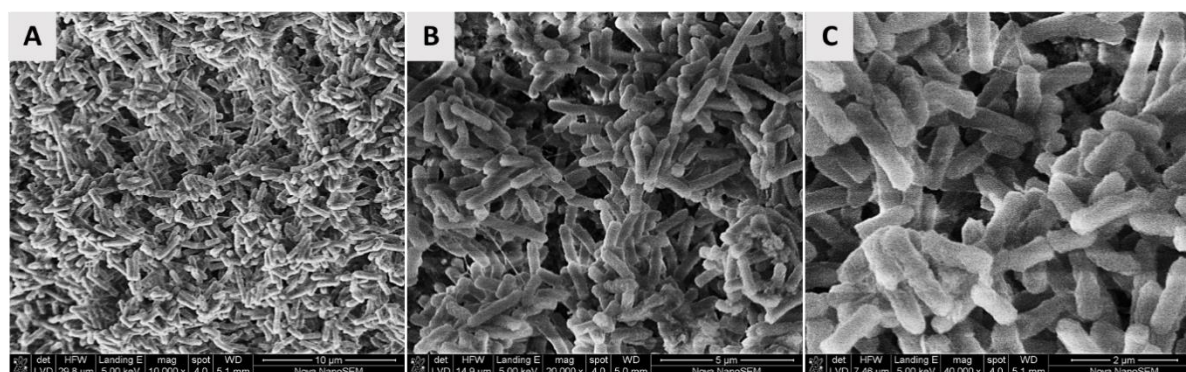


Figure 6-22: SEM images of a section of carrier at 10 h retention time. From Left to Right: A are at 10 000 x magnification. B is at 20 000 x magnification and C is at 40 000 x magnification

The biofilm imaging captured at 6.6 h showed a clear visual of the EPS and connective structure formation at 20 000 x (Image A) and 40 000 x magnification (Image B).

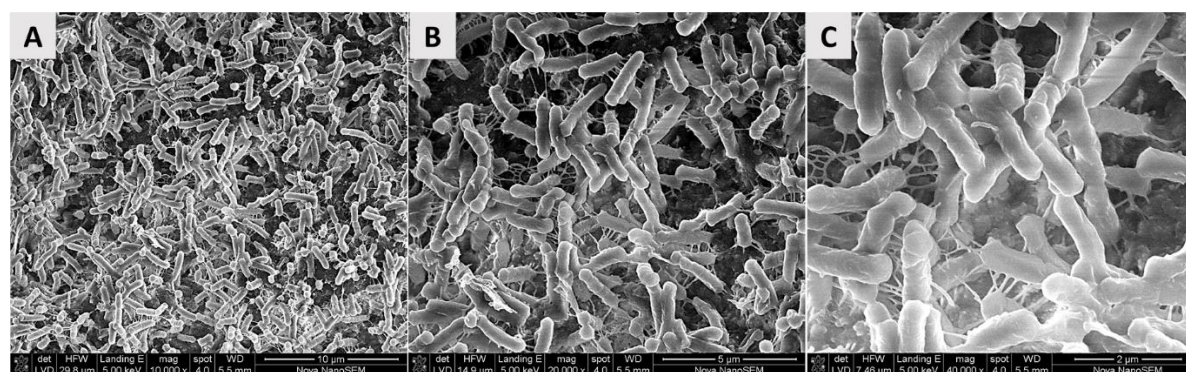


Figure 6-23: SEM images of a section of carrier at 6.6 h retention time. From Left to Right: A are at 10 000 x magnification. B is at 20 000 x magnification and C is at 40 000 x magnification

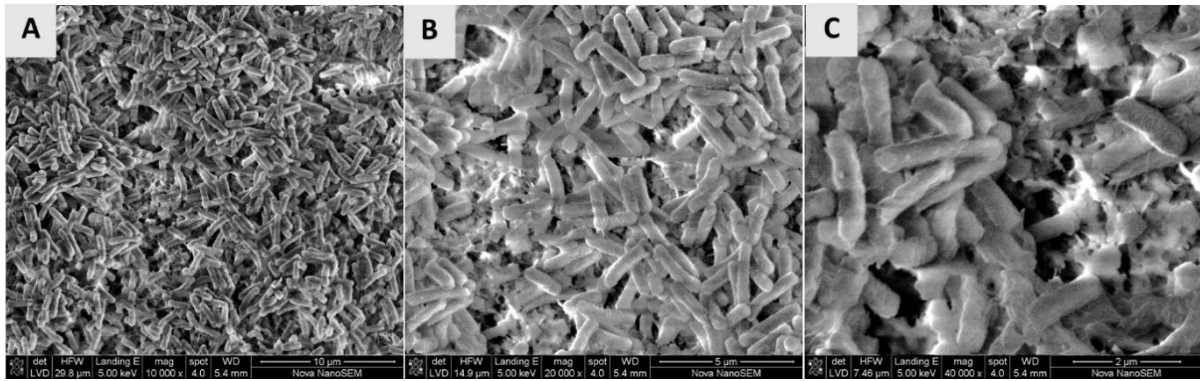


Figure 6-24: SEM images of a section of carrier at 4 h retention time. From Left to Right: A are at 10 000 x magnification. B is at 20 000 x magnification and C is at 40 000 x magnification

Figure 6-24 from 4 h showed a noticeable increase in the density of the attached cells from 6.6 h, especially in Image B (20 000x) and Image C (40 000x), along with further increase in density of the EPS structures. This result is significant as it signifies an increase in biofilm formation, and biomass retention within the reactor.

