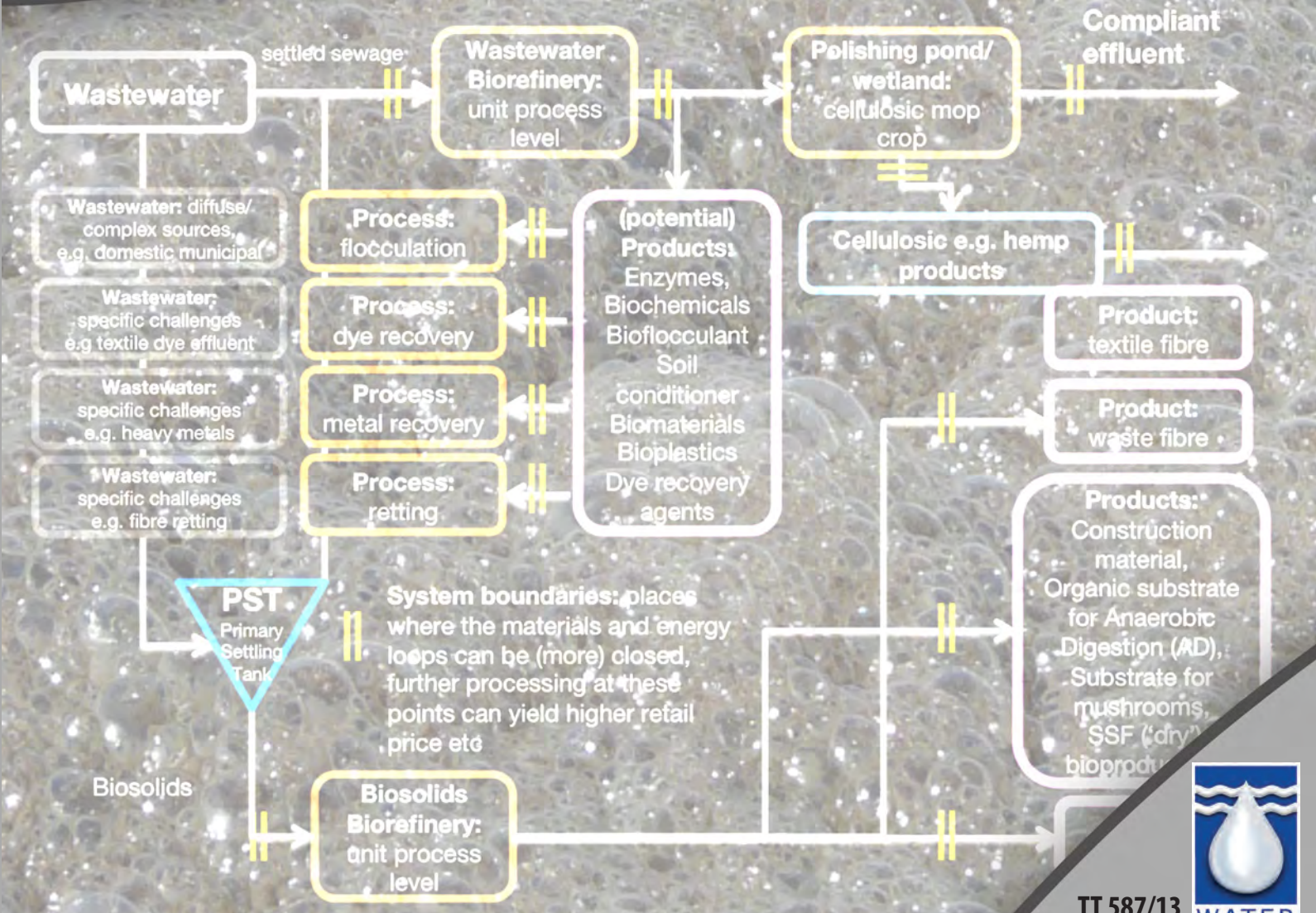
INTRODUCING THE WASTEWATER BIOREFINERY CONCEPT:

A scoping study of poly-glutamic acid production from a *Bacillus*-rich mixed culture using municipal wastewater

Bernelle Verster, Ziningi Madonsela, Sanet Minnaar, Brett Cohen & Susan TL Harrison



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Introducing the wastewater biorefinery concept:

A scoping study of poly-glutamic acid production from a *Bacillus*-rich mixed culture using municipal wastewater

Report to the
Water Research Commission

by

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Executive Summary

Wastewater treatment plants represent a source of nutrients for microbial growth and product formation. In an approach in which bioresource productivity is maximised, it is desirable to not only achieve water treatment to the desired standard, but at the same time to harness the value in these resources. Wastewaters are a source of nutrients such as C, N and P. Macro-nutrients typically comprise the major share of the operating costs of commodity bioprocesses, such as the production of alcohols, organic acids and polymers. The nutrient loads in municipal wastewaters are dilute, but add up to significant daily loads because of the massive volumes generated in urban populations. Bioprocessing to reduce these nutrient loads in wastewater while producing a range of byproducts have conventionally included biogas and compost, produced with minimal modification of existing plants.

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

In such systems, the tension between the environmental biotechnology focus on resultant water purity and the industrial biotechnology focus on the additional products needs to be resolved. In this approach, it is accepted that water quality will take precedence; however, by introducing the product focussed unit operation within the multi-unit process, combined optimisation has potential to allow concomitant achievement of both goals.

In this project, we explore the broader system needs for such a wastewater biorefinery approach and consider the specific case of PGA production in which we aim to use the nutrient component of partially treated domestic wastewater for the production of poly (γ-glutamic acid) (γ-PGA) by enriching the microbial ecology for appropriate *Bacillus* species.

γ-PGA, a polymer of D- and L-glutamic acid monomers connected by amide linkages, is a naturally occurring biopolymer, synthesized by a variety of micro-organisms. Most commonly, γ-PGA production has been studied in *Bacillus* species, such as *B. subtilis* and *B. licheniformis*. *Bacillus* is also associated with domestic wastewater treatment and its enrichment has been associated with improved treatment processes. Potential applications of the γ-PGA are reported in the medical, food, cosmetic, wastewater treatment, plastic and agricultural and textile industries. In this project, we consider the production of γ-PGA by *Bacillus* species for the partial treatment of domestic wastewater and concomitant production of the polymer for soil improvement and water treatment. This system was selected to ensure that natural selection within the wastewater ecology was achievable.

A number of isolates, 19 in total, were obtained from the Mitchell's Plain WWTP and screened for their growth potential and potential to produce PGA. Isolates showing reproducible growth and evidence of polymer production were selected for further screening in terms of growth. The growth, substrate utilisation and PGA production of these were compared. A sub-set was selected for further research with specific emphasis on media composition in terms of C : N : P ratio and selection pressure of continuous culture under reduced substrate concentration.

In parallel to this, the requirements of the wastewater biorefinery were assessed and nature of wastewater as a source of nutrients described. This formed the basis for the review of reactor design and selection of appropriate reactor types. Following a broad review of bioreactor types, reactors supporting biofilm or aggregated microbial growth are selected to allow decoupling of hydraulic and biomass residence time to facilitate growth on dilute media. The fluidised bed reactor with an aerobic granular sludge (AGS) and the rotating bed contactor were selected for further study.

The fluidised bed reactor with an aerobic granular sludge (AGS) and the rotating bed contactor (RBC) were designed, built and commissioned with an emphasis on simple and affordable construction. These reactors were trialled in the laboratory, using synthetic media and a pure culture of isolate 1, a *Bacillus* species. They were also trialled in the field at Athlone WWTP using wastewater. The latter allowed many challenges to be identified.

The review of industrial ecology and the importance of this paradigm in the design of the biorefinery are reported. Further, key features of the wastewater biorefinery in terms of the process flowsheet are described as is the tension between the goals of bioprocess engineering for maximising product formation and environmental biotechnology for maximising remediation.

To implement the wastewater biorefinery, the roles of the government, industry, knowledge generation centres such as universities and the public need to be acknowledged and integrated. Further information flow and the building of sound relationships between stake holders can facilitate or jeopardise success of the approach. These roles and relationships have been explored through the literature, recent workshops and meetings focussed on WWT and utilities as well as a series of interviews with stake holders.

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Prof Alf Botha	University of Stellenbosch
Ms Pam Welz	Cape Peninsula University of Technology
Prof Brett Pletschke	Rhodes University
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List of students and degrees:

Ziningi Madonsela (2013). Selection of bacterial species from wastewater for potential production of poly (γ-glutamic acid): isolation, characterisation and growth kinetics. MSc dissertation, Department Chemical Engineering, University of Cape Town

Bernelle Verster (2014 expected). PhD thesis, Department Chemical Engineering, University of Cape Town

List of conference papers and presentations:

B. Verster organised the Waterbus Schools Awareness and Education, 8 October 2010 during this project, a trip to look at the water system in the Bergriver catchment, and build connection between students and industry professionals (funded by WISA).
www.supernews.co.za/protecting-water-resources-by-empowering-young-citizens/
IWA YWP newsletter, March 2011 (pdf)

B. Verster presented at the 7th renewable Resources and Biorefineries (RRB) Conference, 8-10 June 2011, Bruges, Belgium. Presentation: "Producing poly-glutamic acid from wastewater, using *Bacillus* – considerations when moving from bioprocess to environmental engineering."

B. Verster presented at the Young Water Professionals (YWP) conference, 3-5 July 2011, Pretoria, South Africa, poster: "Producing poly-glutamic acid from wastewater, using *Bacillus* – making a financially viable business case with social and industrial benefit"

B. Verster presented at the African Utility Week, Expo Centre, Johannesburg, South Africa – 21-23 May 2012, presentation – "Building ecosystems not empires"

Prof STL Harrison presented at TEDxCapeTown 2012, Baxter Theatre, Cape Town, South Africa, on 21 July 2012 "Integrating bioprocesses"
www.tedxcapetown.org/video/sue-harrison-integrating-bioprocesses
(youtube: http://youtu.be/o0hk3BO-Ox4)

B. Verster presented at TEDxCapeTown 2011 on 16 April 2011, Ratanga Junction, Cape Town, South Africa, "Musings of a water maverick"
www.tedxcapetown.org/video/musings-water-maverick
(youtube: http://youtu.be/fedfRr2SBgs)

B.Verster was the main organiser of Moola for Amanzi Business Concept Competition, attended by the (then) Chair for the United Nations Secretary-General's Advisory Board on Water and Sanitation (UNSGAB), His Royal Highness Willem Alexander Prince of Orange and the Deputy Minister of Department of Water Affairs.

The competition was launched at the Small Wastewater Treatment conference held in East London in November 2010 and the final was held at the UN World Water Day, held in Cape Town, 2011.

Coverage for Moola for Amanzi in WISA magazine: Water & Sanitation Africa May-June 2011, in editorial (p3), President's comment (p7), article 'Innovation pays off' (p80-85) Greendrinks, St Josephine's Mill, 19 September 2011, public presentation "Tame is not sustainable" (merahmas.co.za/blog/tame-is-not-sustainable)

WISA WC branch meeting, 20 February 2013, presentation "Bacillus, Biofilms and Wastewater Biorefineries. A Love story ... sort of."

CeBER Research day (internal) 22 August 2012 presentation "Survival of the Fittingest"

http://merahmas.co.za/blog/ceber-research-day-2012

Forbes Africa, August 2012, p96-97, article "Poop scoop, how to make money from waste" by Sumitra Nydoo" (merahmas.co.za/blog/forbes)

Mail & Guardian Young South Africans 2012, released in M&G issue 22 June 2012 Vol 28(25)

<u>More info on the Moola for Amanzi business concept completion:</u>

The Moola for Amanzi Business Concept Competition was launched at the Small Wastewater Treatment conference held in East London in November 2010 and was a competition 'by young professionals, for young professionals'. Its aim was to build confidence and partnerships among young water entrepreneurs, help communicate their ideas in a way that makes business sense, and encourage the industry to think a little differently.

The competition, which was part of a bigger initiative – the Dutch-SA water partnership – aimed to generate high quality investment proposals addressing water and sanitation issues and build awareness in the public eye, the water sector and sectors outside conventional water-related industries, so that business can go hand in hand with access to clean and affordable water.

Anyone was eligible to enter – students, SME's, informal settlement communities, municipalities. Big ideas, small ideas, technological ideas, social solutions, IT solutions, non-profit ideas: any idea in water was welcome. The prize money totalled to Euro 15 000 (sponsored by the Dutch government), and some support to implement the winning idea was on offer.

Online exposure:
http://blip.tv/watercube/moola-for-amanzi-the-team-behind-it-4931568
http://blip.tv/watercube/mark-wells-and-david-oldfield-win-moola-for-amanzi-4931355
http://www.supernews.co.za/moola-for-amanzi/
http://trees.co.za/home/announcements/item/ftfa-wins-second-place-inmoola-for-

amanzi-battle-of-water-concepts-competition.html
http://earthworksmagazine.co.za/features/moola-for-amanzi-the-emonti-green-hub-concept/
http://www.waternetwork.co.za/projects/waterweek-moola-for-amanzi-report.html

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1 Introduction

1.1 The Need for Wastewater Treatment

1.1.1 Introduction

In earlier centuries, water could be regenerated through natural systems, notably rivers and wetlands. Population growth and industrial processes overwhelmed the self-purification capacity of rivers in the 18th century, leading to more structured wastewater treatment developments (Rehm 2009; George 2008). Lately, water treatment for re-use is generating more interest as water resources become less available and of poorer quality. In addition, plants that were designed to only remove COD – or the energy load mostly associated with carbon – now need to remove nitrogen- and phosphate-containing nutrients too. There is also a growing awareness of other chemicals that may be harmful to human and environmental health that must be specifically removed. The quality of water treatment in South Africa is regulated through the National Water Act (Act No. 36, 1998).

Wastewater treatment currently remains an end-of-pipe solution, separated from the waste generation. This report argues for an approach that sees wastewater as an opportunity to recover valuable products, including water and nutrients. Using the water as a connector of interlinking industries, wastewater biorefineries could aid in improving the function of the system as a whole. Hence the consideration of wastewater treatment systems in terms of a step in the increasingly closed system of resource utilisation is of interest, using the approach of industrial ecology. This project seeks to test such an approach through considering the development of biotechnological routes to treat wastewater while generating secondary economic value through product formation from the components present in wastewater.

1.1.2 Municipal Wastewater Treatment

The nutrient loads – carbon, nitrogen and phosphate in particular – in domestic municipal wastewaters are dilute, but add up to significant daily loads because of the large water volumes used in urban population centres. Bioprocessing can reduce these nutrient loads in wastewater in a variety of environments, while producing a range of by-products, either for on-site use or for sale. Process constraints include the variable quantity and quality of waste resources, and the need to operate in a non-sterile environment.

Examples of these conventionally include biogas and compost which are produced with minimal modification of existing plants. If treatment works are designed as bioproduction facilities, on the other hand, the range of by-products that can be produced increase. These ‘wastewater biorefineries’ have potential to become cost-competitive by using waste streams as raw materials as well as through reduction in energy costs by non-sterile operation.

These processes have the additional benefit of contributing to job creation, and can fund capacity building. While being theoretically feasible and desirable, the implementation of such technologies will require appropriate training and community understanding.

1.2 Introducing the Wastewater Biorefinery Concept: Integrating Environmental Biotechnology and Bioprocess Engineering for Combined Wastewater Treatment and Product Recovery

In this project, a combined approach between

- environmental engineering: the improvement of the natural environment or remediation of polluted sites, and
- bioprocess engineering: the development of processes for the manufacture of products from biological materials,

is used to produce a valuable bioproduct while partially remediating the waste stream used to produce it (Kamm et al. 2006). From a design perspective the two objectives are of equal importance, but process performance requires the top priority to be given to treating the wastewater (Table 1). This constraint is relaxed by considering the proposed process as a unit process in the context of a complete nutrient removal treatment works, hence not acting as the only treatment process employed.

Table 1: Historic differences between environmental and industrial biotechnology (adapted from Kleerebezem and van Loosdrecht 2007)

	Environmental biotechnology	**Industrial biotechnology**
History	Wastewater treatment	Product formation
Basis	Catabolism	Anabolism
Biomass	Mixed culture (sludge)	Specific strains of microorganisms
Process type	Continuous	Batch or fed-batch
Process models	Lumped black box models	Lumped black box models, Omics-based metabolic network models
Process objectives	**Minimize effluent substrate concentrations**	**Maximize productivity of product**
Substrates	Mixed substrates (waste)	Pure and well-defined substrates, complex substrates as byproducts (dependent on value of product)
Process establishment	Ecological selection by process operation	Specific microorganisms and, frequently, genetic engineering

Appropriate products for production from wastewater need to fulfil a defined role in the microbial ecology (allowing natural selection) and must be easily recoverable. The process can then be engineered to select for this product by imposing stress conditions under which the product provides the most competitive advantage for the microorganism. Under the dynamic conditions prevalent in wastewater, microbial populations change with conditions; however, because of the selection pressure imposed, the product is continually selected. In addition, the product must be produced and its recovery effected while maintaining a robust treatment system, able to withstand shock loads and with potential to be retrofitted into existing plants.

Wastewater biorefineries aim to improve the industrial ecology in the wastewater industry, by partially closing materials and energy cycles to promote economic and environmental sustainability (Kleerebezem & van Loosdrecht 2007). As a case study, the project focusses on demonstrating the ability to produce valuable products from dilute waste streams, with less emphasis placed on the performance and application of the produced material at this stage.

1.3 Refined Project Focus

The original project sought to investigate biopolymer production from microorganisms *Natronoccocus occultus* and *Bacillus licheniformis*, using municipal wastewater. *Natronococcus* was included as an extremophile that was considered capable of dealing with the 'extreme' environment of the wastewater. On further investigation it was decided that the wastewater environment need not be considered as extreme, and that the nature of the wastewater environment is better suited to *Bacillus* species than the halophile *Natronococcus occultus*. *Bacillus* overall proved to be a more appropriate organism to use, as outlined in Chapter 1.

As the project progressed, it became clear that the approach of using synthetic wastewater to produce biopolymer from *Bacillus* is perhaps too simplistic. The mindset around wastewater biorefineries needed significant interrogation, and the approach to reactor design needed to be reconsidered with the additional requirement of adequate product recovery. The project thus took on a more comprehensive character in terms of the concept of wastewater biorefineries.

The scope of the project was thus adapted to look at the concept of wastewater biorefineries as well as to investigate an appropriate reaction system to form part of a broader domestic wastewater biorefinery, using a generic approach. The chosen model reaction system, presented as an example application, consists of three parts: Poly-glutamic acid as a model biopolymer, *Bacillus* as a model organism, and domestic municipal wastewater as model dilute substrate.

Wastewater biorefineries are most suitable for products that fulfil a defined role in the microbial ecology (allowing natural selection) and that are easily recoverable. In addition, as an introduction to the concept, products that may play a role in the

functioning of the treatment works are favoured as they may be able to produce materials required for plant operation, from its own waste resources.

Poly-glutamic acid (PGA) is a suitable product for a wastewater biorefinery: it is synthesized by a variety of organisms, including bacteria, archaea and even a eukaryote. Generally, PGA has two main ecological functions in microorganisms: the released PGA acts as a persistence factor, increasing resistance to harsh environments, and the anchored PGA serves as a virulence factor in disease-causing *Bacillus*. Most research on PGA production has been focused on *Bacillus* species, namely *B. subtilis* and *B. licheniformis* (Candela and Fouet 2006). Although PGA has favourable properties which have led to renewed interest in the biopolymer, it is still a considerable challenge to produce in an economically feasible manner on a large industrial scale. For these reasons, PGA has been identified for possible production as a commercially valuable by-product of the proposed wastewater biorefinery.

Apart from serving an ecological role to the microorganisms present in the wastewater, PGA is also the bioproduct of choice because of its usefulness as a polymer, including as hydrogel, flocculant and soil conditioner, as well as in the medical, food, cosmetic, plastics, agricultural and textile industries (Shih & Van 2001). In addition, it can serve as a transient depot for ammonia (Potter et al. 2001), with recovery of nitrogen into a solid being important in improving the nitrogen environmental cycle (Galloway 2010). Because the concept of wastewater biorefineries is still new, the economics of the process and the distribution chain for the final product are not well developed; therefore producing materials required for plant operation from its own waste resources is attractive to reduce the risk inherent in the wider external market for PGA-related products.

The aim of this project is, therefore, to develop the wastewater biorefinery concept through considering the production of crude, N-rich PGA from wastewater as a potential wastewater biorefinery product. The greater proteinaceous component, associated with the increased nitrogen content in the bio-flocculant, and its water-retention properties can be used for soil conditioners under appropriate conditions. Taking this approach also means that less downstream processing is required, as water flocculants and soil conditioners do not need to be of very high purity. Finally, the applications for this polymer are scalable. Potentially, as downstream processing methods for PGA mature, larger volumes of PGA and PGA of higher purity can be produced competitively from wastewater biorefineries, allowing its use in other fields. This will ensure that sustainable economic growth is possible with continued innovation.