6.5.3 Effects of biomass retention on performance

In a traditional CSTR where there is no biomass retention, the relationship between dilution rate and biomass and residual concentration can be illustrated by the left-hand graph in Figure 6-25. At $D \sim 0$, nearly all the substrate is consumed, and the biomass concentration is at a maximum. As $D > \mu_{\max}$, S_R increases more rapidly and x tends to zero. The point at which x is zero and S_R is at a maximum, is where cell washout occurs (Doran, 1995; Stanbury et al., 1995).

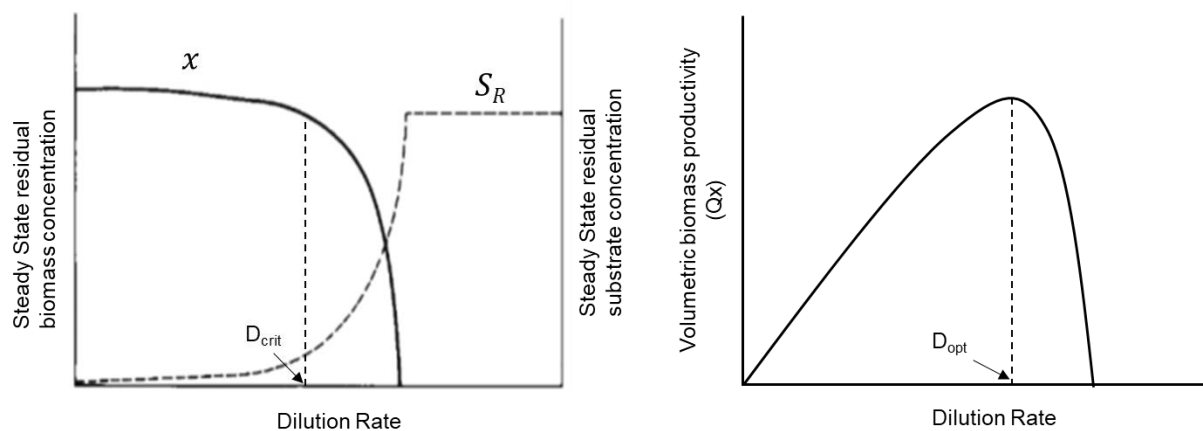


Figure 6-25: The effects of dilution rate on steady state biomass and residual substrate concentration in a CSTR (LH graph) as well as the effects on volumetric biomass productivity. Adapted from (Doran, 1995)

The biomass productivity as a function of the dilution rate shows that there is a point where biomass productivity is at a maximum. Typically, D_{opt} is very close to D_{crit} and operating at D_{opt} is not always practical due to small system changes resulting in large fluctuations in biomass and substrate concentrations. However, the flexibility around D_{opt} can be manipulated through the introduction of biomass retention such that at $D \gg D_{\text{crit}}$ cell washout does not occur. With this understanding about the impact of operating with biomass retention, the results of the MBBR were assessed and compared to the graphs in Figure 6-25.

The results in Section 6.5.2 were used to calculate the key performance characteristics of the MBBR in terms of productivity and yield for biomass and γ -PGA production. These results are summarised in Table 6-6.

Operating the MBBR at the calculated $\mu_{\max}$, resulted in γ -PGA productivity between 0.94 and 1.37 g/L/h. A study by Jiang et al. (2016) achieved a productivity of 0.94 g/L/h and similar studies in other reactor configurations using *Bacillus* species (see Table 2-3) show that the calculated productivities are realistic results for *Bacillus subtilis*.

Table 6-7: Summary table of steady state yields, productivity and loading rates, at each retention time.

Retention time	h	24	21	15	10	6.6	4	2
Dilution rate	h^{-1}	0.042	0.048	0.067	0.100	0.152	0.250	0.500
OLR _G	g/L/h	0.417	0.476	0.667	1.000	1.515	2.500	5.000
	molC/L/h	0.014	0.016	0.022	0.033	0.050	0.083	0.167
OLR _{CA}	g/L/h	0.250	0.286	0.400	0.600	0.909	1.500	3.000
	molC/L/h	0.008	0.009	0.012	0.019	0.028	0.047	0.094
OLR	molC/L/h	0.022	0.025	0.035	0.052	0.079	0.130	0.260
Q _x	g/L/h	0.156	0.147	0.193	0.297	0.458	0.519	0.839
Q _P	g/L/h	0.367	0.444	0.616	0.939	1.372	2.667	7.519
	mol[PGA] _n /L/h	0.003	0.003	0.005	0.007	0.011	0.021	0.058
Y _{X/S}	g/g	0.040	0.043	0.054	0.057	0.064	0.023	0.266
Y _{P/S}	g/g	0.094	0.129	0.171	0.181	0.193	0.117	2.381
	mol[PGA] _n /molC	0.0139	0.0192	0.0255	0.0272	0.0287	0.0172	0.2536

The experimental results for the MBBR are shown in Figure 6 26, and, when compared with Figure 6 25, demonstrate the key differences between a system with biofilm attachment, and one with a suspended culture. Theoretically, with no biofilm attachment (i.e., a suspended culture), it would be expected that at dilution rates of $> 0.152 h^{-1}$, biomass concentrations would rapidly approach zero as substrate concentration increased to reach the feed substrate concentration. As expected, with biofilm attachment, biomass concentration did not show any washout until the dilution rate was increased to 0.25 and $0.5 h^{-1}$. Even at a dilution of 3-fold $\mu_{\max}$, the planktonic biomass concentration remained at approximately half the maximum planktonic biomass concentration, suggesting constant re-seeding from the retained biomass.

Morikawa et al. (2006) reported that γ -PGA was a major EPS substance in biofilm formation in *Bacillus subtilis* and showed that there was a clear correlation between biofilm development, and γ -PGA formation. This is a significant finding for this research. Product yields increased with increasing dilution rates, as well as the level of biofilm formation on both the carriers and walls of the bioreactor.

Biomass yields ($Y_{X/S}$) were lower than the room temperature batch experiments for the majority of the continuous run (24h to 4h) owing to biomass retention on the carriers. The room temperature cultivations in Chapter 4 achieved average biomass productivities of approximately $0.288 \pm 0.007 g_x/g_s$, which agrees with the calculated theoretical maximum. These lower biomass yields are explained by the increased biofilm formation in the reactor. The progress was monitored by the fermentation culture characteristics as biomass retention was not measured with time; thus, the results are reflective of planktonic cells.

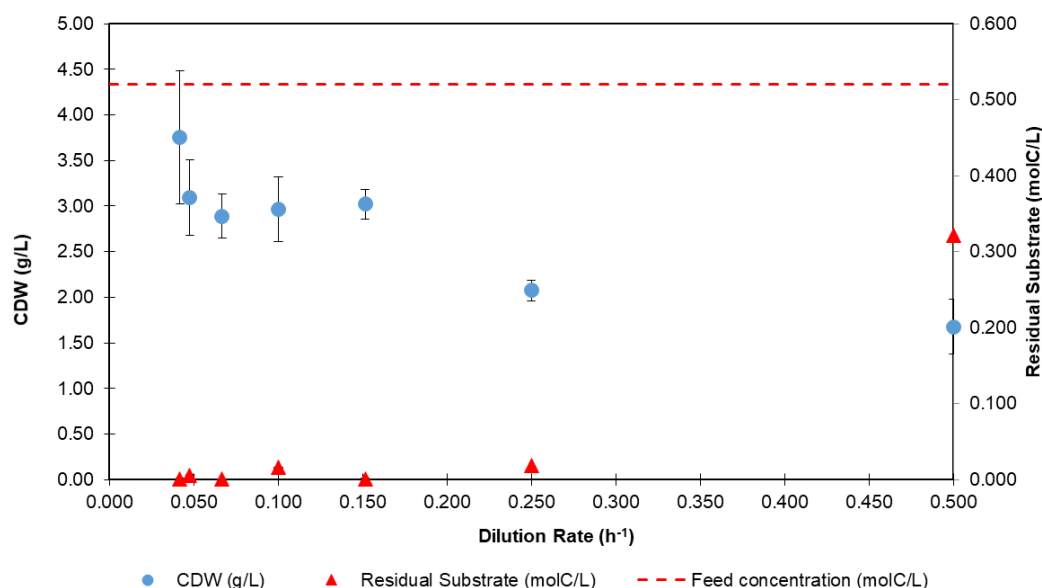


Figure 6-26: Effect of increasing dilution rate on the steady state biomass concentration and residual substrate concentration

As the dilution rates increased towards rates $\sim$ three times great than D_{crit} , the planktonic cell concentrations tended towards a concentration of approximately 1.5 g/L. An increase in the residual feed concentration was also observed. The effects of the biomass retention can be seen in Figure 6-26, because under suspended cell culture operating at $D \gg D_{crit}$, the planktonic cell concentration would sharply decrease and not taper off.