Apart from the capacity of *Bacillus* to produce PGA, the species is also found to occur naturally in all fractions of wastewater. The role of *Bacillus* is recognised in improving the efficiency of wastewater treatment (Gerardi 2006). *Bacillus* is a ubiquitous genus of bacteria, and widely studied as an industrial workhorse for the production of enzymes and biological detergents, and as a laboratory organism for

genetic studies. Some species of *Bacillus* are pathogenic, and some are opportunistic pathogens. *Bacillus* is also the model organism for biofilm studies (Branda et al. 2006; Palkova 2004).

The effectiveness of *Bacillus* in establishing dominance and playing a positive role in wastewater treatment is strongly dependent on the type of reactor employed. No scientific studies investigating the factors that influence *Bacillus* dominance have been found during this project. However, *Bacillus* dominance in the reactor system may be increased through exploiting two characteristics of the species (Sung et al. 2007):

- The presence of a recycling stream that would exploit *Bacillus*'s spore-forming ability to re-inoculate the reactor.
- A surface where organisms that produce extracellular polymers (EPS) can attach to form a biofilm.

These potential attributes, in addition to its recognised role in the production of PGA, led to the selection of *Bacillus* as the micro-organisms of interest for this study.

The existing knowledge on biological nutrient removal of wastewater, and bioremediation potential of using domestic municipal wastewater as substrate made it an attractive choice. The social impact and advantages of financial sustainability of municipal wastewater treatment works made domestic wastewater (settled sewage) a favourable candidate to consider as substrate source for PGA production.

In order to achieve this reactor system as a suitable case study, the project needed to identify suitable reactor designs to provide the appropriate growing environment while allowing for product recovery. The feasibility of enriching the complex wastewater environment with the correct bacterial communities needs to be explored. The production of PGA from this enriched culture needs to be demonstrated, first using a synthetic wastewater substrate, then real wastewater. In order to place this system in context, the impact of biorefineries on the wider environment needs to be considered and particular aspects of biorefineries explored.

The report is structured to present both the specific application of PGA production and the development of the wastewater biorefinery concept in terms of the intercalation of reactor choice and product recovery as well as the challenges to its implementation. The biorefinery concept is introduced in ***Chapter 1***. In ***Chapter 3*** a literature review on PGA production from waste resources is presented, while in ***Chapter 1*** a discussion on *Bacillus* in the context of wastewater is presented, and *Bacillus* is characterised through growth studies on synthetic wastewater. ***Chapter 1*** provides more information on municipal wastewater, its suitability and challenges as considered from a biorefinery perspective. This concludes the discussion of the chosen reaction / product system.

In ***Chapter 1***, existing reactor configurations that extract value from biological waste resources are highlighted to show the environment and product recovery mindset

required for the chosen system. In ***Chapter 1*** bioreactors for bioproduction using wastewater are discussed through the requirements and guidelines for selection of reactors that allow for optimisation of the overall system, rather than individual process units. In ***Chapter 8***, initial experimental observations using laboratory scale reactors for PGA production using the characterised *Bacillus* on site are presented, and challenges identified in the municipal wastewater environment that affect biorefinery function discussed.

In ***Chapter 9***, the chosen reactor designs are placed into context within the wastewater biorefinery flowsheet, and the industrial ecosystem that could ensure overall effluent compliance and complementary bioproduction illustrated.

Reflection on the real world challenges facing wastewater biorefineries, and their potential role in wastewater treatment, from an operational, logistic and social perspective is provided in ***Chapter 10***.

In the final ***Chapter 11***, conclusions are drawn with some remarks on the concept, the approach used, some guidelines and recommendations on future work required.

2 Wastewater biorefineries: an Introduction

There is significant overlap in the technologies and compatibility with respect to those bioprocesses conventionally used in wastewater treatment and those bioprocesses for bioproduction of products of value. The clear goal of this project is the identification of the requirements of each, and the implementation of a "total process" design. This should lead to an optimisation strategy for maximising efficiency of waste treatment and of bioresource utilisation in terms of economic, environmental and social impact. This section gives an overview of the systems thinking approach and specific requirements to be delivered for wastewater biorefineries to become reality.

2.1 Defining Wastewater biorefineries

In this project a combined approach integrating environmental engineering (the improvement of the natural environment or remediation of polluted sites) and bioprocess engineering (developing processes for the manufacturing of products from biological materials) is followed to produce a valuable bioproduct while improving the waste stream used as the production media and raw material (Kamm et al. 2006). From a design perspective the two objectives are equal, but process performance requires the top priority to be given to meeting the water quality specifications of the final water stream. By positioning the production unit operation for the product of value within the integrated biorefinery, embedded in the nutrient removal treatment works, these combined objectives can, at least theoretically, be met.

Using waste materials as raw substrate for a valuable bioproduct reduces the environmental burden of the process producing the waste while increasing resource productivity. Where the process has potential to allow cost-recovery or even profitable business models, this provides scope to effect environmental burden relief in wastewater treatment in a financially sustainable manner. As such, the wastewater biorefinery provides a means to strive towards increased closed material flows in our anthropological systems to slow resource depletion, reduce assimilatory waste burden and thereby contribute to improved environmental performance, ideally with social and economic advantage. While the risks and perceptions associated with this approach are discussed in Section 10, the mindset and so-called 'worldview' of the biorefinery approach is further analysed here.

The wastewater biorefinery is established to maximize biomass productivity by ensuring that, not only is the wastewater treated to the necessary standard (yielding the outgoing water product), but that components removed from this wastewater are converted to products of value (economically or socially or both). Due to the nature of the dilute wastewaters, highly energy intensive production processes are not appropriate. Because of the non-sterile nature of the wastewater environment, it is

best to select micro-organisms for product formation that also fulfil a role in the microbial ecology, thereby providing a selective pressure for their dominance. It is also beneficial to select culture conditions and product to contribute a selective advantage to the microbial community of interest. Hence, wastewater biorefineries are not suitable for all bioproducts.

2.2 Key characteristics of wastewater biorefineries

The concept of a wastewater biorefinery is built around the overarching ability to function under significant infrastructural limitations and adverse conditions and, through its operation, to improve these. Key characteristics to achieve this include:

- The ability to manage complex and poorly defined influent streams of great diversity in both composition and concentration that vary with time (dynamic in nature);
- Low cost treatment of resources-in-transition ('wastes') in a non-sterile, dynamic environment (Polprasert 2007);
- Minimal energy requirements in its operation;
- Robust, resilient systems that can absorb shocks (like shock loads and power failures) and interact favourably with the external and internal environment (or 'Nature') (Todd 2003);
- The potential to function with clearly communicated (biological) understanding to allow 'franchising' to stakeholders, and allow the construction of functional business models as part of an industrial symbiosis;
- The ability to be equally or more efficient at delivering related ecosystem services (Turpie & Malan 2010) than conventional bioproduction processes or wastewater treatment works;
- The ability to give additional benefits with respect to competing 'conventional' options, such as
    - a smaller footprint,
    - shock resilience,
    - an ability to generate multiple co-products;
- Dual purpose: the ability to reduce the overall cost in the production of valuable (bio)products (Kleerebezem & van Loosdrecht 2007) while restoring natural capital (Hawken et al. 1999);
- Cost effective production of Bioproducts, thereby being economically competitive with conventional competing processes without the need for subsidies.

In addition to these key characteristics, design of systems that use renewable energy, such as sunlight, and gravity flow systems is expected to improve the competitiveness of biorefineries from the environmental, social and economic perspectives. Development of systems that require lower energy density delivery

and are designed to cope with circadian rhythms, seasonal changes, power failures and other unexpected events requires a “sense and respond” approach in preference to designing systems that aim for increased predictability and control.

2.3 Process constraints inherent in the wastewater context

Before the “systems thinking” approach is considered, it is critical to have a realistic overview of the process constraints inherent in wastewater handling. In the approach to this project, it is acknowledged that waste streams typically contain variable quantity and quality of nutrients, limiting process optimisation. Further, wastewater composition is not optimal for conventional bioproduction. The dilute and variable nature of the waste stream affects productivity as well as biomass resilience. Biomass retention can improve productivity by increasing kinetic rates, providing a more consistent environment for the active biomass, and de-coupling biomass and hydraulic residence time thereby preventing biomass washout (Wuerts et al. 2003). This allows a smaller process unit footprint, providing more lee-way to include polishing steps to ensure effluent compliance.

Some 50-80% of the cost of conventional bioprocesses for the production of commodity products such as ethanol or bulk polymers is associated with the raw material (Doran 1995). Using a waste material has the potential to reduce this cost. In the case of wastewater, however, the dilute and variable nature of the waste stream necessitates the use of additional process units either in pre-treatment or during product recovery, with an associated increase in capital and operating cost. With careful reactor design, this trade-off can be optimised to produce an integrated system competitive with conventional bioprocesses. Using waste materials as raw substrate for a commodity bioproduct reduces the environmental burden of both processes producing the waste and product. It may allow cost-recovery or even profitability in wastewater treatment providing scope to effect environmental burden relief in financially sustainable ways.

Reactor design is the main area that can be optimised for bioproduction from wastewater resources. Reactor design for wastewater treatment is fairly well developed. There are two main challenges with the current approach to reactor design, however. Firstly, optimisation of the reactor as an isolated unit does not ensure that the overall system functions optimally. A systems approach, where other processes are considered in combination with the reactor design stage, can lead to greater functionality and productivity. Secondly, the reactor is typically the primary source of energy utilisation, especially when where aerobic reactors are operated at low substrate concentrations (Harding et al. 2013a,b). With increasing energy costs and concerns about consistent energy supply, this is an area that needs re-design.

The unit processes for downstream processing (DSP) are well developed, with the mode of separation dependent on a small number of principles such as solubility, volatility, size, charge, surface properties etc., hence DSP is easily adapted to any

chosen reactor. To effect efficient DSP on a large scale in a cost efficient manner, the primary objective of the DSP is to provide the product in a different phase to the bulk material to enable easy recovery, while reducing the amounts of unwanted components. The reactor should contribute positively to taking the product into a different phase. When the reactor is designed toward product recovery, the loading on these DSP unit operations may be lowered and their efficiency increased, with associated reduction in the cost of product production as well as reduction in maintenance and down-time.

2.4 Combining different approaches requires facilitative tools

The approach, combining

1. environmental engineering centred on the improvement of the natural environment or remediation of polluted sites, and
2. bioprocess engineering centred on developing processes for the manufacturing of products from biological materials,

which is required to produce a valuable bioproduct while improving the waste stream used to produce it, is illustrated in Figure 1 (Kamm et al. 2006). Despite the equality of the two branches in concept, process performance gives top priority to treating the wastewater.

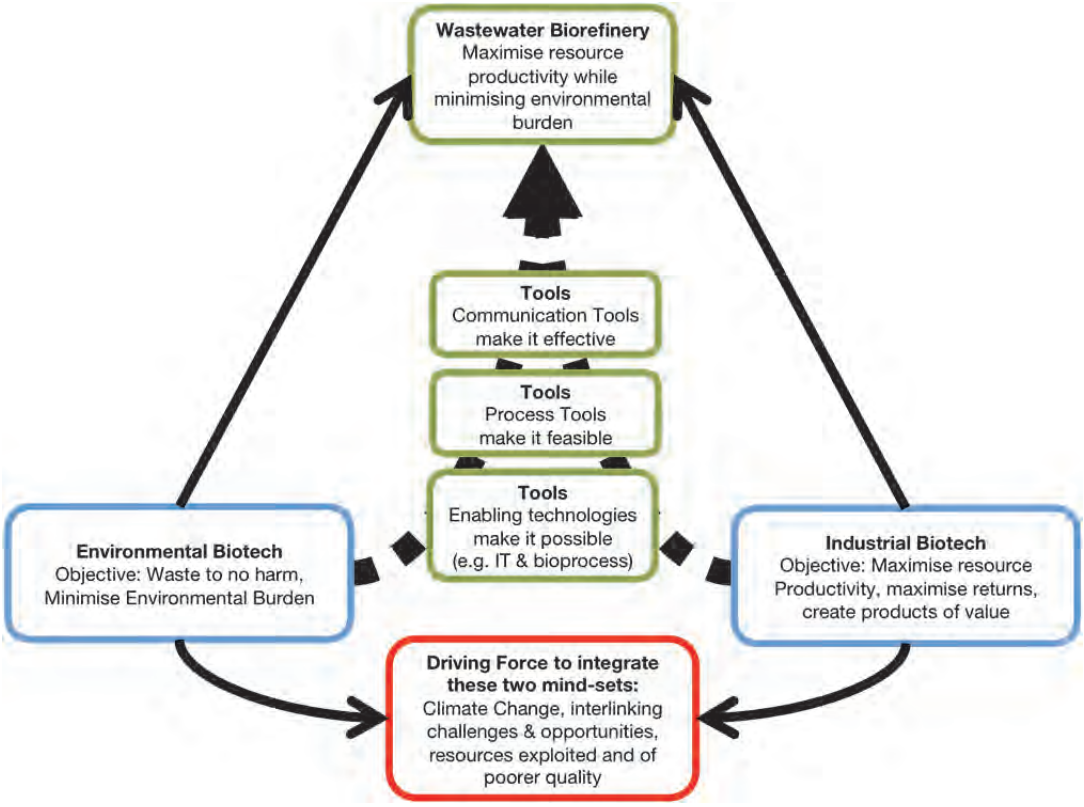


Figure 1: The interaction between environmental biotechnology and industrial biotechnology in creating the wastewater biorefinery (Kamm et al. 2006)

The tension between environmental biotechnology and industrial biotechnology is evident in the desired outcome – clean water on the one hand, and productivity on the other. Conventionally these have to be traded off against each other. Despite some examples of potential symbiosis, for example, the biofuels industry, these different paths seldom cross directly. Initially this apparent conflict is addressed by regulating for increased compliance of waste streams, which initiates innovation in the area. Three main barriers hamper progress, however.

The most apparent barrier is the range of possible products that can be generated from waste (Technology barriers/enablers). The preliminary literature review for this project (Verster et al. 2010) gave an overview of products that are known to be produced from waste in a commercially viable and environmentally responsible way, and examples relevant to this project are discussed briefly in Chapter 1. In this project, the focus is on extracellular polymeric substances (specifically poly-glutamic acid (PGA)) produced by *Bacillus* species as motivated in Section 4.1.2 PGA plays a role in serving an ecological function in wastewater treatment, which supports its value towards contributing to *Bacillus* dominance and hence higher potential productivity in a mixed culture. The potential uses of PGA in industry, most notably as bioflocculant and soil conditioner, are reviewed in Section 3.2.

Additional barriers are a lack of appropriate process tools to measure and track performance of the units in the context of the wider biorefinery, and the difficulty of communicating between different disciplines from the two sectors with traditionally different objectives. In order to address these barriers, systems tools need to be employed. Some of these approaches are highlighted in the next section.

2.5 Systems tools

Several tools exist that can be used to inform the design of wastewater biorefinery systems. These tools carry different names, like ‘systems thinking’; ‘industrial ecology’, ‘biomimicry’ and ‘circular economy’, and have different nuances and focal points, but all contribute to a better understanding of the system under study. Some specific benefits of these tools as they relate to wastewater biorefineries are outlined below.

2.5.1 Biomimicry

Biomimicry (‘bios’ – life, ‘mimicry’ – to imitate) is a methodology inspired by nature to inform process design, with special emphasis on a systems approach (Benyus 1997). The “Challenge to Biology” (Figure 2) makes use of a design spiral that uses a set of questions to inform the final design in context of the wider environment (© the Biomimicry Institute).

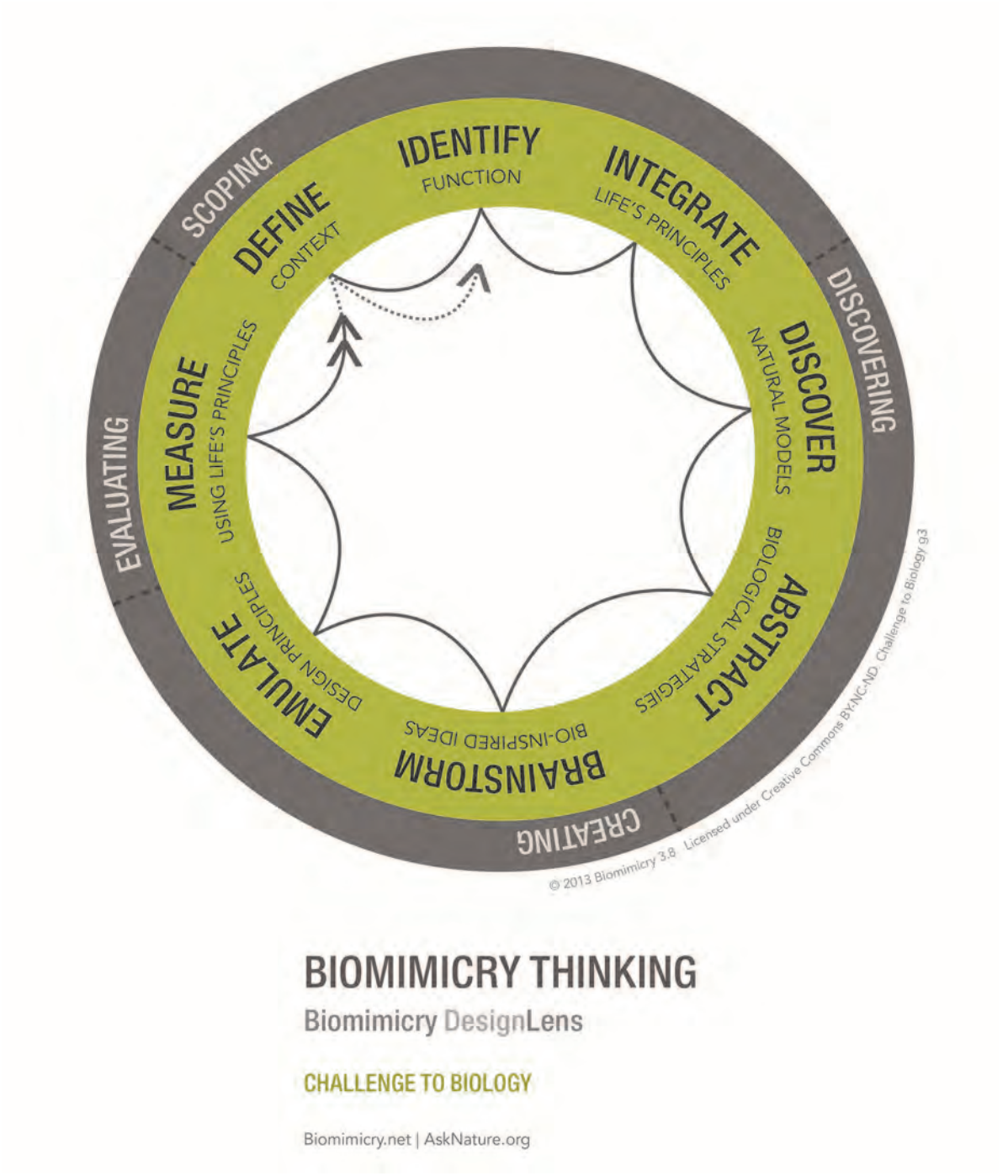


Figure 2: Biomimicry Methodology: Challenge to Biology.

The important aspect of biomimicry to highlight here is the iterative nature of the design process, and the requirement to comply with ALL of life's principles (Table 2, Figure 3). While a deep biomimicry approach was not used in this project, the concept of wastewater biorefinery has a natural link to the biomimicry design approach, as it has to comply with Life's principles by virtue of the process constraints discussed in Section 2.3.

Table 2: Following environmental constraints – Life's Principles: Design Lessons from Nature (Benyus 2008)

Evolve to Survive	Adapt to Changing Conditions	Be Locally Attuned and Responsive	Be Resource-Efficient	Use Life-Friendly Chemistry	Integrate Development with Growth
Replicate strategies that work	Maintain integrity through self-renewal	Use readily available materials and energy	Use multi-functional design	Do chemistry in water	Self-organise
Integrate the unexpected	Embody resilience through variation	Cultivate cooperative relationships	Use low-energy processes	Break down products into benign constituents	Build from the bottom up
Reshuffle information	Incorporate diversity	Leverage cyclic processes	Recycle all materials	Build selectively with a small subject of elements	Combine modular and nested components
	Use feedback loops		Fit form to function		

Figure 3: Life's Principles – a design tool

2.5.2 Industrial Ecology and Industrial Symbiosis

Industrial symbiosis (IS) is a subset of industrial ecology and can be defined as the on-the-ground practice of sharing of information, services, utility, and by-product resources among one or more industrial actors in order to add value, reduce costs and improve environmental performance. Industrial symbiosis relies on collaboration and the synergistic possibilities offered by geographic proximity. Industrial symbiosis may otherwise be defined as:

> *"A co-operation between different industries by which the presence of each of them increases the viability of the others and by which the demands from society for resource conservation and environmental protection are taken into consideration"*

While industrial symbiosis refers to a practice of information and resource sharing, the term Industrial Cluster refers to the actual collection of industries or other players within a close geographic proximity which have any form of synergistic relationships, developed for purely economic, or for environmental and social, reasons.