The effects of the biofilm attachment on the productivity of biomass and γ -PGA can be further illustrated in Figure 6-27. The linear relationship between volumetric productivity and dilution rate was shown for both biomass and γ -PGA. Once again, the role of biomass retention was evident with the 4h and 2h retention times. Instead of the values tending to zero, productivity continued to increase despite there being evidence of a decreased planktonic cell concentration (decreasing OD_{600} and CDW).

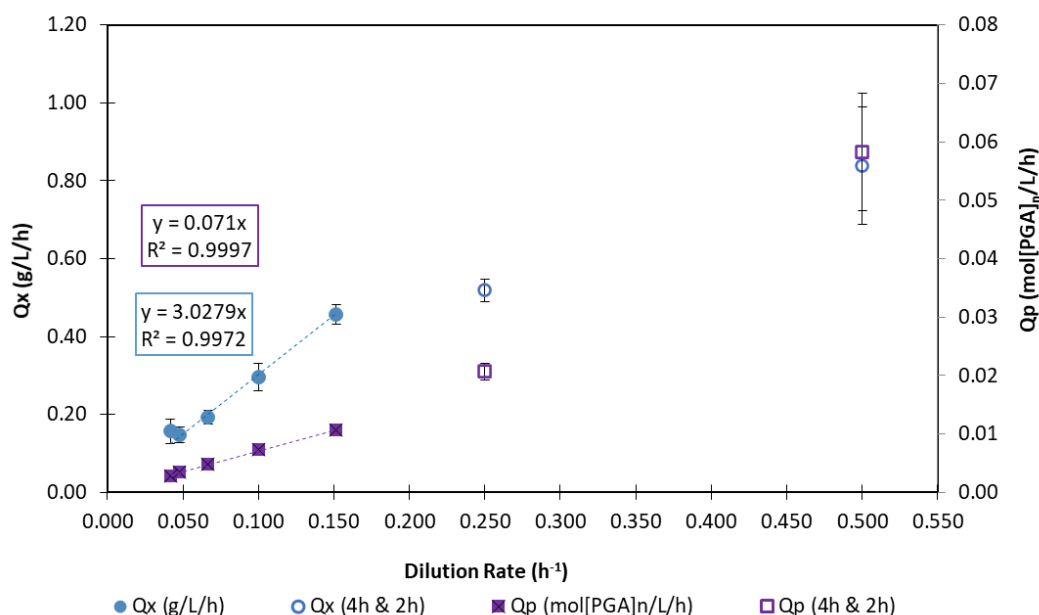


Figure 6-27: Relationship between volumetric productivity of biomass and γ -PGA with dilution rate

A plausible explanation for these results at the higher dilution rates, is that significant biofilm development is occurring, resulting in higher-than-expected productivities and yields of γ -PGA. The evidence for increased biomass retention is shown in the SEM imaging for 4h retention time, relative to

the imaging for 6.6h, 10h and 24 h. In Figure 6-28 (A), the substrate utilisation is shown to decrease from $\sim 99.9\%$ at 0.042 h^{-1} to $\sim 95\%$ at 0.500 h^{-1} . This is a relatively small decrease given that the dilution rates had increased almost 10 fold, and is again further evidence

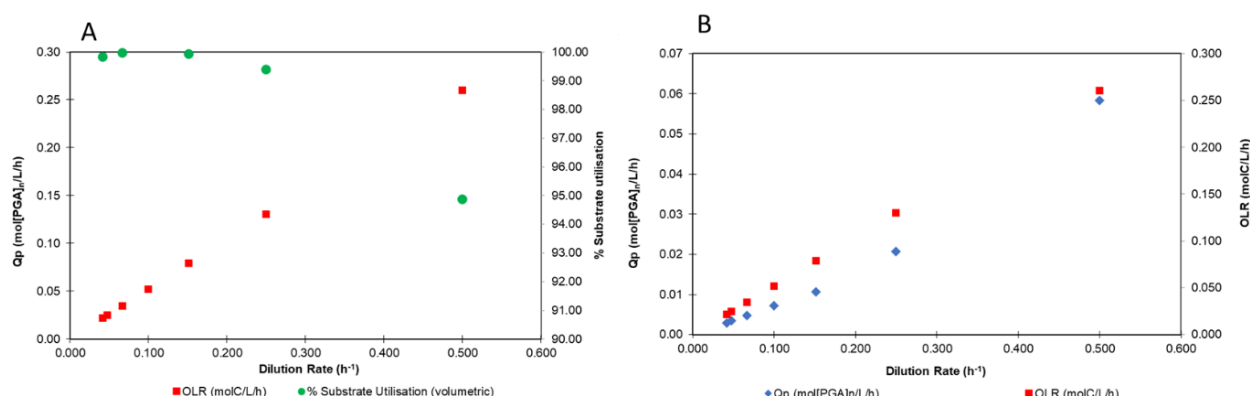


Figure 6-28: Relationship between dilution rate, % substrate utilisation and volumetric PGA productivity (A) and volumetric productivity and OLR (B)

6.6 Conclusions

The key aim of this chapter was to demonstrate that through biofilm development on the carriers, the biomass and hydraulic residence time in the reactor could be decoupled resulting in the reactor being able to achieve high productivities and nutrient removal at high hydraulic loads.