A key concept related to industrial ecology and clustering is that of the "circular economy" which implies a closed loop of materials, energy and waste flows in the economy. The development of the closed system is critical to both the maximisation of resource productivity and the minimisation of waste burden.

Industrial ecology approaches have had limited success when planned from scratch. In addressing whether wastewater biorefineries should be developed as new greenfields projects or retro-fitted into existing complexes using a brownfield approach, the benefits of the latter are widely accepted to outweigh a top-down approach in practice. Co-location of symbiotic plants through policy decision is rare and has seldom been shown to achieve maximal environmental benefit (van Leeuwen et al. 2003; Ster and Ott 2004). The introduction of incentives, facilitation and promotion instruments have provided the most impact towards achieving industrial ecology principles. This is further supported by the infrastructure already available to the site e.g. transport of wastes to the site. Van Berkel (2006) considers the impact of facilitation and promotion as much more effective than greenfields planning.

When considering industrial complexes that view wastewater as a core component, the industrial partners may need to be viewed in a different way. Rather than looking at industries as independent producers, consumers and decomposers, an alternative approach can consider industries as having all three of those characteristics to some extent. This provides opportunity to internalise waste management to a degree, and to identify with each component as and when they relate to industrial partners, creating more autonomy (paradoxically), flexibility and scope for potential partnerships. This can be seen to occur already as industries start cleaning their own water and increase their re-use cycles based on financial incentives. As a local example, South African Breweries' Newland brewery commissioned an anaerobic

digestion wastewater treatment plant in 2005 to reduce the organic burden transported to the municipal waste-water treatment works. By integrating energy recovery from the biogas into the brewery, significant minimisation of environment burden was attainable (Cohen 2006). This was achieved through using the biogas to power the boiler. Subsequently, expansion of this energy source has been sought through digestion of waste solids and suspensions (Hoffman 2012). Further the grading of water use and re-use in the brewery in terms of the water quality requirement is routine practice.

2.5.3 Other examples of systems thinking approaches: Natural Capitalism, Blue Economy

Systems thinking approaches tend to transcend the technological solutions and focus instead on organisational and societal interventions required to effect systemic change. A reference work that draws some parallels with the natural world and may serve as a good introduction to systems thinking is 'Biomatrix: A systems approach to organisational and societal change' by Dostal et al. (2005). Two specific approaches are highlighted in the following sections.

2.5.3.1 Natural Capitalism

The conservation of Natural Capital is at the central point of sustainable development and requires a paradigm shift from the 20th century custom of treating natural capital as a "free good" (Harrison and Dennis 2004). Biomimicry is nested within a larger emerging discipline of Natural Capitalism, which takes the methodology of Biomimicry and the current approach of increased resource efficiency, and places them in a context of a changing economic climate where restoration of natural capital is valued. The principles of Natural Capitalism is summarised in Table 3.

Table 3: Natural Capitalism principles (Hawkins et al. 2010)

Radical Resource Productivity	Benefits include slower resource depletion, lower pollution, potential increases in employment
Bio-mimicry	Redesign industrial system along biological lines: eliminate the concept of waste, change the nature of industrial processes and materials (as outlined in Table 2), enable constant re-use of materials in continuous closed cycles, eliminate toxicity
Service and Flow Economy	Replace goods and purchases with services and flow, by rewarding consumer and producer, through for example hire versus sale.
Investing in Natural Capital	Reverse planetary destruction through investments in processes that create conditions conducive to life.

The Natural Capitalism approach fits with wastewater biorefineries in the greater emphasis it places on the nutrients and energy in wastewater to be a contributor to

resource productivity, and the restoration potential that wastewater biorefineries can have on the broader environment. It also assists in clarifying how the flow of water between industries can act as a connector in a 'service and flow economy', allowing for shared benefit.

2.5.3.2 Blue Economy

The concepts of Biomimicry and Natural Capitalism have been taken even further and with greater emphasis on economic benefit in the 'Blue Economy' (Pauli 2010). Pauli argues that the current economic model ('Red Economy') is unsustainable due to the reliance on forms of capital that do not exist (debt) and do not acknowledge the value of ecosystems and natural resources. The 'Green Economy', or the first attempts at sustainability lacked impact because of the reliance on consumers having to pay a higher cost for lower quality products to achieve environmental benefit that could not be directly observed or relied on. The principles behind a Blue Economy are highlighted in Table 4, and rely on market forces as well as regeneration for the preservation of ecosystems. It goes beyond sustainability – which implies no change – to a constantly changing system that maintains balance.

As an interface between human activities and the natural environment, wastewater treatment works are the ideal pioneers for this approach on an industrial scale. It is worth noting, however, that many of the technologies and products highlighted in the Blue Economy has failed to scale thus far. The possible reasons and implications for this are reflected on in Chapter 10.

Table 4: Guidelines for Enterprises based on the Blue Economy

Promote Life
Strengthen Resilience
Rely on what is available
Build on sustainable practices
Work within the flow of physics
Offer innumerable opportunities to learn
Adapt to changing conditions
Respond to basic needs
Build community
Instil a sense of responsibility beyond oneself
Generate jobs
Create multiple revenues
Provide challenges

3 Poly-Glutamic Acid Production on Waste Resources: A Literature Review

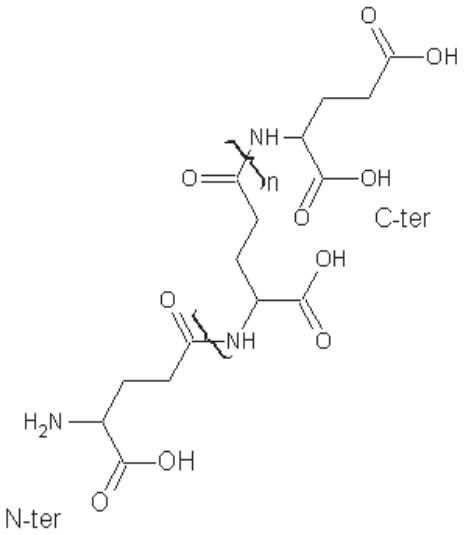
3.1 Introduction

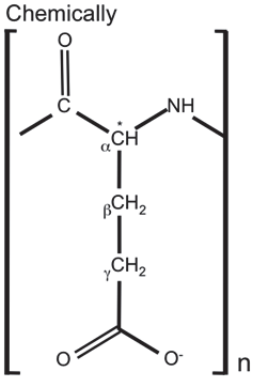
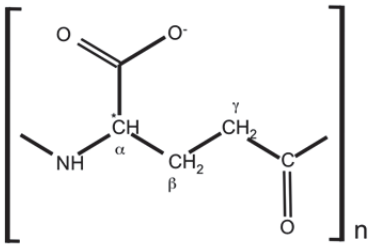
Poly-glutamic acid (PGA) production has been reported on both waste resources as well as in a conventional bioprocess utilising well defined media resources. Typically, high nutrient concentrations have been used in conventional bioprocesses, relative to those in wastewater. Where waste resources have been used, the medium has typically been supplemented with higher quality substrate. Further, the recovery of the PGA reported from complex waste resources is laborious, leading to a high cost of the final product. In this section, the state-of-the-art knowledge of PGA production from both waste resources and defined resources is reviewed, and it is argued that dilute wastewater is a preferable substrate for cost-effective PGA production.

3.2 PGA as a Commodity Product

PGA is a highly anionic polymer for which a great range of applications have been suggested, using the free polymer, hydrogels or nanoparticles composed of the polymer (Buescher & Margaritis 2007). Poly-glutamic acid (PGA) is part of the mucilage formed during the fermentation of soy beans to produce *natto*, a traditional Japanese foodstuff (Birrer et al. 1994; Shih & Van 2001, Figure 4). Chemically produced PGA is coupled through the 'alpha' carboxylic acid group, while bacterially produced PGA is coupled through the 'gamma' carboxylic acid side chain (Figure 5). γ-PGA is the focus of this project.

Figure 4: Poly-glutamic acid (PGA) is produced during fermentation of soybeans to produce natto (Shih & Van 2001)



	poly-α-(glutamic acid)	poly-γ-(glutamic acid)
CAS (sodium salt)	26274-79-0	26274-79-0
Preferred method of production	Chemically	Microbially
Structure		

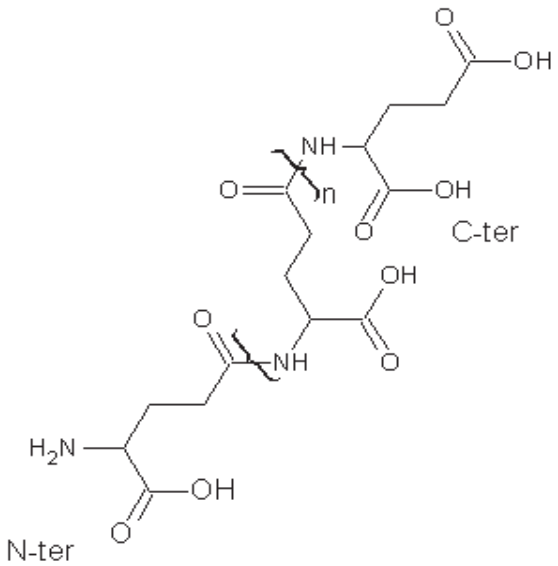


Figure 5: Two isoforms of poly-glutamic acid (adapted from Buescher & Margaritis 2007) and the structure of poly-(γ-glutamic acid) (Adapted from Candela and Fouet 2006)

PGA is water soluble, biodegradable, edible and non-toxic toward humans and the environment. Potential applications of PGA and its derivatives have been researched in a broad range of industrial fields such as food, cosmetics, medicine and water treatment. PGA offers a wide variety of applications including use as thickener, humectant, bitterness relieving agent, cryo-protectant, sustained release material, drug carrier, curable biological adhesive, biodegradable fibre, highly water absorbable hydrogel, biopolymer flocculant, heavy metal absorber and animal feed additive (Shih & Van 2001). With chemical crosslinking, it becomes a hydrogel suitable for applications like biodegradable, disposable baby nappies and water retaining soil conditioners (Choi & Kunioka 2004). Buthelezi et al. (2009) have reported on bio-flocculant properties of extracellular polymeric substances (EPS) isolated from *Bacillus* both in terms of clarifying water and removing microbial load. Taniguchi et al. (2005) reported on PGA's flocculant properties. Similarly, its use as a soil conditioner is favourable (Kinnersley et al. 1994; Koskan et al. 1998; Potter et al. 2001).

Its widespread application is limited by cost, which is the result of the use of relatively expensive substrates, method of production and downstream processing. Current literature on PGA production from *Bacillus* is restricted to the use of monoseptic culture conditions on defined media at high substrate concentrations (Huang et al. 2011; Jiang et al. 2010). A low cost production route for these applications is thus desirable.

3.3 Biochemistry of γ-PGA production

Biosynthesis of γ-PGA in bacteria has been widely reported. Goto and Kunioka (1992) studied the pathway for γ-PGA synthesis in *B. subtilis* IFO 3336. They proposed that γ-PGA is produced mainly from the citric acid and ammonium sulphate found in the medium via the tricarboxylic acid or TCA cycle. Citric acid is metabolized to isocitric acid and then α-ketoglutaric acid (or 2-oxoglutaric acid), a direct glutamate precursor.

This α-ketoglutaric acid is further processed through one of two pathways: In the absence of glutamine, the glutamate dehydrogenase (GD) pathway, commonly found in microorganisms and first reported by Stadtman (1966) is used. In this pathway, L- glutamic acid is synthesized from α-ketoglutaric acid and ammonium sulphate, catalyzed by GD (Equation 1):

$$2-oxoglutaric\ acid + NH_3 + NAD(P)H + H^+ \xrightarrow{\text{GD}} L-glutamic\ acid + NAD(P) \qquad \text{Eqn 1}$$

In the presence of L-glutamine, the glutamine synthetase (GS) and glutamine-2-oxoglutarate aminotransferase (GOGAT) pathway is used, in which reaction of α-ketoglutaric acid and L-glutamine is catalyzed by GOGAT to form L-glutamic acid,

and L-glutamine is regenerated from L-glutamic acid and ammonium sulphate is catalyzed by GS (Equations 2 and 3) (Holzer 1969).

$$2-oxoglutaric\ acid + L-glutamine \xrightarrow{GOGAT} (2H) \qquad 2L-glutamic\ acid \quad \text{Eqn 2}$$

$$L-glutamic\ acid + NH_3 \xrightarrow{ATP} L-glutamine + ADP + P_i \quad \text{Eqn 3}$$

The proposed schematic for γ-PGA biosynthesis is illustrated in Figure 6.

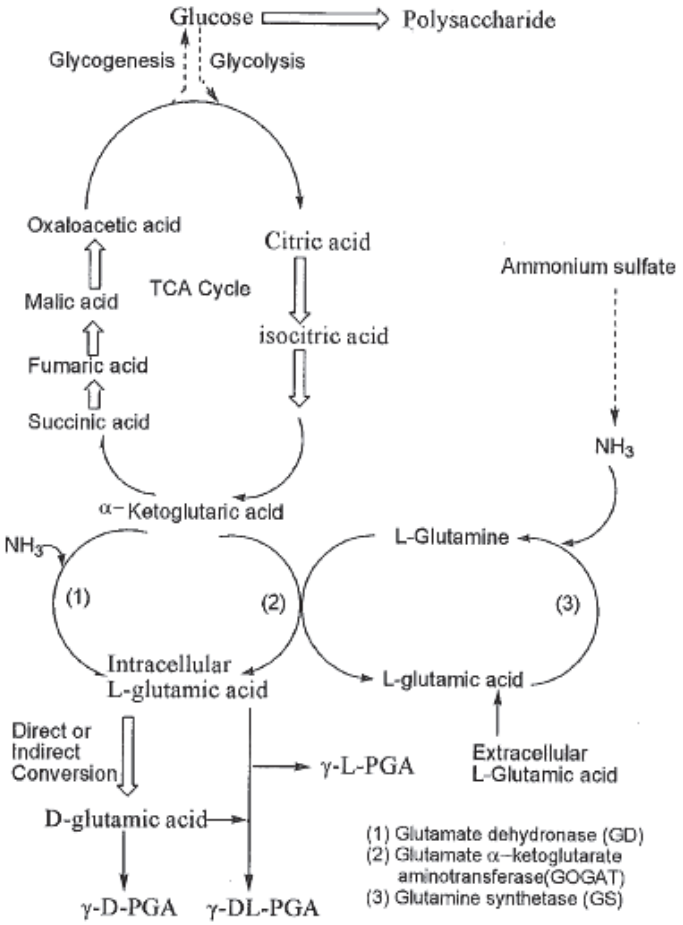


Figure 6: Biosynthesis mechanism for γ-PGA via the TCA cycle (modified from Shih and Wu 2009)

3.4 PGA as a By-product of Wastewater Treatment

Poly-glutamic acid (PGA) was chosen as the bioproduct for consideration in this project because it has potential use for on-site application in the impure form of the polymer. The process constraints of the wastewater treatment stream also may be advantageous for its production, as described below.

3.4.1 Low substrate concentration

Potter and co-workers (2001) encountered difficulties in PGA production using concentrated waste resources, where decreasing PGA yields were obtained using increasing concentrations of manure. The low substrate concentration of wastewater thus has potential benefits for PGA productivity.

At high substrate concentrations *Bacillus* may experience substrate stress, leading to foaming and stress compounds forming (manifested by a pink colour and strong acrylate smell). Substrate inhibition is well recognised in microbial cell culture and is typically overcome by the use of intermediate starting concentrations of the inhibiting substrate, followed by fed-batch operation to provide the substrate at the rate of use. Huang et al. (2011) followed the fed batch route for PGA production and obtained good results with the highest PGA concentration reported of 101.1 g/L, a productivity of 2.19 g/L.h and a yield of 0.57 g PGA/g total substrate. High concentrations of PGA lead to a viscous broth (Yong et al. 2011) which impairs effective mass transfer. In addition, the very high oxygen requirement associated with rapid utilisation of the carbon source may not be attainable under all reactor configurations. By controlling the substrate delivery in the fed-batch system, the oxygen requirement can be controlled within its supply rate (Doran 1995). Alternatively, the combination of the dilute substrate found in many wastewaters with biomass retention could circumvent this challenge, making this approach worthy of investigation, hence included as a focus of this project.

Using dilute waste sources may reduce these inhibitory effects, if the reactors and downstream processing are designed to accommodate the dilute product formation. The use of dilute medium with its associated lower productivity, on the other hand, is only really justified if the water is remediated through the process.

3.4.2 Non-sterile conditions

The production of PGA from fermented beans (Shih & Van 2001) implies that PGA can be produced in semi-sterile conditions, hence showing promise for effective PGA production from non-sterile sources like wastewater. To achieve this, the polymer needs to fulfil an ecological function to provide *Bacillus* with a competitive advantage. Work by Chen et al. (2005) on swine manure supports this possibility (Section 3.5.2). Buthelezi et al. (2009) isolated wild-type *Bacillus* bacteria and showed that material isolated from the strain was effective in flocculating river water (Figure 7), suggesting that it provides biological advantage in certain environments (for example capturing floating food particles).

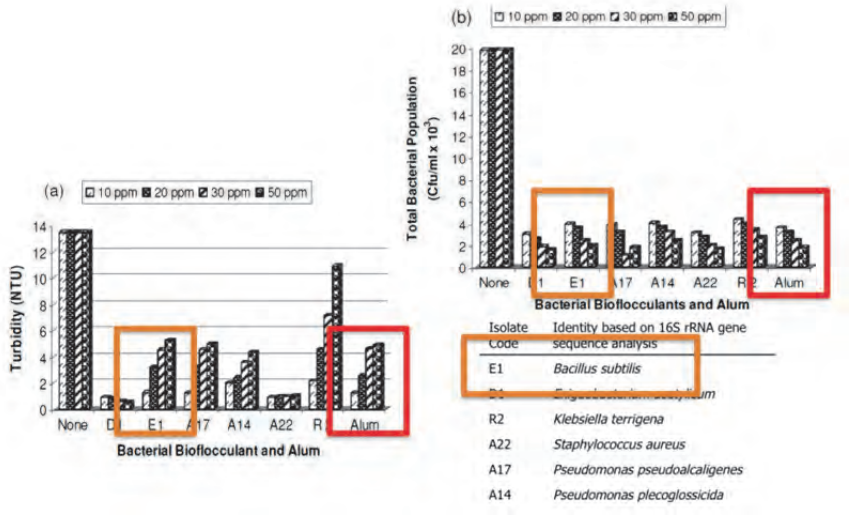


Figure 7: Removal of (a) turbidity and (b) microbial load in river water by different bacterial bio-flocculants, adapted from Buthelezi et al. (2009), emphasis on isolated *Bacillus* strain.

The organism producing PGA needs to be able to establish dominance. Park et al. (2008) suggest *Bacillus* is capable of achieving such dominance under suitable reactor conditions.

3.5 PGA Production on Waste Resources

3.5.1 Production of PGA using submerged medium fermentation

Potter et al. (2001) produced PGA using *Bacillus* growing on liquid manure as the carbon and nitrogen source. The goal of their study was to reduce the amount of ammoniacal-nitrogen in the manure, and thereby its escape into the atmosphere. Synthetic media contained 16 g/L $(NH_4)_2SO_4$. This was sequentially replaced with filtered and autoclaved liquid manure (chemical composition: 0.46% (w/w) nitrogen, 0.32% (w/w) ammonium N, 0.27% (w/w) P_2O_5, 0.29% (w/w) K_2O, and 3.9% (w/w) dry matter). The amounts of PGA were significantly reduced in the presence of increasing concentrations of manure. The C:N ratio of the medium was improved by supplementing with other carbon sources; sucrose, gluconate and glucose gave the best results. PGA production started after the cells entered the stationary growth phase (correlating with Morikawa et al. 2006), but overall values were poor, reaching maximum PGA concentration of 2.2 g/L under fed-batch conditions, and most of the ammonia reduction occurred due to evaporation. The authors conclude that the treatment of liquid manure to improve the culture conditions and control of the process may be too costly and too sophisticated to be accepted by farmers.