Using SEM imaging, the biofilm attachment was visually demonstrated with a noticeable increase in cell attachment density from 24 h (0.042 h^{-1}) to 6.6 h (0.152 h^{-1}) and further increase in density at 4 h (0.250 h^{-1}). The presence of EPS on the carriers could clearly be seen.

The steady state results at each retention time showed that the MBBR was in stable operation between 24 h and 6.6 h with the biomass, OD_{600} and residual substrate concentration relatively constant despite the increasing flowrates i.e., the planktonic biomass productivity, substrate utilisation rate and PGA productivity increase with increasing flowrate. This robust operation with increasing flowrates rates is evidence of stable operation. Continued increase in biomass productivity, substrate utilisation rate and PGA productivity at dilution rates that exceed μ_{max} indicate that biomass retention time was decoupled from hydraulic residence times, allowing effective substrate conversion and γ -PGA productivity to be sustained.

The productivities showed a continual increase with increasing organic loading rate, deviating from the theoretical relationship for suspended cultures, and as expected due to biomass retention. The key reason for this observation is that there was significant biofilm development within the bioreactor and on the biofilm carriers. Despite a small decrease in the substrate utilisation ($\sim 5\%$), and the measured planktonic cells having a decrease in concentration (50% decrease from maximum cell dry weight), the retained biomass was still utilising up to 94% of the substrate and had increasing productivity rates thereby demonstrating the value that biomass retention in both nutrient removal and product formation

The MBBR was successfully able to demonstrate that at dilution rates higher than D_{crit} , nutrient removal and product formation was achieved through biomass retention.

7 Conclusions and Recommendations

The overall aim of this research was two-fold. First, a suitable reactor technology currently used for wastewater treatment was selected that is suitable for the manufacture of a value-add product in a wastewater biorefinery set-up. The selected reactor needed to demonstrate the required bioreactor characteristics for operation of a bacterial bioreactor for application in a WWBR. The short-listed reactors included the aerobic granulated sludge reactor (AGS), the rotating bed contactor (RCB) and the moving bed bioreactor (MBBR). Based on this, the final selected reactor technology was the MBBR and a lab-scale reactor was constructed. It was used for cultivating *Bacillus subtilis*, a known producer of the commodity product γ -PGA and isolated from wastewater treatment plants, for its ability to produce γ -PGA and biomass under continuous, non-sterile conditions.

7.1 Batch and fed-batch STR studies

The *Bacillus subtilis* species, Isolate 1 isolated from the Mitchell's Plain WWTP, used in this research was first tested in STRs for its ability to grow using lower substrate concentrations than those tested by Madonsela (2013) at room temperature conditions, in order to simulate the fluctuating environmental elements that would be experienced within a WWBR reactor operated with limited temperature control. The aim of these experiments was also to determine an approximate $\mu_{\max}$ under the same conditions that were to be tested in the continuous MBBR. This $\mu_{\max}$ would provide an estimate to the critical dilution rate under continuous steady-state conditions.

The STR was operated under batch conditions at 37 °C and room temperature. The 37 °C batch achieved an overall biomass yield and maximum biomass productivity of 0.277 g_x/g_s and 1.246 g/L/h. These results are in agreement with a yield coefficient of 1.36 g_x/g_s calculated by Madonsela (2013) for the same isolate, but using double the medium concentration. The room temperature batch runs achieved an average overall biomass yield and maximum biomass productivity across all three runs of 0.288 ± 0.007 g_x/g_s and 0.423 ± 0.109 g/L/h. Complete substrate utilisation of glucose was achieved in all cultivations. The $\mu_{\max}$ for each batch run was calculated and provided critical retention times of 10 h, 6.6 h and 5.3 h for RTB1, RTB2 and RTB3 respectively and 2.6 h for 37°C. These results provided key process parameters for the design of the MBBR operation.

Fed-batch runs were performed to evaluate improvement in overall biomass yields and productivity. γ -PGA was quantified at the start and end of the fed-batch to evaluate if there was an increase in productivity. The average overall γ -PGA yield for both runs was calculated to be 0.667 ± 0.135 g_x/g_s. The overall average biomass productivity was increased from 0.071 ± 0.007 g/L/h under batch conditions to 0.192 ± 0.011 g/L/h under fed-batch conditions.

7.2 Evaluation and selection of suitable WWBR reactor technology

The reactor design for a WWBR is influenced by several factors. The criteria for evaluating reactor technologies were developed by Verster et al. (2014). These criteria interrogate different bioreactor types against the key requirements in terms of design, product selection and operability. A high-level analysis of current wastewater treatment reactor technologies was evaluated for their suitability against these key criteria detailed in . A shortlist of the five most suitable reactors was selected against a matrix that assesses the reactors for highest biomass retention and ease of product recovery. Following this, the five reactors were assessed in a comprehensive review to understand the principle of operation, their current role in wastewater treatment plants in South Africa and main advantages and disadvantages.

The key criteria were refined to focus on design priority criteria and operability criteria. The five selected reactors were then assessed against these five criteria and further short-listed. A SWOT analysis of the

three most suitable reactors showed that the MBBR was the most suitable, with the lowest risk and threats for application in a WWBR. The threats and weakness discussed were easy enough to remedy without significant risk or cost.

The MBBR was selected to investigate the effects decoupling of solid and hydraulic residence through biofilm retention, on the reactor performance and productivities as well as how retrofittable into the technology is into existing infrastructure where Activated Sludge is the primary technology.

7.3 Evaluating proof of concept in a MBBR

A lab-scale MBBR was constructed at 7 L total volume. Investigations into the effects of the MBBR carrier and aeration rate on the oxygen transfer showed that carrier fill percentage of 40% and aeration rates of 3 to 5 L/min would provide the highest k_{La} . Hydrodynamic studies confirmed that there were no dead volumes, and the reactor was sufficiently mixed with mixing times being significantly shorter than the retention times.

Following successful biofilm attachment studies, the reactor was operated continuously for a total run time of 870 hours at decreasing retention times of 24h, 21h, 15h, 10h, 6.6h, 4h and 2h. The calculated μ_{max} in Chapter 4, corresponding to retention times between 10 h and 6.6 h were expected to trigger washout of planktonic cells, allowing investigation of the impact of biofilm attachment on operating at D_{crit} , and at dilutions rates higher than D_{crit} .

The effect of biomass retention was successfully demonstrated, and the biomass and hydraulic residence times were decoupled. These conclusions can be drawn based on the result that biomass and γ -PGA productivities continued to increase beyond D_{crit} of 0.152 h^{-1} . Operating beyond 0.250 h^{-1} resulted in partial planktonic cell washout (decreasing OD_{600} and increasing residual substrate concentration). An interesting result was that the γ -PGA yield and productivities were considerably higher despite the evidence for cell washout. A likely reason for this was that at $D \gg D_{crit}$, the high hydraulic load caused attached biofilm on the reactor wall to become dislodged. γ -PGA is a major component of biofilm and thus the concentrations in circulation increased.

Overall, the hypothesis that the MBBR would demonstrate the proof of concept for a WWBR reactor was demonstrated; complete substrate depletion of carbon at high dilution rates $D > D_{crit}$ was achieved, as well as production of γ -PGA at increasing productivities through the decoupling of solid and hydraulic residence times.

7.4 Recommendations

1. The MBBR studies can be improved, and further feasibility studied, by operating under conditions that more closely mimic the environment of a WWBR. This would include using a reactor medium that is representative of the concentrations expected in a municipal or industrial WWTRP. Alongside this, operating the reactor in a more mixed-culture environment to improve the understanding of selection pressures for niche products. Optimisation work should be done to find the greatest substrate utilisation rates and highest γ -PGA formation rates. The product and biomass yields have scope for improvement through optimisation of the oxygen mass transfer to limit operating at low dissolved oxygen concentrations.
2. Further work is required in understanding the best method for downstream processing for the recovery γ -PGA from the bulk liquid and biofilm that is both cost-effective and scalable. Quantifying the amount of biomass in the attached biofilm and not just the planktonic biomass, will aid in understanding and modelling the system dynamics and effects of biomass retention on reactor performance.
3. The construction and operation of a larger-scale MBBR (20 - 50 L) is recommended to demonstrate scalability of this process and further show the potential of WWBRs for the treatment of wastewater, and production of valuable products. Along with this, quantify the mass balance and flows for improved modelling.
4. A techno-economic feasibility study is recommended to understand the economic viability of producing γ -PGA using municipal or industrial wastewater, from retro-fitted AS reactors in a South African context.

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Appendix A Detailed Methodology

A.1 Biological Analyses

A.1.1 SEM Analysis of biofilm attachment

- Samples are fixed by placing in 2.5% glutaraldehyde for approximately 8 hrs in a fridge.
- Rinsed with pH 7 sodium phosphate buffer.
- Rinsed with distilled water
- Dehydrated by taking the samples through an alcohol series consisting of 30%, 50%, 70%, 90%, 95% and 100% alcohol. The samples are placed in the alcohol for about 10 mins for each step. In the case of small samples (e.g., cells or bacteria), the stages of preparation up to here can be done in Eppendorf tubes with a gentle centrifuge between each stage. After dehydration, the samples can then be filtered onto filter paper and glued onto an aluminium stub
- Critical Point dried (CPD) or with small samples (e.g., cells on filter paper), dried using HMDS
- Mounted on a stub covered with carbon glue
- The stub is sputter coated with gold palladium alloy or carbon
- The stub is then placed in the scanning electron microscope (SEM)
- Images are recorded and stored.