Producing PGA in submerged medium fermentation exhibits problems resulting from the rheological behaviour of the culture. On increasing PGA production and thereby viscosity, mixing, heat transfer and oxygen supply were reduced (Doran 1995); this increases manufacturing costs and limits the maximum γ-PGA concentration

achievable and thus the product quality (Huang et al. 2011). Conventional bioprocessing aims to circumvent this challenge through fed-batch approaches, a technique which limits substrate concentration and hence growth rate, in turn limiting the need for high mass transfer rates (Doran 1995). In the current project, a different approach, especially suited to wastewater, is proposed i.e. to use a dilute substrate concentration and retained biomass. This allows the decoupling of the hydraulic residence time (containing the substrate) from the solid residence time (containing the biomass). Biofilm reactors are one example of this approach, and solid substrate fermentation is a more extreme example.

3.5.2 Production of PGA using solid substrate fermentation

Solid Substrate Fermentation (SSF) is a good alternative to submerged medium fermentation (SmF) to utilise waste while minimising the large volumes of water required for conventional bioprocessing. SSF refers to aerobic, anoxic or anaerobic bioprocesses that occur in a low water activity environment (Pandey et al. 2011). It is discussed in more detail in Section 7.3.3.2.

Chen et al. (2005) used SSF consisting of a combination of swine manure (62.3% w/w, dry weight basis), soybean cake (25%, a by-product of soy oil or biodiesel production), wheat bran (5.0%), glutamic acid (5.0%), citric acid (2.5%) and $MnSO_4.H_2O$ (0.2%) and obtained high yields of PGA (6.0% w PGA / w dry medium). The open-air, compost-style experiment yielded 4.5% PGA, indicating potential viability in a non-sterile environment. In a related optimization experiment by the same group, a maximum PGA yield of 83.6 g/kg of dry substrate was obtained under laboratory conditions, using soybean cake powder and wheat bran as main substrate (Xu et al. 2005). By utilizing swine manure and agro-industrial materials as media, SSF achieves lower manufacturing costs, but also higher product concentrations with simpler process and less pre-processing energy than SmF. Product recovery was poorly addressed in this work, using the conventional method of PGA recovery – addition of four volumes of cold ethanol to precipitate the polymer, followed by centrifugation. This method of product recovery remains unfeasible for economic bulk biochemical production.

Yong et al. (2011) used diary manure supplemented with monosodium glutamate production residues to produce PGA in SSF conditions. The optimal SSF medium (20 g substrates with 50% initial moisture) for producing γ-PGA was determined to contain 5.51 g dairy manure compost, 1.91 g soybean cake, 0.57 g corn flour, 2.15 g monosodium glutamate production residues, 1.5 g wheat bran, 0.5 g rapeseed cake, 0.1 g citric acid, 0.05 g $MgSO_4.7H_2O$ and 0.03 g $MnSO_4.H_2O$. In this medium the strain produced up to 0.0437 g γ-PGA per gram of substrates (4.37%) when cultured for 48 h at 37 ˚C. Energy costs and product recovery were not addressed as the media was autoclaved and the PGA was recovered by addition of four volumes of cold ethanol followed by centrifugation.

While PGA production on biosolids using SSF was not considered in this project, the approach followed sets the scene for further investigation into solid substrate fermentation, by considering the waste (both the water, as in this project, and the biosolids – for SSF) as valuable, by decoupling the biomass from the substrate which allows a wider range of bioprocessing options, and considering the reactor design needed when wastes are beneficiated. Solid substrate fermentation forms a key component of the wastewater biorefinery concept, and is briefly investigated in Section 7.3.3.2.

3.6 Considerations in development of PGA production process

In this context, multiple aspects need to be considered when developing a process. Two aspects are highlighted here.

3.6.1 Efficient product recovery

Purifying a more dilute product is proportionately more expensive. Current product recovery of PGA involves addition of four volumes of ethanol to the broth followed by precipitation. The precipitate is re-suspended and the PGA is purified using gel chromatography. Ethanol precipitation is excluded as a process option in this project both because the large amounts of ethanol required are too expensive and, at low concentrations, precipitation is limited by physical constraints. It becomes even more critical to address downstream processing in reactor design to reduce these limitations. The main consideration here is to decouple the biomass from the dilute solution, in other words to retain the biomass, without impairing product recovery.

3.6.2 Biomass retention

At low substrate concentrations, growth rates are constrained to low values, hence death rates and consumption of substrate for microbial maintenance are significant. Further, the dependence of growth rates on substrate availability is typically first order. PGA production is not genetically related to biofilm growth, which continues even when the PGA-producing genes have been removed (Morikawa et al. 2006). Under high substrate concentrations, in standing culture conditions, PGA formation is growth-associated (Morikawa et al. 2006, Section 4.4.2). This implies that the biofilm attachment/detachment kinetics can be decoupled from PGA production in reactor operation, even while the biofilm environment may favour the dominance of PGA producers. Designing the reactor to incorporate both biomass retention and product recovery is discussed in Chapter 1.

4 Growth Studies of *Bacillus* for PGA Production

This chapter considers the organism selected for PGA production and explores the best conditions to achieve its production.

4.1 *Bacillus* and its role in wastewater treatment

4.1.1 The role of *Bacillus* in wastewater treatment

Wastewater microbiology traditionally considered microbial populations only in terms of general characteristics, for example their electron donor requirements, substrate requirements, phosphate accumulating, nitrifying and denitrifying abilities (Henze et al. 2008), and not the specific bacterial species involved. This approach has utility when discussing unit process operation, but the tendency of bacteria to fulfil multiple roles is not reflected adequately when reporting on a specific genus or species. The development of molecular technologies is slowly starting to complement this approach, but literature on the role of *Bacillus* in wastewater is still scarce. *Bacillus* is thought to play an important role in soil health (Gerardi 2006), since it is found in healthy soils and contributes to nutrient provision through, for example, nitrification. From the soil it finds its way into water bodies where it contributes to nutrient removal.

Bacillus species are used widely in industry for natural pest control; for example, BT (*Bacillus thirungiensis* spores) products for protection of citrus harvests (Zhuang et al. 2011), enzyme and surfactant production in detergents, and oil pollution bioremediation. They are expected to have a similar utility in the wastewater environment (Gerardi 2006).

With the development of molecular biotechnologies, more species-specific identification of the microbial population dynamics have become possible. Park et al. (2008) reported on the relative dominance of endospore-forming bacteria, and notably *Bacillus*, in rotating biological contactors, a type of fixed-biofilm reactor typically used in wastewater treatment. Their study compared both culture-dependent and culture-independent (partial 16S rDNA sequence analysis) methods; both methods concluded that *Bacillus* was an abundant genus. Their results indicate that endospore-forming bacteria like *Bacillus* and *Clostridium* successfully colonized and dominated the Rotating Activated Bacillus Contactor (RABC) process without a specific objective to achieve this.

Jackson et al. (2009) published work on bioremediation of metal contamination in the Plankenburg River in the Western Cape. They used a suspended biofilm carrier bioreactor system placed next to the river, with river water directed to flow through it, and found that *Bacillus* was well represented as a potential metal-tolerant genus, along with *Pseudomonas* sp. and *Sphingomonas* sp. While bacterial communities were dynamic during the bioreactor run due to environmental factors, *Bacterium*

PTO3, *Pseudomonas* sp., *Variovorax* sp., *Bacillus* sp., *Sphingomonas* sp. and *Brevundimonas* sp. were present throughout the bioreactor run.

4.1.2 *Bacillus* and PGA

Morikawa et al. (2006) studied biofilm formation using a *Bacillus* strain that produces PGA (Figure 8). They isolated a *B. subtilis* strain producing high levels of PGA from an oilfield. Increasing concentrations of glycerol (0-80 g/L, optimal PGA production at 50 g/L) and dissolved $MnSO_4$ (0-1000 mM, optimal PGA production at 1000 mM) increased PGA formation; this is supported by data reported by Ko & Gross (1998) and Du et al. (2005). This relationship was found with both suspended culture (Ko & Gross 1998 and Du et al. 2005) and biofilm studies (Branda et al. 2006). While these studies were done under high substrate concentration, this correlation shows that the effect may be concentration-independent. Morikawa et al. also found that γ-PGA was produced in a growth-associated manner in standing culture and floating biofilms were formed. However, γ-PGA was produced in a non-growth-associated manner in shaking culture conditions, leading to the hypothesis that PGA may be only loosely associated with the biomass.

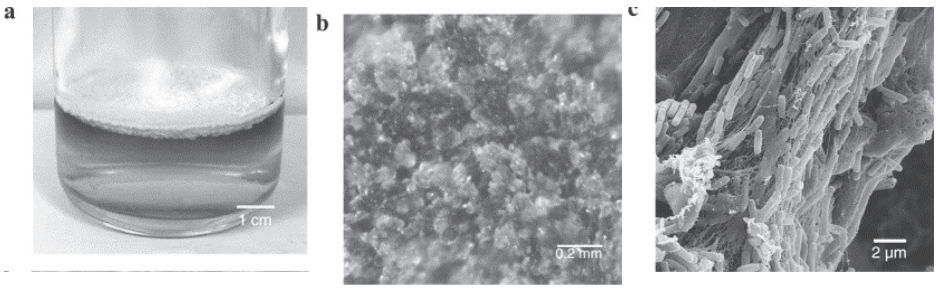


Figure 8: Biofilm formed by B. subtilis wild type (a) Side view; (b) optical microscopic observation; (c) scanning electron microscopic observation (Morikawa et al. 2006)

Morikawa et al.'s work suggests that PGA plays a role in protecting the cell from high osmolarity caused by the high glycerol concentration (and possibly the oil in the environment from which the organism was isolated), and gives protection from high metal concentrations, presumably through chelation using the carboxyl groups in PGA. PGA's role in protecting against high osmotic pressure is supported by its production by alkalophilic organisms like *Natronococcus* (Niemetz et al. 1997). Stanley and Lazazzera (2005) investigated the genetic differences that affect PGA production. The regulators identified in PGA production also affect biofilm formation, and the authors suggest that these two processes are regulated by osmolarity, high cell density, and phase variation. Shih & Van (2001) suggest that PGA is a carbon and nitrogen storage compound, but no work investigating this claim could be found. If one role of PGA is as a storage polymer, this would be significant in providing PGA-producing organisms with a selective advantage in the microbial community engineering approach.

Branda et al. (2006) conducted a study on PGA production in *Bacillus* using a series of knock out mutants and observing colony morphology microscopically. The supporting data is presented in Figure 9. The wild-type organism on the left, termed 'Wild', has not been genetically modified. In the pgs mutant the genes responsible for PGA formation have been eliminated, so that no PGA formation can occur. As can be seen the colony biofilm morphology is virtually unchanged. In a mutant where the genes responsible for sporulation have been disrupted, colony (hence biofilm) formation is impaired, but the colony is still mucoid, indicating functional PGA production. The colony on the right, *spo0Apgs* has both sporulation and PGA production genes disrupted and has lost its mucoidy.

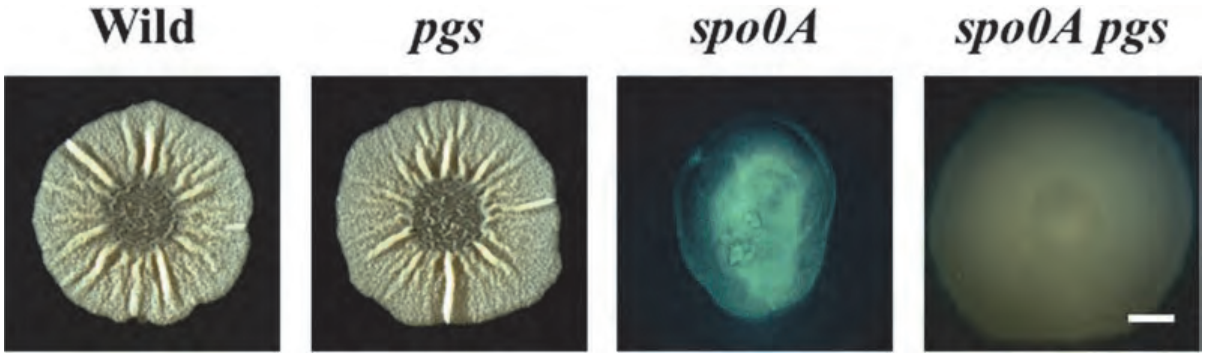


Figure 9: The role of γ-poly-glutamic acid in colony development. Measure bar is 5 mm. (Branda et al. 2006). 'Wild" = wild-type organism; 'pgs' = mutant with genes responsible for PGA formation deleted; "spo0A' = mutant with genes responsible for sporulation disrupted; '*spo0Apgs'* = mutant with both sporulation and PGA production genes disrupted.

The *spo0A* mutant shows an inverse correlation between PGA and sporulation. PGA production is linked to colony mucoidy. If the *pgs* gene for PGA is disrupted, this mucoidy is lost. The gene for sporulation, spo0A, somehow represses PGA formation, and *spo0A* mutants recover their mucoidy. The genes involved in sporulation are also important for biofilm formation.

The authors concluded that 'poly-γ-glutamic acid does not contribute significantly to the extracellular matrix in the wild strain'. This is consistent with the findings of Stanley & Lazzazera (2005). Hence it is apparent that PGA and biofilm formation are two distinct processes, controlled by the genes responsible for sporulation. The relation between these findings and the growth-associated PGA production in standing culture, hypothesised by Morikawa et al., is not clear yet. However, it provides insight into the relationship between biofilm production, sporulation, and PGA production, which could be leveraged in reactor design to decouple biomass production (through biofilm growth) and PGA production, increasing PGA productivity.

4.2 The Growth Matrix for *Bacillus*

4.2.1 A suitable growth matrix for *Bacillus licheniformis*

The growth matrix used by literature for PGA production is shown in Table 5. Because of the broad similarity to synthetic wastewater (Table 6; Table 7) used by Henze et al. (1992) and in order to compare PGA productivity to existing literature, the composition of this growth matrix was not altered. The fundamental limitations this imposes on process optimisation with regards to using real wastewater are acknowledged, but exploring this complexity further lies outside of the immediate scope of this project. Should this project be successful, this will be investigated in future research.

Table 5: Modified Medium E (Birrer et al. 1994)

Substrate	**g/L**	**trace elements**	**g/L**
Glutamic acid	20	MgSO4.7H2O	0.5
Glycerol	80	MnSO4.H2O	0.104
Citric acid	12	FeCl3.6H2O	0.04
NH_4Cl	7	CaCl2.2H2O	0.15
K_2HPO_4	1.5		

Table 6: Example of the sub-fractions in the readily biodegradable COD in raw wastewater (Henze 1992)

	g COD/m^3	**N content g N/g COD**
Acetic acid	25	0
Higher VFA's	10	0
Alcohols (ethanol methanol)	5	0
Lower amino acids	10	0.14
Simple carbohydrates	10	0

Table 7: COD composition of synthetic wastewater (Cokgor et al. 1998)

Component	**Fraction (% COD)**
Acetic acid	41
Propionic acid	17
Ethyl alcohol	8
Glutamic acid	17
Glucose	17

4.2.2 Metabolic considerations

All substrates in this project are considered to be readily biodegradable COD (RBCOD) as the synthetic wastewater consists of simple sugars and amino acids. This assumes that all COD is available for first order consumption, without relying on secondary processes or pre-hydrolysis.

4.2.2.1 Glycerol as a gluconeogenic substrate:

Glucose is the model 'ideal' substrate used in metabolic studies in bacteria. Glycerol has a similar thermodynamic yield per carbon, but not all organisms have the required enzymes to metabolise it effectively through a gluconeogenic pathwayF. In addition to this metabolic constraint, bacteria do not utilise the substrate efficiently, but can have futile cycles (Wolfe 2005). *Bacillus* has a high maintenance metabolism, and is known to waste substrate through futile cycles (Zamboni et al. 2003). Ko & Gross (1998) reported poor growth and sub-optimal PGA yield when glucose was added to the medium. Good growth on glycerol was observed, as long as this was not the only substrate (Ko & Gross 1998).

4.2.2.2 Glutamic acid and citric acid as Krebs cycle intermediates:

To generate biomass and secondary products from the Krebs cycle, metabolites need to be fed into the Krebs cycle. Pyruvate, and its precursors, of which glycerol is one, contribute only energy, and leave as NADPH and CO_2. Some organisms, including *Bacillus*, can bypass this pyruvate shuttle through the malic enzyme complex (Sauer & Eikmanns 2006). PGA production does not only occur through the Krebs cycle, but the alternative route of production is still somewhat controversial (Shih 2001, Ashiuchi 2002, Cromwick & Gross 1995a).

The metabolic pathway illustrated in Figure 10 indicates possible direct routes from substrate to PGA, as well as requirements for NADPH. Note that it is an incomplete representation, as the Pentose Phosphate Pathway (PPP) has several routes depending on the oxidation state and number of carbon atoms in the substrate molecules. The Emden Meyerhof Parnas (EMP) pathway is also just one of several pathways.

4.2.3 Metals concentration

Table 8 indicates the required metals for optimal PGA production using *Bacillus*. Cromwick & Gross (1995) investigated the effect of Mn and Mg on PGA productivity and came to the conclusion that Mg was needed for PGA production and Mn had an effect on the stereoselectivity. Anecdotal evidence from a company promoting a wastewater treatment technology based on increased *Bacillus* populations in the community also indicated an increased requirement for magnesium. It is expected that PGA production requires these metals. Preliminary experimental results in this project support this observation, as very poor growth was obtained on medium with low, or no metals present.

Table 8: Trace metals of Medium E (Birrer et al. 1994)

	MW	**g/L**	**mmol/L**
$MgSO_4.7H_2O$	246.4696	0.5	2.029
$MnSO_4.H_2O$	169.0138	0.104	0.615
$FeCl_3.6H_2O$	270.2928	0.04	0.148
$CaCl_2.2H_2O$	147.0136	0.15	1.020

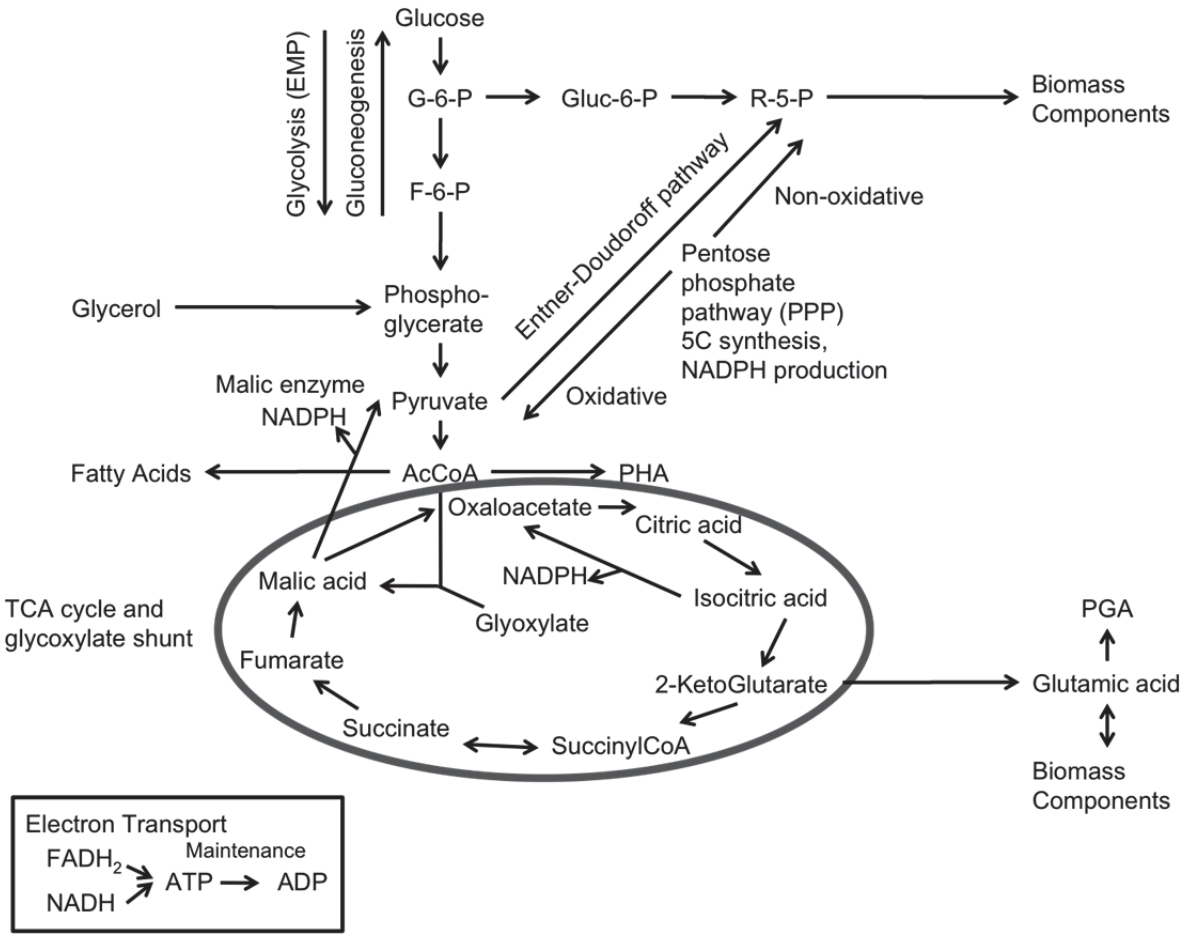


Figure 10: A much simplified metabolic pathway, showing the production of PGA. (Adapted from Wolfe 2005)

Wastewater contains a wide variety of metals, depending on the streams discharging into the receiving plant. Domestic, settled wastewater has a lower metal content, and these may not be bio-available. Dosing may then be required to optimise PGA production, keeping in mind that the effluent must comply to the General Authorisation Standards (discussed in Section 5.5.2). In this project the focus is maintained on the four metals listed in Medium E (Table 8).