A.2 Chemical Analyses

A.2.1 HPLC Sample Preparation.

5. Wash and prepare HPLC vials by soaking in a dilute acid bath and sonicating for one hour.
6. Place vials in oven overnight to ensure they are sufficiently dry.
7. Prepare mobile phase solution of 5 mM H_2SO_4 (276 μL of 98% H_2SO_4 in 1000 mL dH_2O) solution.
8. Filter using a 0.22 μm filter paper and vacuum filter. Degas in a sonicator bath for a minimum of 30 minutes.
9. Prepare cocktail of glucose, glycerol and citric acid at 10 g/L, 0.5 g/L and 6 g/L respectively in a volumetric flask.
10. Once sufficiently mixed and dissolved, make up to the volume line.
11. Make serial dilutions of the standard (1x, 2x, 4x, 8x, 10x, 20x dilution) with dH_2O .
12. To prepare the samples, thaw supernatant and vortex microfuge tubes to ensure they are well mixed. Dilute sample appropriately with dH_2O and vortex again.
13. Filter the standards and samples through a 0.22 μm syringe filter into the HPLC vials and place in the autosampler.
14. Place blank samples (filtered mobile phase) before cocktail standards and after the standards as well as between every third sample.

A.2.2 γ -PGA UV Spectrum Method

1) Preparation of pH 7 sodium phosphate buffer

15. To prepare 1000 mL of pH 7 sodium phosphate buffer, combine 8.660 g of Na_2HPO_4 (base) and 5.382 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (acid) in 500 mL of deionised water in a volumetric flask, stirring continuously on a magnetic stirrer until fully dissolved.
16. Make up the remainder with deionised to the demarcated line on the volumetric flask.
17. Store in fridge overnight.

2) Preparation of standard solution of γ -PGA

18. A standard solution of 1 g/L γ -PGA is prepared by accurately weighing out to a minimum of two decimal places, and dissolving the γ -PGA in a volume of water less than the total volume of the volumetric flask.

Once fully dissolved, make up the volume to the demarcated line ensuring any powder rinsed from the sides of the flask.

19. Keep stirring the solution for a minimum of 30 minutes until fully dissolved.
20. Store standard solution in the fridge.
21. Standard curves are generated by making serial dilutions from 0.2 g/L to 0.04 g/L (see Section - 319644896.635.-319644896 for exact dilutions made)

Appendix B Batch and fed-batch cultivation of *B. subtilis* in a traditional CSTR

B.1 Feed pump Calibration Curves

The feed pump (Pump A) on the New Brunswick fermenter was calibrated before being used and the flowrate of the feed pump adjusted accordingly, to ensure that 2.5 L of feed was pumped at the rate of the fastest glucose utilisation rate (g/h) of the batch cultivations. The calibration at each setpoint was done in triplicate, by dispensing 10 mL of liquid into a 10 mL measuring cylinder. The figure and table below are an example of the pump calibration curve. The calibration was repeated for each fed-batch experiment, using the same pump head tubing.

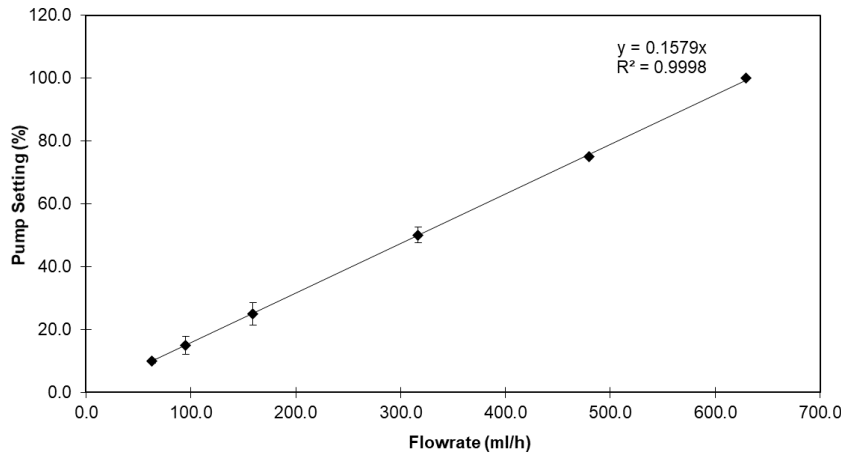


Figure. B.1-1: Feed pump calibration curve of Pump A of New Brunswick system.

Table. B.1-1: Summary of pump setting and corresponding flowrate

Pump Setting	Average time (s)	Flowrate (mL/h)
10	570.5	63 ± 0.01
15	378.7	95 ± 0.7
25	226.0	159 ± 2.5
50	113.7	317 ± 3.8
75	75.0	480 ± 0.0
100	57.2	629 ± 3.8

Appendix C Production of γ -PGA with cell retention in a continuous MBBR

C.1 Calibration of feed and effluent pump

The four-headed pump used in the continuous MBBR experiments was calibrated using half strength MME and was repeated in triplicate for each pump setting. Double heads for both the influent and effluent pumps were required, in order to meet the flowrate demands of retention times. Figure. C.1-1 shows the pump heads used. When the pump head tubing required replaced mid-run, care was taken to ensure that the same sized pump head tubing was used.

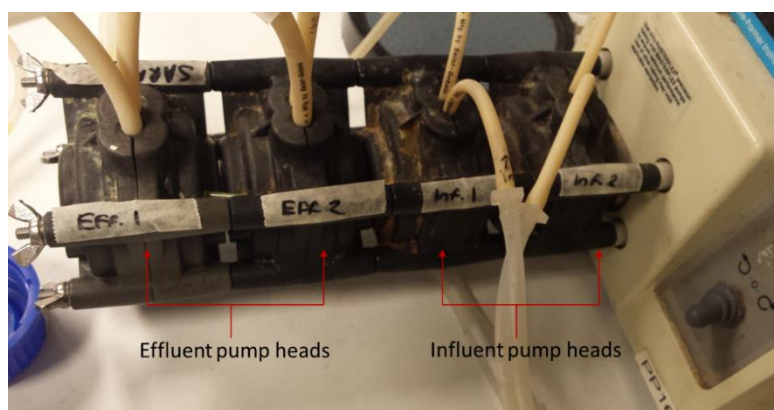


Figure. C.1-2: Photograph showing the four-headed pump used in the MBBR experiments.

Table. C.1-1: Calculated flowrates and pump settings from influent pump head for required retention times.

Retention time (h)	Flowrate (mL/h)	Pump Setting
24	229	1.58
22	250	1.64
15	367	1.98
10	550	2.50
6.6	833	3.31
5.5	1000	3.78
4	1375	4.85
2	2750	8.76

C.2 Influent Pump Heads

The figure and table below show the pump calibration curve that was used to determine pump settings for the required flowrates.

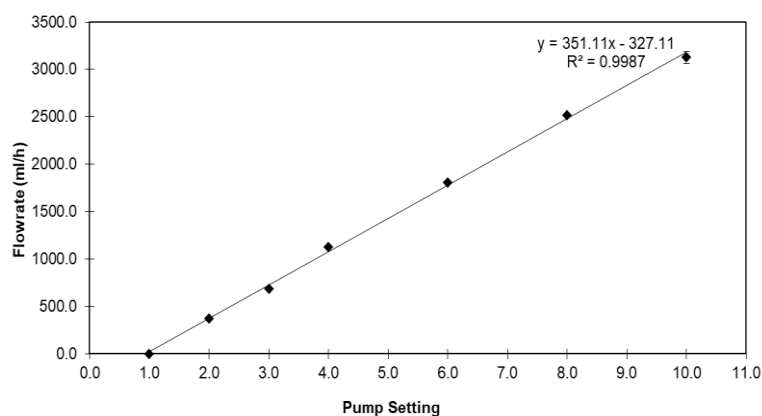


Figure. C.2-1: Influent pump head calibration curve

Table. C.2-1: Raw data used to generate the influent pump calibration curve. The average time is reported for a volume of 20 ml dispensed.

Pump Setting	Average time (s)	Flowrate (mL/h)
10	23.0	3129 ± 65
8	28.6	2519 ± 14
6	39.8	1811 ± 10
4	63.7	1131 ± 5
3	52.3	689 ± 5
2	97.3	370 ± 2

C.3 Effluent Pump Heads

The figure and table below show the pump calibration curve that was used to determine pump settings for the required effluent flowrate

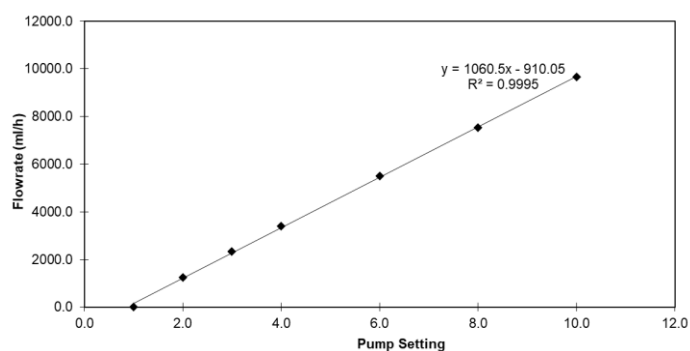


Figure. C.3-1: Effluent pump head calibration curves

Table. C.3-1: Raw data used to generate the influent pump calibration curve. The average time is reported for a volume of 20 ml dispensed

Pump Setting	Average time (s)	Flowrate (mL/h)
10	18.6	9653 ± 4
8	23.9	7538 ± 8
6	32.7	5504 ± 3
4	53.0	3399 ± 3
3	77.0	2338 ± 0
2	143.3	1256 ± 1