In future research, it is expected that the presence of other metals may have a beneficial effect on PGA production, as the PGA may be produced naturally to either sequester and inactivate harmful metals, or be used to increase the bioavailability of required metals. This is supported by observations by Jackson et al. (2009), where *Bacillus* was a dominant organism in metal bioremediation of the Plankenberg River. PGA produced in sites where metal sequestration through PGA has been achieved, cannot be used in applications where metals are not allowed, for example, soil conditioners. These aspects will not be considered in detail in this project.

4.2.4 Growth matrix calculations

4.2.4.1 Rationale

From literature values, PGA production has improved by a factor of two in 15 years (Table 9). Jeong et al. (2010) shows a promising concentration of 48.7 g PGA per litre, and yield of 0.61 g PGA per g substrate. The substrate used, however, was not cost effective. Glutamic acid and yeast extract were the main ingredients. The Jeong medium showed the highest N and P ratio with regards to C content. In order to explore this hypothesis that the conventional medium is N and P limited, the molar compositions of C, N and P were calculated and different ratios investigated on a qualitative basis. The significance of these preliminary findings to wastewater treatment suggested that *Bacillus* has a greater requirement for N and P, and hence could have evolved scavenging mechanisms to remove these nutrients from solution. PGA may have a direct role to play in this mechanism.

Table 9: Comparison of literature brag values for PGA production (compiled from various literature sources)

Authors	**Bacterial strain**	**PGA conc. (g/L)**	**Biomass conc. (g/L)**	**Time for apparent max prodn (h)**	**Productivity (g PGA / g biomass / h)**	**Biomass yield (g PGA / g biomass)**	**Substrate yield (g PGA / g Carbon)**	**Starting CNP ratio, normailised to N**		
	Medium E							**13.7**	**1**	**0.011**
Cromwick et al. 1996, pH 6.5, high aeration	*B licheniformis* ATCC 9945A	23	4	50	0.12	5.8	0.29	13.7	1	0.011
Cromwick et al. 1996, pH 6.5, low aeration	*B licheniformis* ATCC 9945A	6	1.6	50	0.075	3.8	0.71	13.7	1	0.011
Bajaj 2009	*B licheniformis* NCIM 2324	26.12	2.3	96	0.12	11.4	0.30	12.2	1	0.022
Yeong 2010	*B subtilis* RKY3	48.7	0.88	25	2.2	55.3	ND	6.3	1	0.031
Shih 2002	*B licheniformis* CCRC 12826	19.62	ND	48	ND	ND	0.082	14.7	1	0.005
Wu et al. 2010	*B subtilis* NX-2	31.7		48			0.32	7.9	1	0.021

4.2.5 Initial growth matrix composition

The basic growth matrix shown in Table 10 With this standardised Glycerol : Glutamic acid : Citric acid ratio, the mol C/L (in this example 2.5) is used to construct C:N:P ratios.

Table 10 uses the literature-derived ‘Medium E’, and normalises the molar carbon concentration in order to calculate C:N:P ratios while allowing comparison with literature data. In order to compare these values to domestic, settled wastewater, further verifying experiments were needed.

With this standardised Glycerol : Glutamic acid : Citric acid ratio, the mol C/L (in this example 2.5) is used to construct C:N:P ratios.

Table 10: Normalised Carbon substrate concentrations, using Medium E substrates

Total Carbon:	**g/mol**	**g/L added**	**mol/L**	**C/molecule**	**mol C/L**
Glycerol	92.09	58.2	0.632	3	1.896
Glutamic acid. H_2O. Na	187.13	12	0.064	5	0.321
Citrate. H_2O	210.14	12	0.057	5	0.286
Total required C substrate		82.2	0.753		2.502

*4.2.5.1 **Nitrogen and Phosphate***

The nitrogen source was NH_4^+, as $(NH_4)_2SO_4$. Glutamic acid makes a small contribution to the nitrogen load, and has been accounted for. Phosphate was added as a sodium or potassium phosphate buffer, originally at a pH close to 6.5 (Table 11) (with the addition of the citric and glutamic acid requiring additional pH adjustment).

Table 11: Phosphate source, stock solution calculation to buffer at pH 6.5

Compound	**MW**	**g/L**	**g/L stock**	**molarity (mol/L solvent)**
Na_2HPO_4 anh	141.9579	0.236	0.47	0.0017
KH_2PO_4	136.0838	1.134	2.27	0.0083

4.2.5.2 Metals

Metals were added in the carbon source stock solution, to maintain a molar relationship with carbon. Using this consideration, the metals decreased in concentration with the decrease in carbon concentration and approximate the metals concentration in wastewater.

4.2.5.3 Future considerations

An important question is the effect of very low substrate concentration on the uptake kinetics. It is expected that low substrate concentrations affect C, N and P uptake in different ways, and hence may change the optimal C:N:P ratio at these concentrations.

The optimisation of the C:N:P ratio is presented in Section 4.3.8, following the description of isolates and effect of operating conditions.

4.3 The Growth Kinetics for PGA Production

To design and optimize a process for poly (γ-glutamic acid) production, it is important to understand the growth kinetics of the various *Bacillus* strains, many of which are ubiquitous in the environment, including in wastewater (Bramucci and Nagarajan 2000; Buthelezi et al. 2009). Additionally, an improved level of understanding of the challenges of using minimal media as a source of nutrients combined with understanding the key bioprocess parameters influencing γ-PGA production will

allow for efficient optimisation of this system (Richard and Margaritis 2004), by providing the information required for design of a cost-effective and sustainable process with nutrient sources such as dilute wastewater streams.

In the present study, microorganisms isolated from a domestic wastewater treatment plant were enriched for *Bacillus* species and screened for the ability to produce γ-PGA. Suitable species were selected based on their γ-PGA production and their growth kinetics. Cultivation conditions for γ-PGA production were optimized for improved growth rates, substrate utilisation, biomass concentrations and subsequent γ-PGA yield, in terms of the ratio of carbon, nitrogen and phosphorus provided.

4.3.1 Bacterial strains

Bacillus licheniformis JCM 2505 was purchased from the Japanese Culture Collection and used as a reference strain. *B. licheniformis* was maintained on tryptone soy agar plates (17 $g.L^{-1}$ tryptone, 3 $g.L^{-1}$ soy peptone, 5 $g.L^{-1}$ NaCl, 2.5 $g.L^{-1}$ K_2HPO_4, 2.5 $g.L^{-1}$ dextrose and 15 $g.L^{-1}$ bacto agar) at 37°C.

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

4.3.1.1 Isolation of γ-PGA-producing strains

The isolation plates were inspected for different colony morphologies and 20 different colonies were selected. After numerous plate re-streaking, pure culture of isolates, 4 and 18, could not be obtained. The remaining 18 isolates were further investigated. Table 12 shows the cell and colony morphologies of these 18 isolates grown on medium MME agar plates.

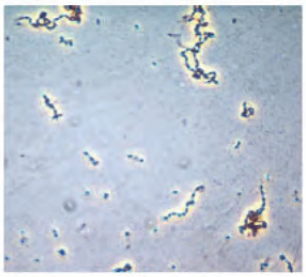
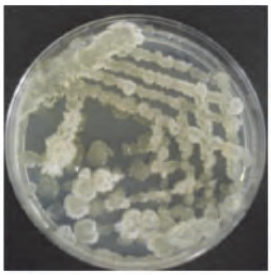
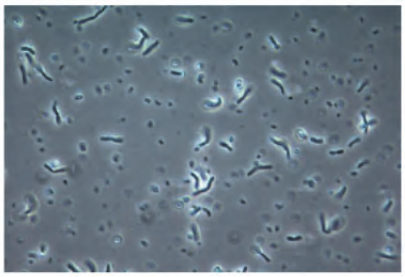
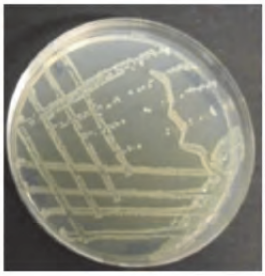
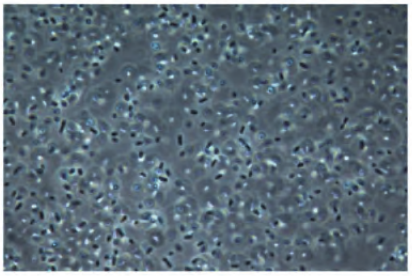
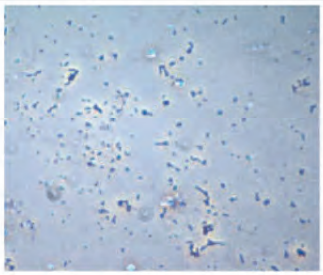
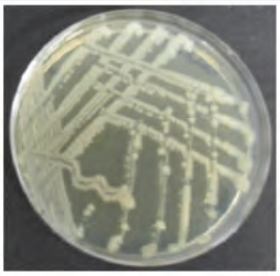
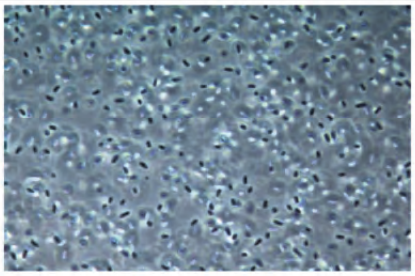
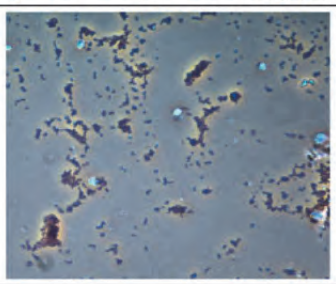
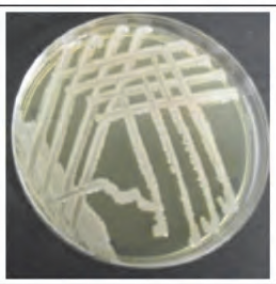
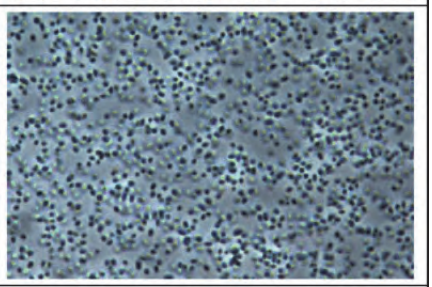
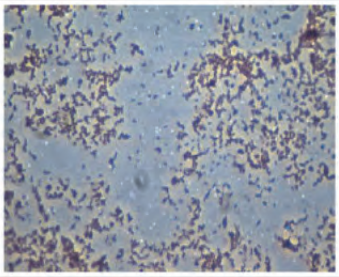
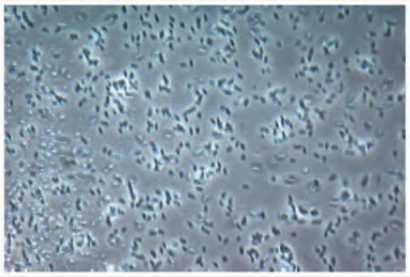
The Gram stains did not yield clear classification in all isolates; however 15 isolates appeared dark blue or purple i.e. Gram positive. These were mostly rod-shaped. Isolates 7, 12 and 14.2 stained pink, indicating Gram negative. These could be

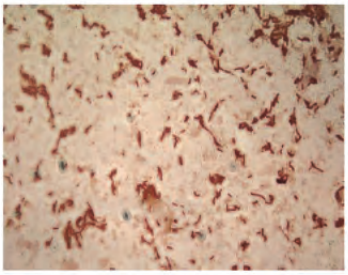
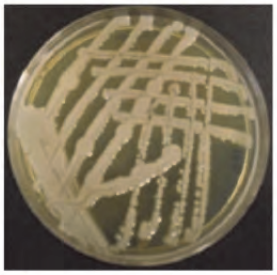
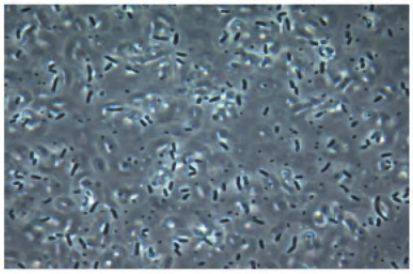
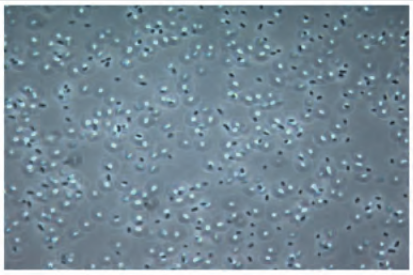
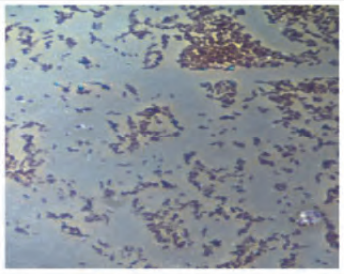
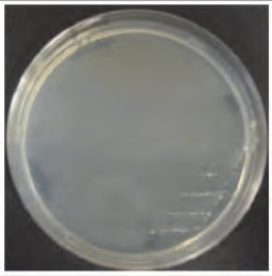
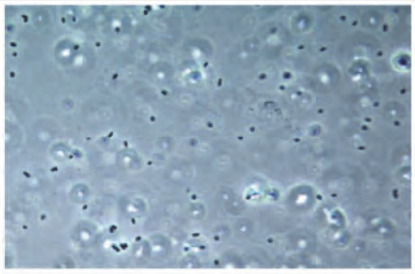
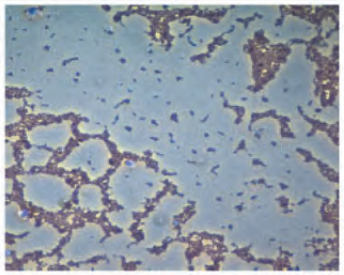
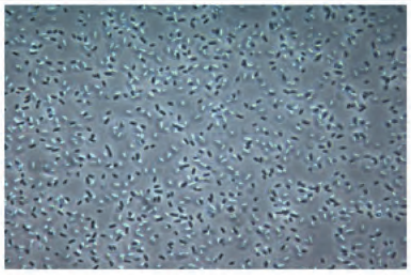
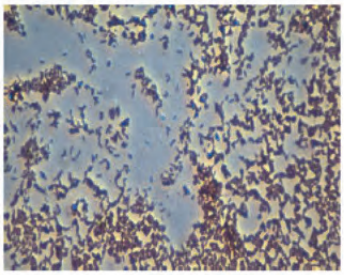
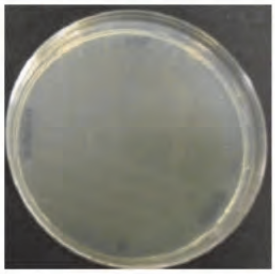
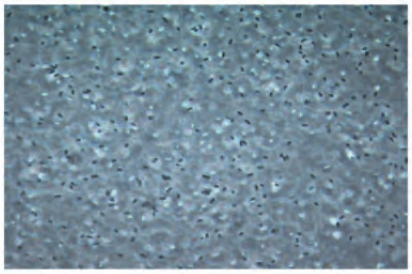
enteric bacteria since they were isolated from a domestic wastewater treatment plant. The long, linked rods of isolate 8 provided the clearest *Bacillus*-like morphology. The Gram stain is a good basic diagnostic tool, but may produce many inconclusive results. To counteract this, the isolates were also grown on MacConkey agar, a Gram negative selective and differential media. All isolates displayed varying degrees of growth, except isolate 11 which did not grow at all on either the isolation or the MacConkey plates. The Gram negative isolates aforementioned did show considerably greater growth compared to the Gram positive isolates on MacConkey agar.

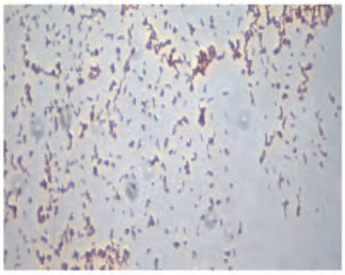
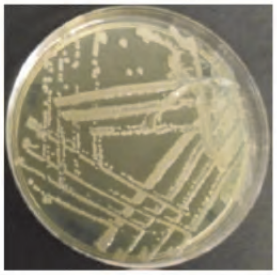
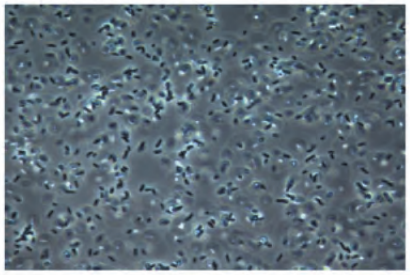
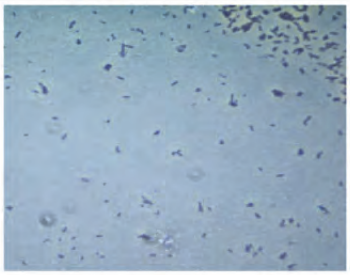
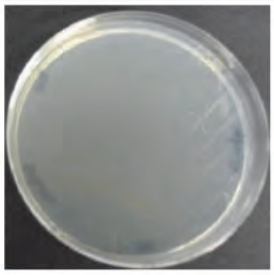
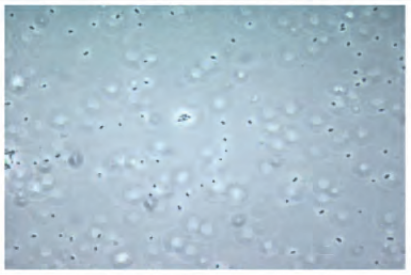
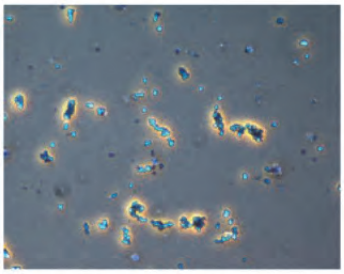
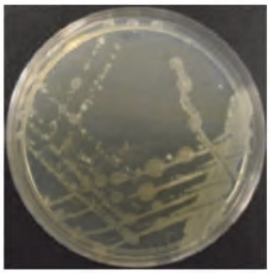
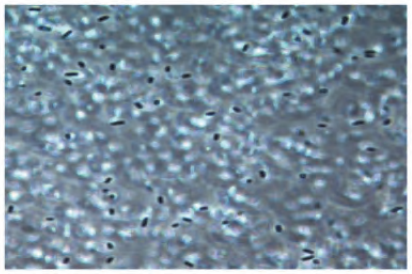
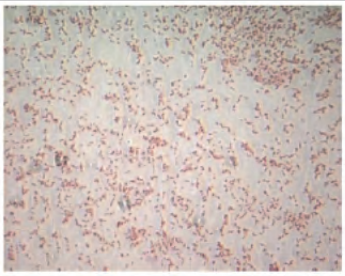
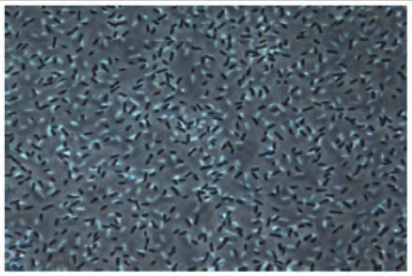
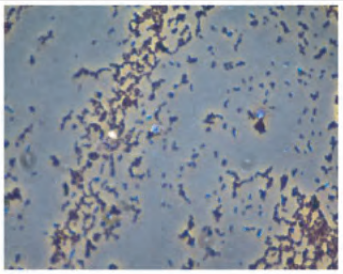
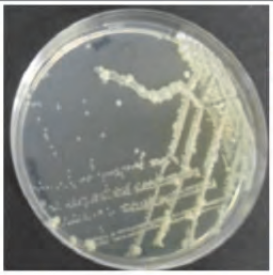
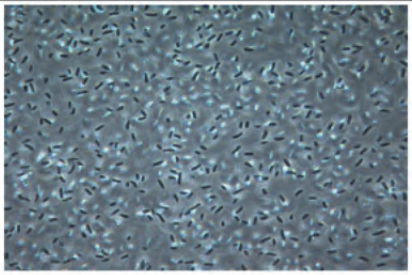
Isolates 1, 2, 7 and 12 were medium to long rods, some comma shaped and some with spores. These are indicators consistent with the *Bacillus* bacterial species (Bergey and Holt 1994). The rod shape was also observed for isolates 3, 6, 8, 9, 10, 11, 13 and 17, but the rods were smaller in size. Medium rods were observed for 14.2, 15, 16 and 19 whilst 14.1 had a slightly longer cell size. Most of the cells were randomly and singly dispersed in the medium, except isolate 1 which had cells in pairs and isolates 11 and 16 which had regions where the cells would aggregate together. Isolate 5 had coccus-shaped cells. The isolates produced similar cell morphologies when grown in medium ME (results not shown).

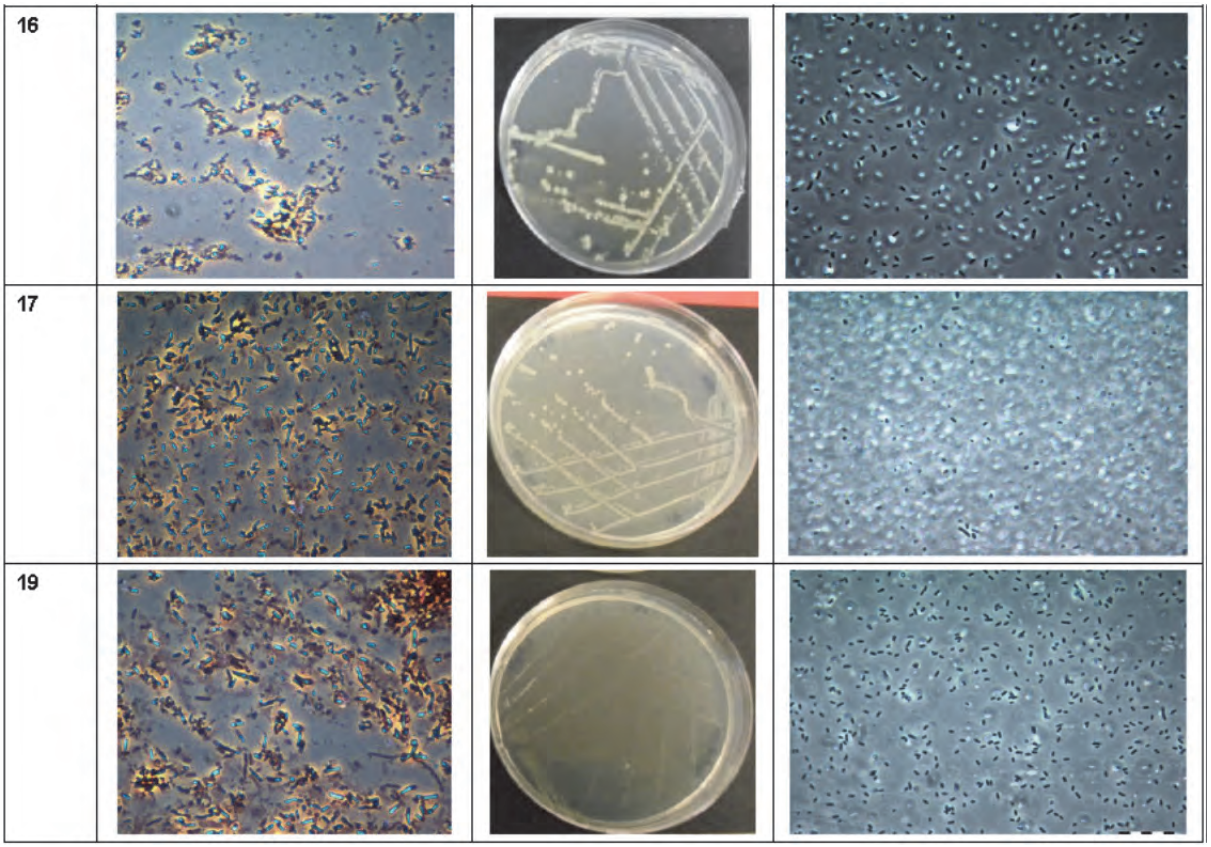
The most mucoid colonies on the isolation plates were isolates 1 and 10. The culture of isolate 6 was very viscous and elastic after the cultivation which is an indicator for the presence of γ-PGA (Yoon et al., 2000).

Table 12: The morphological characteristics of the isolates obtained from the Mitchell's Plain Waste Water Treatment Plant after growth at 37°C for 48 hours on isolation agar plates and MME.

Isolate	Gram stain	Isolation medium	MME
1			
2			
3			
5			
6			

7			
8			
9			
10			
11			

12			
13			
14.1			
14.2			
15			



Note – Due to non-growth in MME, the ME microscope picture is shown for isolate 19

4.3.1.2 Extraction of genetic material from isolated strains

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

4.3.2 Analytical procedures

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

An Adventurer Centrifuge (model no. AR2140, Ohaus Corp., NJ USA) was used to centrifuge 1 mL cell suspension in a pre-weighed Eppendorf tubes. The supernatant was discarded and the harvested pellet retained, washed with phosphate buffer

saline (in $g.L^{-1}$: NaCl 8.00; KCl 0.20; Na_2HPO_4 1.44; KH_2PO_4 0.24; pH 7.4), dried at 80°C for 24 hrs, left to cool in a desiccator cabinet overnight and thereafter measured to a constant weight to determine the dry cell weight.

The concentrations of glucose, glycerol and citric acid were determined by high performance liquid chromatography (HPLC) equipped with an Ultra Violet (UV-Vis) and Refractive Index (RI) detector (Thermo Scientific, Kempton Park, RSA) using a Rezex ROA organic acid H^+ (8%) column (7.8 mm x 300 mm, Phenomenex, California, USA) and 0.01M H_2SO_4 as the mobile phase. A flow rate of 0.5 $mL.min^{-1}$ was used with the column oven set at 80°C.

The γ-PGA analysis was carried out by HPLC (model no. AS3000, Thermo Scientific, Kempton Park, RSA) equipped with a RI detector using a TSKgel G5000 PW_{XL} gel permeation chromatography (GPC) column (7.8 mm x 300 mm, Tosoh, Tokyo, Japan). A 20 µL sample was injected in each run. Samples were eluted with 25 mM distilled water at a flow rate of 0.6 $mL.min^{-1}$ with the column oven set at 35°C. The purified γ-PGA was used as a standard to determine the concentration. Polyethylene oxide standards of known molecular weight were used to construct a calibration curve (Waters, Bellville, RSA) (Zhong et al. 2009).

4.3.3 Kinetic parameters

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

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

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

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

Integration of the Malthus equation (Equation 4) yields the straight line function, given in Equation 5. In the exponential phase where µ remains constant at μ_{max}, a straight line of slope μ_{max} is obtained on plotting the natural log of the biomass concentration as a function of time:

$$\frac{dX}{dt} = \mu X \qquad \text{Eqn 4}$$

$$\ln(X) = \mu t + X_0 \qquad \text{Eqn 5}$$

where X is the biomass concentration, t the incubation time and X_0 is the initial biomass concentration after inoculation.

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

4.3.4 Initial growth studies using *Bacillus licheniformis*

Shake flask cultivations were conducted with *B. licheniformis* JCM 2505 in shake flasks containing medium ME (Figure 11) and medium MME (Figure 12). Cell dry weight values of 1.35 $g.L^{-1}$ and 2.0 $g.L^{-1}$ were obtained after the 48 hour cultivation in media ME and MME, respectively. The pH values were similar in the two cultivations, and ranged between pH 5.73 and 6.27. The substrate analyses indicated that citric acid was not utilised during the 48 hour cultivation. The glycerol concentration decreased from 43.1 $g.L^{-1}$ to 38.7 $g.L^{-1}$, and 40.7 $g.L^{-1}$ to 36.5 $g.L^{-1}$, respectively, for media ME and MME. For medium MME, glucose was fully consumed after 24 hours. The maximum specific growth rate in medium ME and MME were 0.082 and 0.119 h^{-1}, respectively.

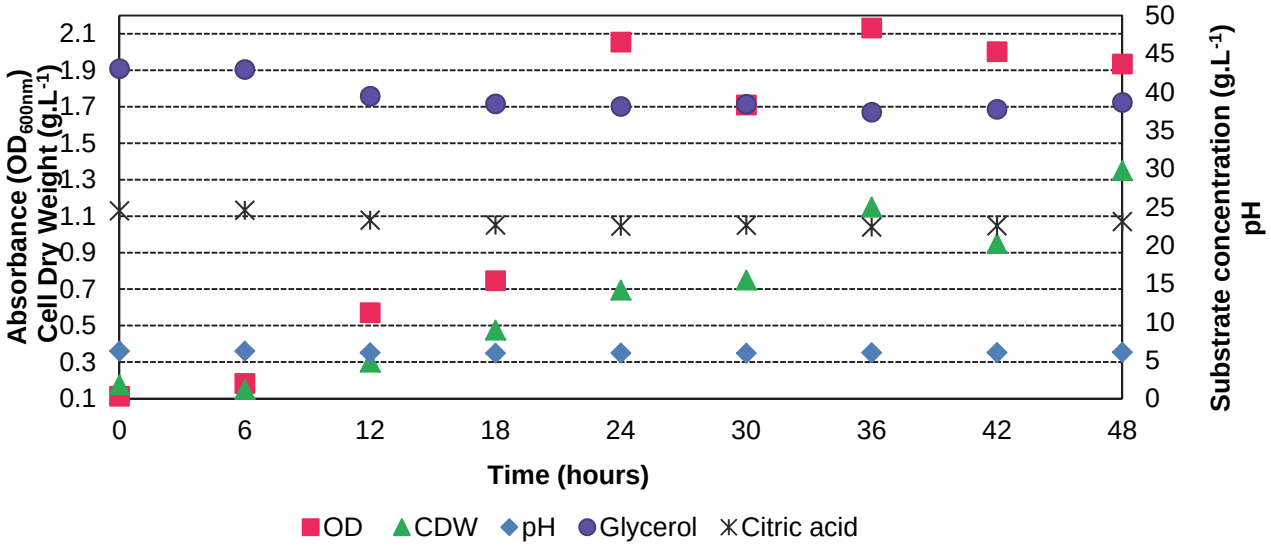


Figure 11: Growth profile for *B. licheniformis* grown in medium ME at 37°C. The averages of duplicate experiments are shown.

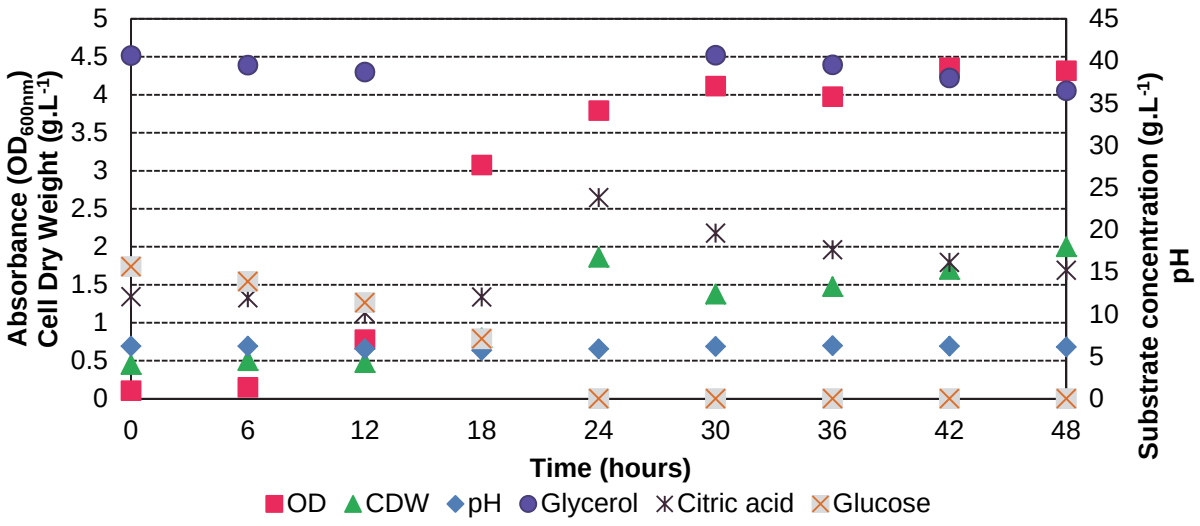


Figure 12: Growth profile for *B. licheniformis* grown in medium MME at 37°C. The averages of duplicate experiments are shown.

4.3.5 Optimising inoculation strategy for *B. licheniformis* JCM 2505

4.3.5.1 Initial screening

B. licheniformis was inoculated from the tryptone soy agar plates into γ-PGA screening medium. This consisted of the following: 20 $g.L^{-1}$ L-glutamic acid, 80 $g.L^{-1}$ glycerol, 12 $g.L^{-1}$ citric acid, 7 $g.L^{-1}$ NH_4Cl, 1.5 $g.L^{-1}$ K_2HPO_4 and mineral salts (0.5 $g.L^{-1}$ $MgSO_4.7H_2O$, 0.104 $g.L^{-1}$ $MnSO_4.H_2O$, 0.04 $g.L^{-1}$ $FeCl_3.6H_2O$ and 0.15 $g.L^{-1}$ $CaCl_2.2H_2O$) as given by Birrer *et al*. (1994) for Medium E (ME). An additional Modified Medium E (MME) was used to screen for microorganisms which overproduced γ-PGA in the absence of L-glutamic acid in the medium (glutamic acid-independent). This medium contained 20 $g.L^{-1}$ glucose instead of L-glutamic acid. Both media were adjusted to an initial pH 6.5 using 5M NaOH. L-glutamic acid was obtained from Sigma Aldrich (Missouri, USA). A pre-inoculum was prepared in either ME or MME by transferring a loopful of colonies from the streaked plates to 10 mL of medium in a 125 mL Erlenmeyer shake flask and incubated at 37°C and 200 rpm for 48 hours. The pre-inoculum was transferred to a 500 mL flask containing 100 mL of the same ME or MME medium. This inoculum was incubated at 37°C and 200 rpm for 16 hours and used to inoculate a sterile 250 mL ME or MME medium contained in a 1 litre flask to obtain a culture with a starting optical density of 0.1 at A_{600}. The flasks were placed on a rotary shaker at 200 rpm and 37°C for 48 hours. All media and glassware was autoclaved at 121°C for 20 min in order to render the equipment and materials free of undesired microbial contamination. Glucose and NaOH were obtained from Merck Chemicals (Wadeville, RSA) and all other medium components were from Saarchem (Honeydew, RSA). The experiments were done in duplicate.

4.3.5.2 Improving the inoculum strategy

In order to improve the growth rate and biomass concentration of *B. licheniformis*, the inoculum strategy was altered. A pre-inoculum was prepared by transferring a loopful of colonies from the tryptone soy agar plates to 10 mL of tryptone soy broth medium in a 125 mL Erlenmeyer shake flask and incubated at 37°C and 200 rpm for 48 hours. The pre-inoculum was transferred to a 500 mL flask containing 100 mL tryptone soy broth. This inoculum was incubated at 37°C and 200 rpm for 16 hours and comprised of the first step in the strategy. The second step involved using this inoculum to inoculate a sterile 250 mL tryptone soy broth medium contained in a 1 litre flask to obtain a culture with a starting optical density of 0.1. The culture was incubated at 37°C and monitored for 48 hours at 200 rpm. Thereafter, this culture was used to inoculate a sterile 250 mL ME or MME medium contained in a 1 litre flask to obtain a culture with a starting optical density of 0.1 in the third step. The culture was incubated at 37°C and monitored for 48 hours at 200 rpm. In the fourth step, 250 mL ME or MME medium contained in a 1 litre flask was also used. The inoculum was obtained from the flask in the third step when the cultivation period had been completed. The same starting inoculum concentration, incubation conditions and duration were used. This was repeated in a similar manner in the fifth

step, using an inoculum from the preceding step. A summary of the protocol is given schematically in Figure 13.

Figure 13: Schematic diagram of the protocol followed to determine growth in a complex and chemically defined medium using the sequential inoculation strategy experiment for *B. licheniformis*.

The effect of growth in a complex medium (tryptone soy broth) was compared to that in a chemically defined medium (ME and MME) in a series of shake flask experiments (Figure 14 to Figure 17). The inoculum was prepared in tryptone soy broth medium. A small sample was used to inoculate the first shake flask also containing tryptone soy broth medium to an A_{600} of 0.1 and incubated to maximum OD (step 2). Three subsequent inoculation and cultivation stages (similar to the first step) were conducted into flasks containing ME or MME, using a sample from the previous step as inoculum. No change in the final maximum OD value was evident after three transfers to MME (Figure 15). In contrast, the maximum OD values achieved in ME decreased after two transfers into medium ME (step 4, Figure 14). This could be due to the depletion of the additional growth factors supplied through the complex medium during the second transfer to a chemically defined medium. Interestingly, the maximum OD value during the third transfer to chemically defined medium (step 5) was again similar to the original maximum values achieved during step 2 and 3 in ME, indicating that the culture was utilising the same amount of carbon in both media and, potentially, adaptation to the minimal medium.

The maximum specific growth rates (μ_{max}) obtained during step 3 and 4 (first two transfers to the chemically defined medium) in both ME and MME was lower than the μ_{max} values measured during growth in the complex medium (step 2) (Figure 16 and Figure 17). The μ_{max} value decreased from 0.184 h^{-1} to 0.05 h^{-1} and 0.08 h^{-1} for ME and MME, respectively, after two transfers (step 4). Maximum specific growth rates were higher in both media after the third transfer (step 5) with values of 0.11 h^{-1} and 0.16 h^{-1} for ME and MME, respectively, indicating that the cultures had adapted to the new medium.

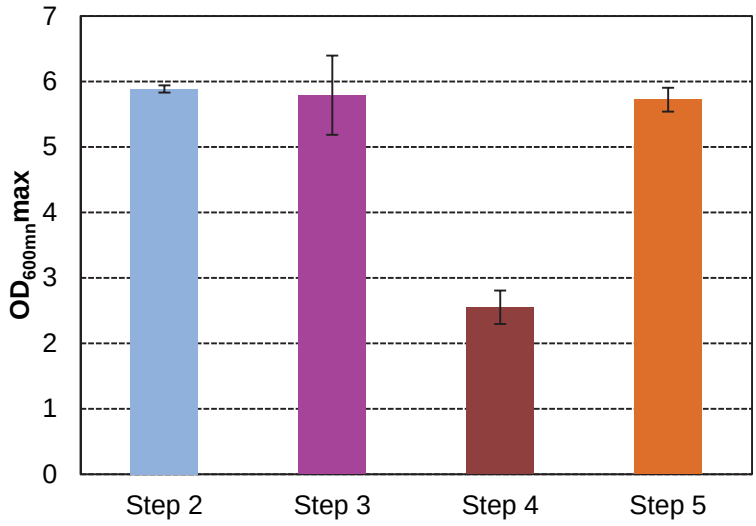


Figure 14: Maximum OD values obtained after growth of *B. licheniformis* at 37°C in shake flasks for 48 hours (average of duplicate experiments). The medium used for Step 2 was tryptone soy broth and for Step 3 to 5 ME.

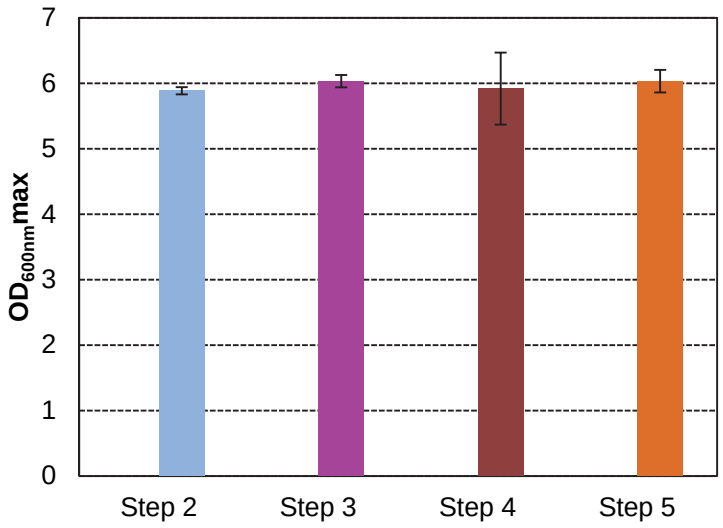


Figure 15: Maximum OD values obtained after growth of *B. licheniformis* at 37°C in shake flasks for 48 hours (average of duplicate experiments). The medium used for Step 2 was tryptone soy broth and for Step 3 to 5 MME.

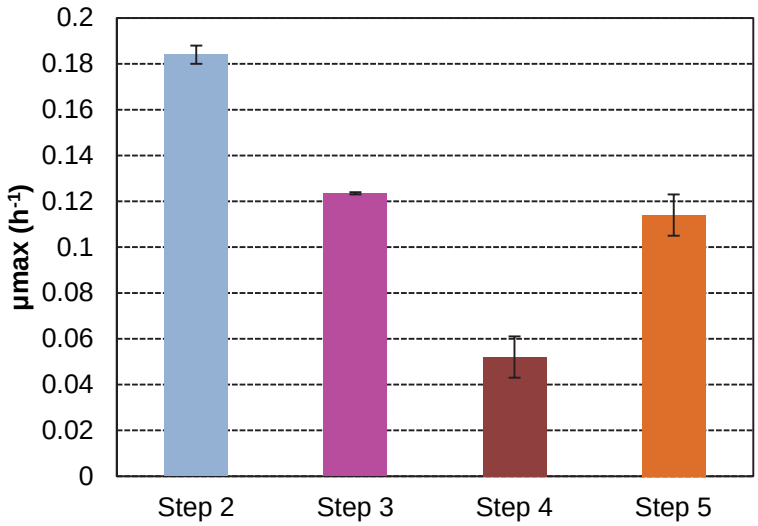


Figure 16: Maximum specific growth rate obtained after growth of *B. licheniformis* at 37°C in deep well plates for 48 hours (average of duplicate experiments). The medium used for Step 2 was tryptone soy broth and for Step 3 to 5 ME.

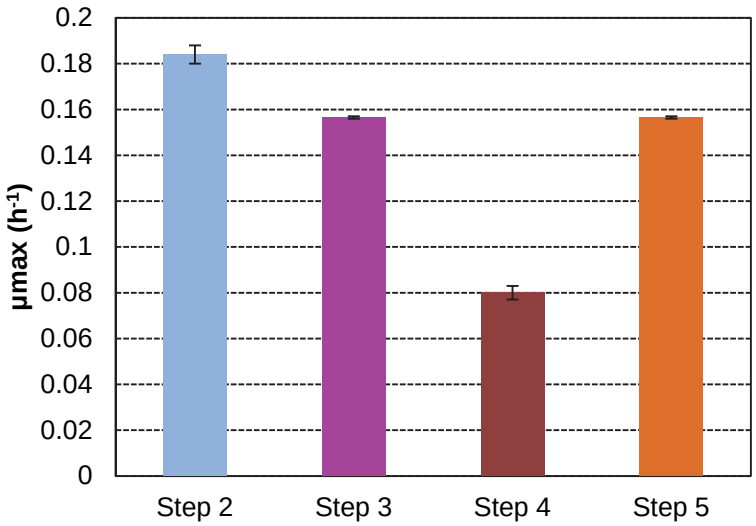


Figure 17: Maximum specific growth rate obtained after growth of *B. licheniformis* at 37°C in deep well plates for 48 hours (average of duplicate experiments). The medium used for Step 2 was tryptone soy broth and for Step 3 to 5 MME.

4.3.6 Preliminary screening of the isolated strains

The isolated microorganisms were inoculated from the isolation medium plates into 10 mL in ME and MME and incubated at 37°C in a rotary shaker (Incoshake LaboTec, USA) at 200 rpm for 48 hours. This was done in a single experiment.

4.3.6.1 Microscopic analysis

To assist in assessing that the culture was pure, gram staining was performed (Steele 1964). Once pure cultures had been obtained, the morphology of the isolates was studied microscopically (model no. BX40, Olympus Optical Company Ltd, Japan). Cultures were also periodically withdrawn and checked for contamination microscopically.

4.3.6.2 Isolate screening for production of γ-PGA

Medium E and Modified Medium E were used for the experiments to screen for γ-PGA production. A pre-inoculum was prepared in either ME or MME by transferring a loopful of colonies from the streaked plates to 10 mL of medium in a 125 mL Erlenmeyer flask and incubated at 37°C and 200 rpm for 48 hours. The pre-inoculum was transferred to a 500 mL flask containing 100 mL ME or MME medium. This inoculum was incubated at 37°C and 200 rpm for 16 hours and used to inoculate 250 mL sterile ME or MME contained in a 1 litre flask or a deep well microtitre plate containing 2 mL ME or MME. All flasks and deep well plates were inoculated to obtain a culture with a starting optical density of 0.1. The flasks and plates were placed on a rotary shaker at 200 rpm and 37°C for 48 hours. Samples were taken every 3 hours for the first 15 hours of incubation and every 8 hours thereafter for the 48 hour incubation period. All shake flask experiments were done in duplicate and deep well plate experiments in triplicate.

4.3.6.3 Selection for good growth

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

The screening experiment was also repeated with MME and the results are shown in Table 14. The final pH values were slightly lower compared to the ME screening experiment, with values ranging between pH 4.54 and 5.77 for all isolated except isolate 19, with a final pH of 6.37. The final pH for *B. licheniformis* was 6.14. Isolates 9 and 19 failed to grow in MME, and isolates 2 and 16 achieved very similar final biomass values (2.4 $g.L^{-1}$ and 2.05 $g.L^{-1}$) when compared to *B. licheniformis* (2 $g.L^{-1}$). Isolates with biomass production above 10 $g.L^{-1}$ included strains 1 (12.35 $g.L^{-1}$), 5, (11.7 $g.L^{-1}$), 6 (30.3 $g.L^{-1}$), 7 (19.6 $g.L^{-1}$), 8 (32.1 $g.L^{-1}$), 10 (15.05 $g.L^{-1}$) and 15 (32.45 $g.L^{-1}$).

Table 13: A summary of the OD values, biomass and pH values of the different isolates and *B. licheniformis* after growth in ME medium in shake flasks for 48 hours.

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

Table 14: A summary of the OD values, biomass and pH values of the different isolates and *B. licheniformis* after growth in MME medium in shake flasks for 48 hours

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

4.3.6.4 Selection through amplification of pgsB, pgsC, and pgsA genes

Primer design

The nucleotide sequence (accession number AB016245) of the three genes which encode a poly-γ-glutamate synthetic system in *Bacillus* was obtained from the National Centre for Biotechnology Information (NCBI) database website (Ashiuchi et al. 1999). Poly (γ-glutamic acid) synthetase forward (PGSF) and poly (γ-glutamic acid) synthetase reverse (PGSR) primers were designed using DNAMAN version 4.13 software (Lynnon BioSoft) to amplify an internal fragment of this gene cluster Figure 18).

Figure 18: The designed primers and their respective binding regions.

Additionally, a multiple sequence alignment was performed using all documented sequences from the NCBI database (GenBank accession numbers JF343561.1, JF343562.1, JF343563.1, HM034756.1, HM034757.1, AL009126.3, CP002183.1, DQ086153.1, AB016245.1, EF066513.1, GQ249061.1, GQ249062.1, HQ599194.1, EF066513.1). The consensus sequence obtained was then used to design an additional set of primers, PGS2F and PGS2R (Table 15) which could bind to the same region. These primers also amplified an internal fragment but annealed to a different region. The two sets of primers were synthesised at the Department of Molecular and Cell Biology, University of Cape Town.

Table 15: The primers and corresponding sequences used for the detection of the presence of the poly (γ-glutamic acid) synthetase gene complex in the isolates

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

Amplification of pgsB, pgsC, and pgsA genes

A polymerase chain reaction (PCR) was used to verify the ability of the selected isolates to produce γ-PGA by detecting the presence of the *pgsBCA* genes. The reaction mixture comprised 1 µL of each primer at a concentration of 0.25 µM, 200 µM deoxyribonucleotide triphosphate mixture, 2.5 mM $MgCl_2$, 2 units Taq polymerase and 2.5 µL reaction buffer (Kapa Biosystems, Cape Town, RSA), and 30 ng of genomic DNA in a 25 µL reaction volume. After an initial denaturing step of 5

min at 95°C, amplification was performed for 32 cycles under the following conditions: 30 s at 95°C, 30 s at 58°C, 30 s at 72°C and a final extension step of 72°C for 3 min. All PCR reactions were carried out in a PCR Sprint Thermal Cycler (Thermo Hyabaid, Middlesex, UK). The PCR product was analysed by loading 12 µL of each product on a 0.8% (w/v) agarose gel (prepared in TAE buffer (40 mM Tris-acetate and 1 mM EDTA) containing ethidium bromide (0.5 $\mu g.mL^{-1}$)) at 70 V for approximately 60 min and viewed under UV light using the Gel Doc system (Bio-Rad Laboratories, California, USA). The presence of a 1.1 kb fragment confirmed the *pgsBCA* gene locus whose product is necessary to confer on a cell the ability to produce γ-PGA.

PCR amplification using primers PGSF and PGSR was carried out to detect the presence of the pgsBCA genes as an indication of γ-PGA production potential. Based on the plate morphology and viscosity, isolate 1 was selected as the positive control and isolate 19 as the negative control. These primers failed to amplify the desired 1.1kb product, even after numerous temperature, time, and cycle optimisation steps, and therefore the presence of these pgs genes were not detected in any of the isolates tested (Figure 19).

Since some isolates had shown characteristics typical to γ-PGA producers such as the highly viscous broth, a second attempt was made to design more optimal primers. PGS2F and PGS2R were obtained and tested. These primers produced multiple bands for most of the isolates (Figure 20). However, some of the isolates did produce the desired fragment. The PCR procedure was then further optimised by varying the PCR cycle parameters. Distilled water was used as a negative control.

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

Isolates 1, 3, 6, 7, 8, 10, 11, 12, 13, 15, 17 and 19 produced the desired fragment size (Figure 20). The thick bands in lanes 1 and 7 show that the pgsBCA gene fragment of isolates 1 and 7 was strongly amplified. Isolates 10 and 12 also produced light bands at 1.1kb. Isolate 6 produced multiple bands, slightly smaller or larger than 1.1kb. Sequencing was done to ascertain that these represent the desired gene fragment.

Isolates 3, 13, 17, 19 could did not grow well in MME (Table 14). Isolate 15 could not replicate the growth shown in Table 14. Isolate 11 and 19 did not produce the desired plate morphology in Table 12.

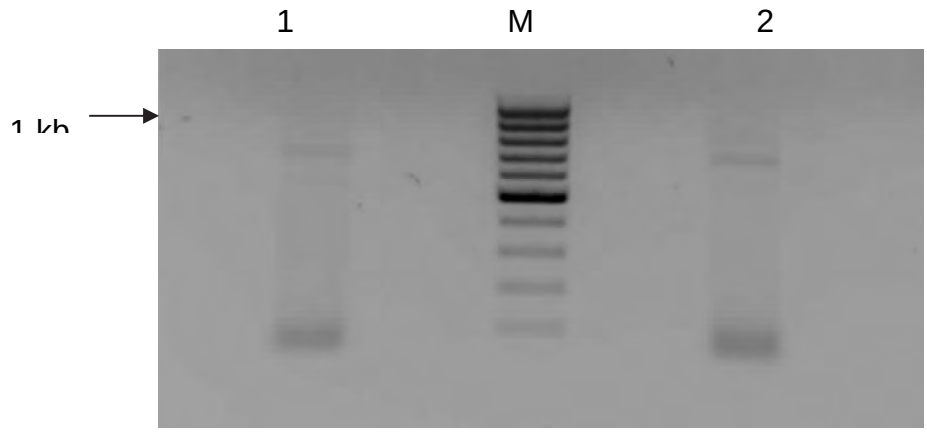


Figure 19: Detection of *pgs* genes by PCR using *pgsBCA* primers PGSF and PGSR. (1) isolate 1; (M) 100bp molecular weight marker; (2) isolate 19.

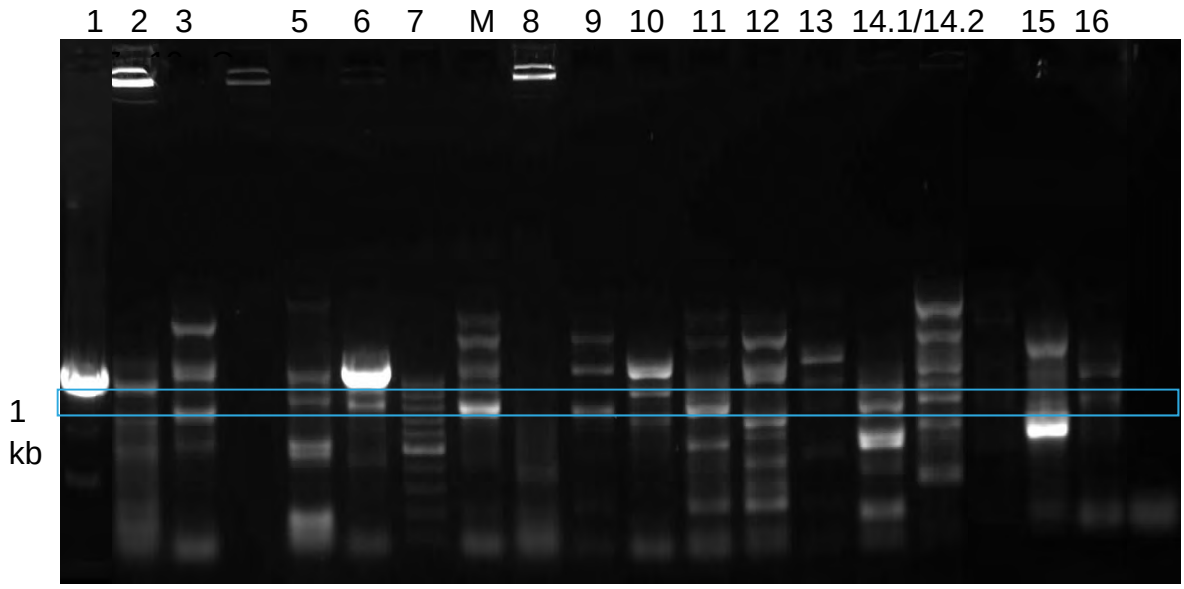


Figure 20: Detection of *pgs* genes by PCR using *pgsBCA* primers PGS2F and PGS2R in the isolates. The lane number correlates with the isolate number. (M) is the 100bp molecular weight marker and (C) the negative control.

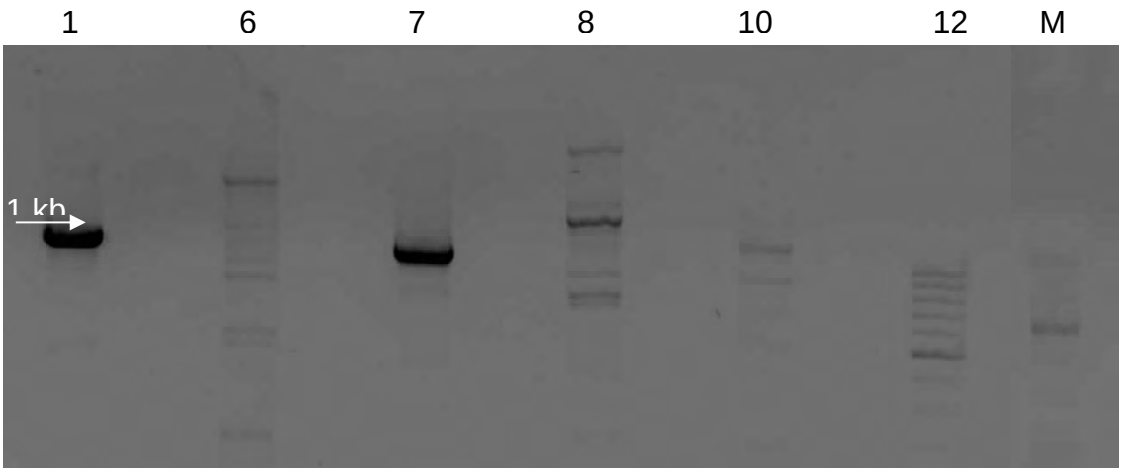


Figure 21: The optimised PCR of the selected six isolates using *pgsBCA* primers PGS2F and PGS2R. The lane number correlates with the isolate number. (M) is the 100bp molecular weight marker.

4.3.7 Screening of selected γ-PGA-producing strains

4.3.7.1 Screening in shake flasks

Based on the results from the first set of screening experiments (Table 13 and Table 14), particularly in MME, the morphological appearance and viscosity of the cultures (Table 12) and PCR experiments (Figure 21), isolates 1, 6, 7, 8, 10 and 12 were selected for further experimentation. The PCR of these isolates is shown in Figure 21. Figure 22 and Figure 23 show the growth curves for the different isolates that were cultivated in shake flask experiments in MME and ME growth media, respectively.

The results were reproducible with repeated experiments. The growth curves for both media follow the expected trend for an active inoculum in a typical batch process. No adaptation phase was observed for isolates 6, 7 and 12, and a very short adaptation phase of 3 hours was observed for isolates 1, 8 and 10 before reaching μ_{max} as the cells were diluted from the inoculum into the same fresh growth medium for the experiment.

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

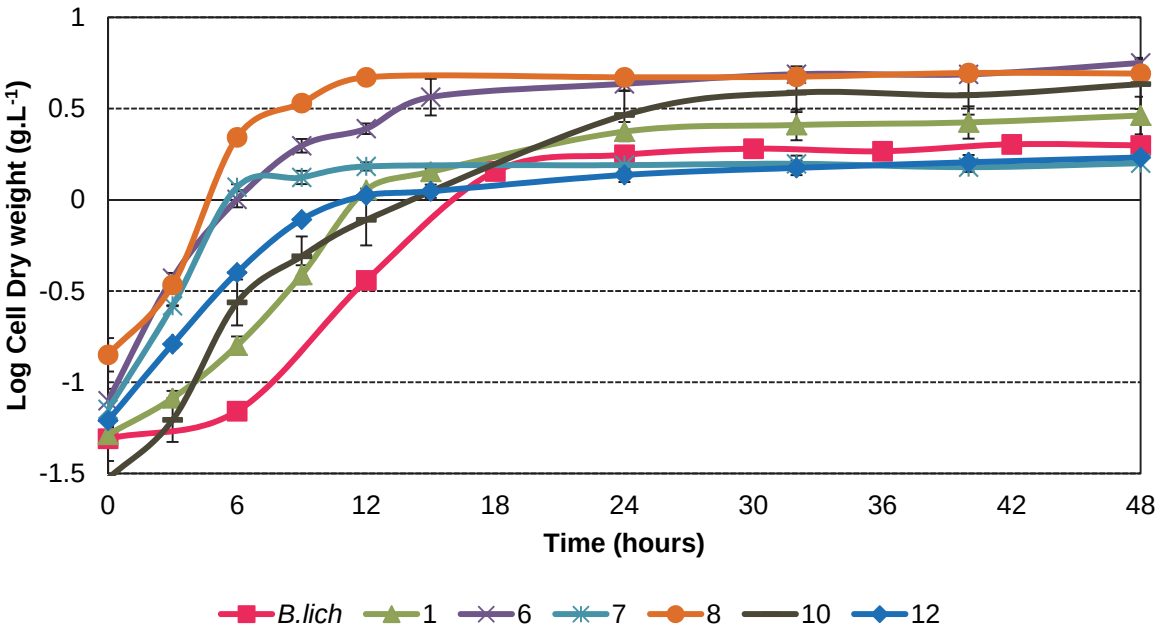


Figure 22: Growth profiles of *B. licheniformis* and isolates 1, 6, 7, 8, 10 and 12 in shake flasks containing MME. The averages of duplicate experiments with standard deviation error bars are shown.

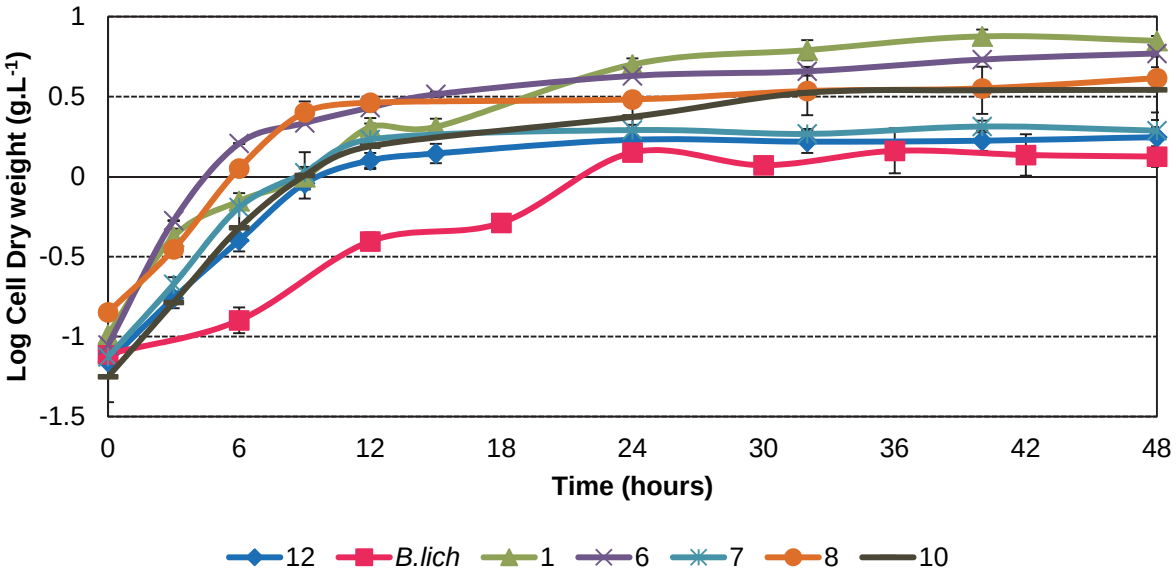


Figure 23: Growth profiles of *B. licheniformis* and isolates 1, 6, 7, 8, 10 and 12 in shake flasks containing ME. The averages of duplicate experiments with standard deviation error bars are shown.

The growth rate, final biomass concentration and change in biomass concentration determined for *B. licheniformis* and the six isolates in shake flasks containing growth media, ME and MME, are shown in Table 16. The maximum specific growth rates

varied between the different isolates and the medium used, and ranged between 0.112 h^{-1} and 0.269 h^{-1}. Whilst isolates 1 and 6 showed highest specific growth rates on ME (with μ_{max} of 0.203 h^{-1} and 0.209 h^{-1}, respectively), isolate 7, 8 and 10 achieved higher growth rates in MME (μ_{max} of 0.202 h^{-1}, 0.269 h^{-1} and 0.214 h^{-1}, respectively). Isolate 12 showed similar specific growth rates on ME and MME, however these growth rates and the maximum biomass concentrations were low, hence it was excluded from further experimentation.

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

4.3.7.2 Screening using deep well plates

Figure 24 and Figure 25 show the summary for growth of the selected isolates in deep well plate experiments in MME and ME respectively. Similar to the shake flask experiments, the growth curves for both growth media followed the expected trend for an active inoculum in a typical batch process. No adaptation phase was observed for isolate 8 and a very short adaptation phase of approximately 3 hours was observed for isolates 1, 6, 7 and 10 (Figure 24). The exponential growth lasted for approximately 9 hours. The maximum biomass concentration was maintained in the stationary phase with the expected death phase not evident in the culture period.

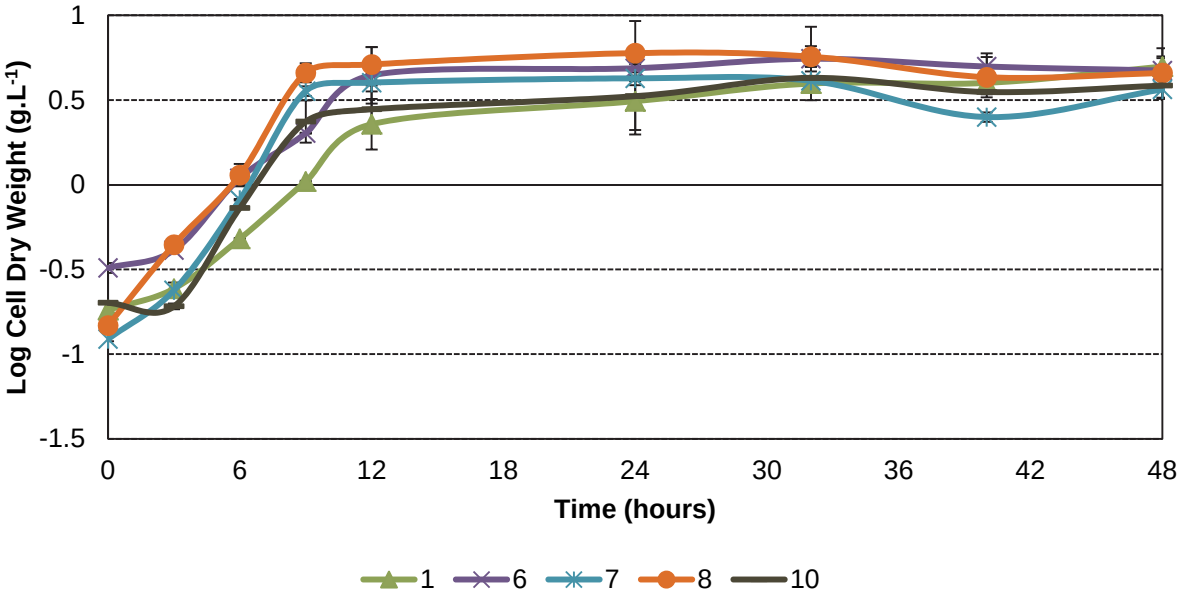


Figure 24: Growth profiles of *B. licheniformis* and isolates 1, 6, 7, 8 and10 in micro titre deep well plates containing MME. The averages of triplicate experiments with standard deviation error bars are shown.

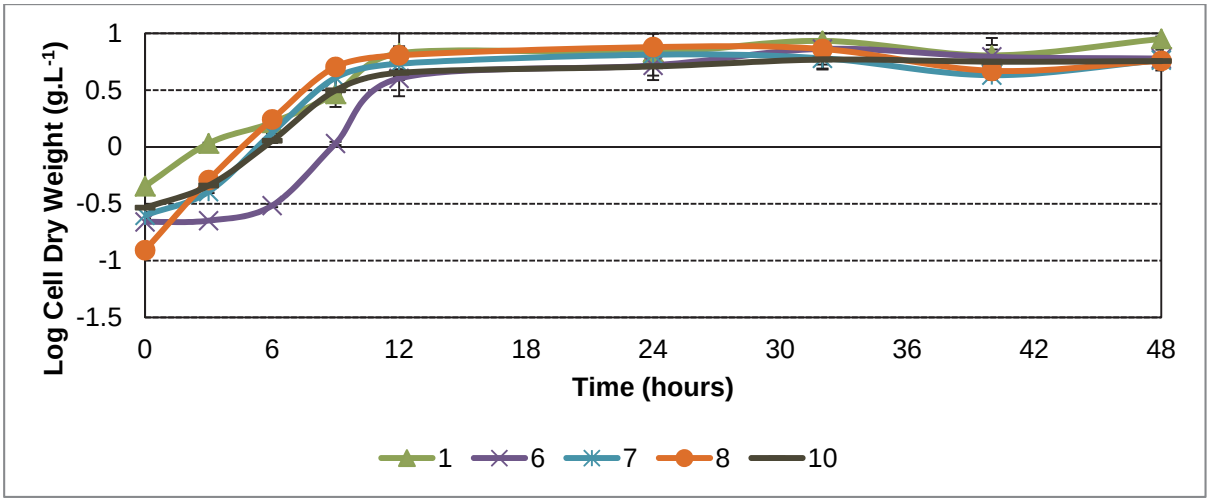


Figure 25: Growth profiles of *B. licheniformis* and isolates 1, 6, 7, 8 and 10 in micro titre deep well plates containing ME. The averages of triplicate experiments with standard deviation error bars are shown.

The growth rate, final biomass concentration and change in biomass concentration calculated for the different isolates and *B. licheniformis* grown in deep well plates containing growth media, ME and MME are shown in Table 17

Under the cultivated conditions of the deep well plates, the μ_{max} of the isolates ranged from 0.101 hr^{-1} to 0.196 hr^{-1} when grown in MME. When the isolates were grown in ME, the μ_{max} ranged from 0.101 hr^{-1} to 0.191 hr^{-1}. The maximum biomass ranged from 4.25 $g.L^{-1}$ to 5.98 $g.L^{-1}$ when the isolates were grown in MME, and 5.88 $g.L^{-1}$ to 8.94 $g.L^{-1}$ when grown on ME. Isolate 1 yielded the greatest biomass of 8.94 $g.L^{-1}$ when grown in medium ME. Isolates 6, 7 and 8 had the highest μ_{max} values (0.186 h^{-1} in ME, 0.196 h^{-1} in MME and 0.191 h^{-1} in ME, respectively).

4.3.7.3 Comparison of biomass growth in shake flasks and deep well plates

When comparing the growth kinetics of the isolates obtained from shake flasks and deep well plates (Table 16 and Table 17 respectively), the deep well plates generally produced higher biomass. ME favoured improved biomass, with isolate 1 producing a maximum biomass of 8.94 $g.L^{-1}$ compared to 7.08 $g.L^{-1}$ in the shake flask. Isolates 6, 7 and 8 also produced high biomass amounts of 7.30 $g.L^{-1}$, 6.51 $g.L^{-1}$ and 7.57 $g.L^{-1}$ in the deep wells, whilst the shake flasks produced slightly lower values, in some cases, of 5.89 $g.L^{-1}$, 2.06 $g.L^{-1}$ and 4.13 $g.L^{-1}$ respectively.

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

Table 16: Summary of the kinetic parameters for the different isolates after growth in shake flasks in ME and MME: The average of triplicate experiments are shown,

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

Table 17: Summary of the kinetic parameters for the different isolates after growth in micro titre deep well plates in ME and MME: The averages of triplicate experiments are shown,

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

4.3.7.4 Substrate utilisation

Substrate utilisation is shown in Figure 26 to Figure 29. The glycerol utilisation in the experiments in shake flasks containing MME and ME was less than 13.5% (Figure 26 and Figure 27, respectively). In general, the citric acid utilisation was also poor in MME, with less than 11% utilisation for most isolates, except isolate 12 where 41.2% citric acid was used (Figure 26). A big variation in citric acid utilisation was noted in the shake flasks containing ME, and ranged between 0% and 84%. The glucose utilisation in shake flasks with MME was the highest for isolates 1, 8, 10 and *B. licheniformis* with values above 93% utilisation. Isolates 6, 7 and 12 achieved glucose utilisation less than 56%.

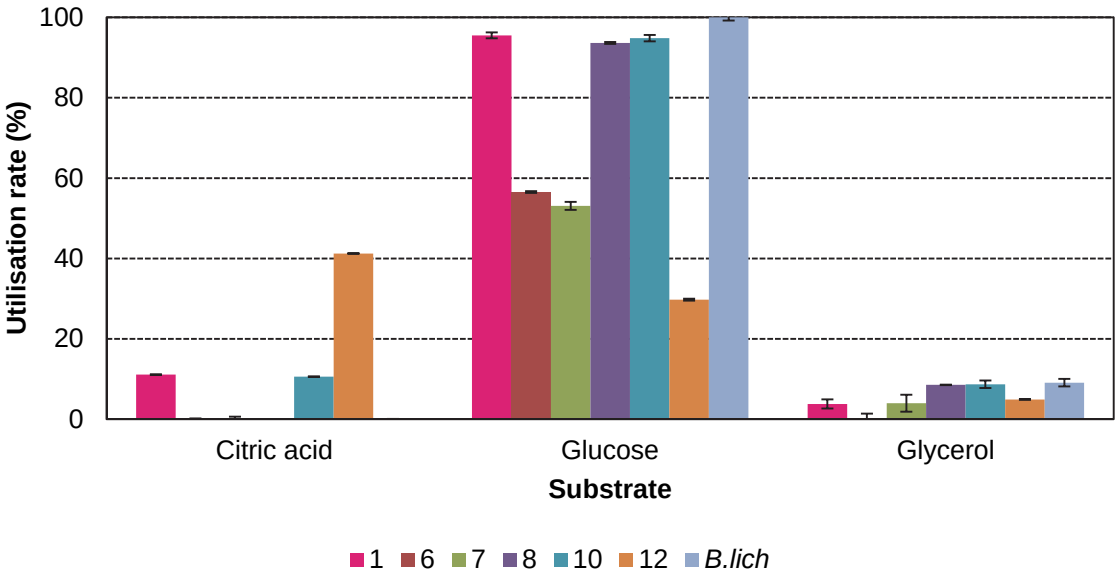


Figure 26: Substrate utilisation rates of *B. licheniformis* and isolates 1, 6, 7, 8, 10 and 12 in shake flasks containing MME, shown as average of duplicate experiments and standard deviation error bars.

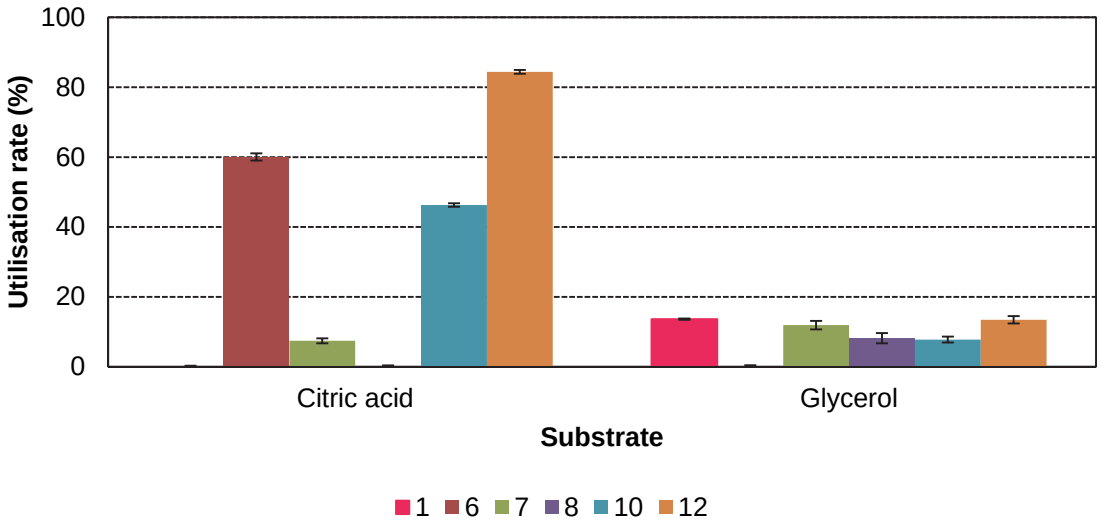


Figure 27: Substrate utilisation rates of *B. licheniformis* and isolates 1, 6, 7, 8, 10 and 12 in shake flasks containing medium ME. The averages of duplicate experiments with standard deviation error bars are shown.

In the deep well plates using MME (Figure 28), a similar trend was noted for the citric acid and glycerol utilisation, when compared to the shake flask cultivations using the same medium. Low utilisation rates were reported, with utilisation values less than 16.6% and 11.8% for glycerol and citric acid, respectively. The cultivations in deep well plates using ME (Figure 29) indicated low citric acid utilisation (less than 7%) for all isolates except isolate 8 with 82.6% citric acid utilisation. Isolates 1 and 8 achieved high glycerol utilisation in deep well plates using ME (above 79%), whereas

the other isolates achieved glycerol utilisation of less than 11%. In the deep well plates with MME, all isolates utilised glucose well (utilisation rates above 79%) except isolate 1 which did not utilise the glucose in the medium.

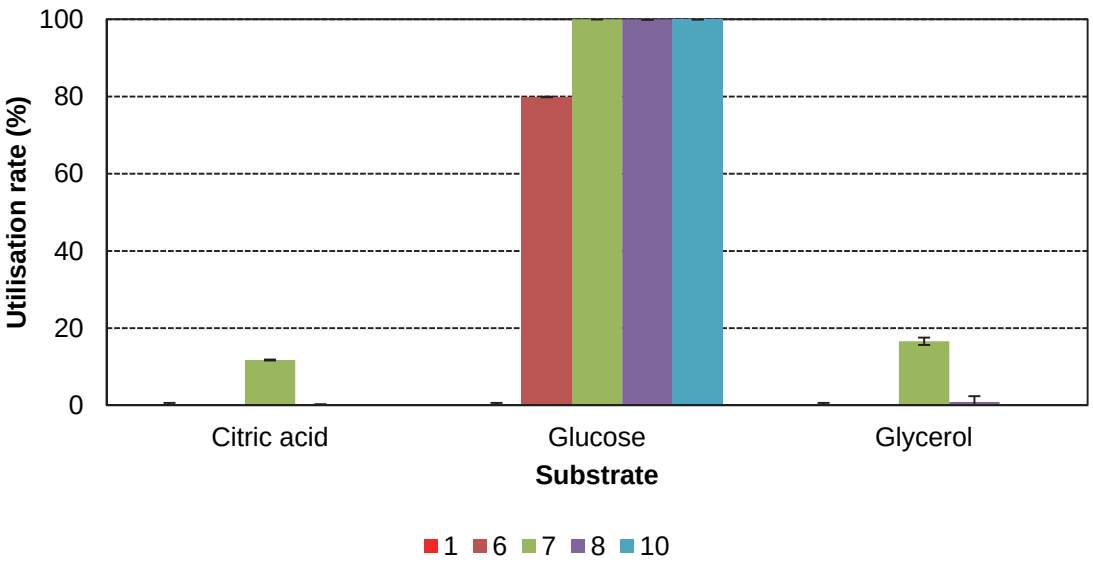


Figure 28: Substrate utilisation rates of *B. licheniformis* and isolates 1, 6, 7, 8 and 10 in micro titre deep well plates containing MME as averages of triplicate experiments with standard deviation error bars.

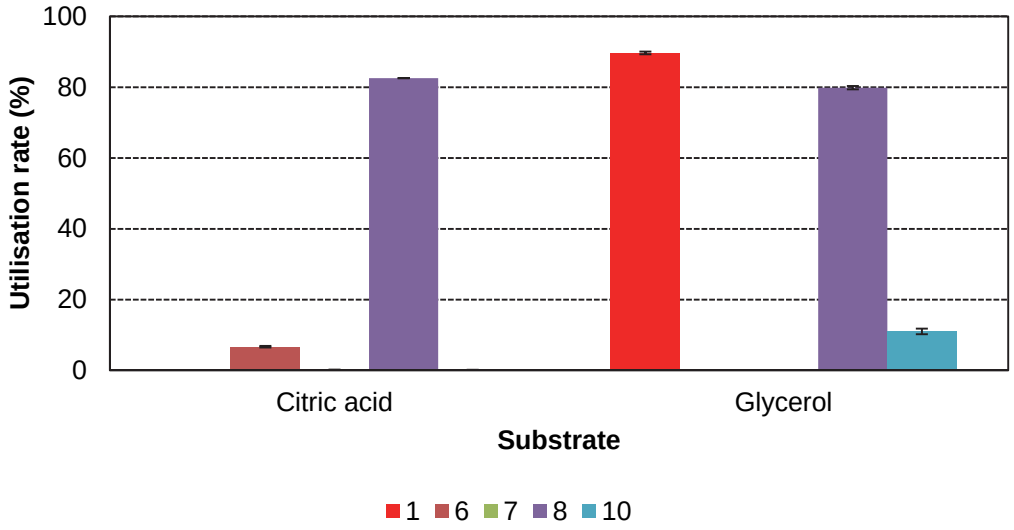


Figure 29: Substrate utilisation rates of *B. licheniformis* and isolates 1, 6, 7, 8 and 10 in micro titre deep well plates containing ME as averages of triplicate experiments with standard deviation error bars.

4.3.8 Optimisation of growth matrix in terms of the C:N:P ratio

4.3.8.1 Experimental system for qualitative assessment of C:N:P ratio using a factorial design

Deep well plates were used for the preliminary investigation of C:N:P ratio, using Isolate 1 (described in Section 4.3.1) at C concentrations of 1 and 2 M. The ratios used are given in Table 18, with media concentrations of 1 M and 2 M based on carbon. The medium used in these preliminary studies is a modification of Medium E, presented in Table 5, based on the work of Birrer et al. (1995) and others. Table 5 provides a representative sample of an example of the substrate concentration in well A1 (C:P:N of 5:1:0.01) from Table 18.

Table 18: C:N:P ratios for preliminary microwell experiments

	1			**2**			**3**			**4**		
	Carbon: 5			**10**			**50**			**Blanks for C, N, P**		
	C	N	P	C	N	P	C	N	P	C	N	P
A	5	1	0.01	10	1	0.01	50	1	0.01	0	1	0.5
B	5	1	0.05	10	1	0.05	50	1	0.05	10	0	0.5
C	5	1	0.10	10	1	0.10	50	1	0.10	10	1	0

Table 19: Substrates in well A1 (CNP: 5:1:0.01) from Table 18 at 1 M carbon as example of substrate composition

Substrate	**g/mol**	**mol/L**	**mol C/L**	**g/L**
Glycerol	92	0.295	0.886	27.2
Citric acid. H_2O	210	0.019	0.114	4
Total C substrate		0.314	1	31.2
$(NH_4)_2SO_4$	132	0.2	-	13.2
K_2HPO_4	174			
$NaH_2PO_4.12H_2O$	358			
Total P substrate		0.002	-	0.348

4.3.8.2 Optimisation of growth matrix in terms of C:N:P ratio using a factorial design

The glucose modified medium E (MME) was optimised for optimal carbon, nitrogen and phosphorus ratios for high growth rate and biomass production. A Plackett-Burman factorial design was used to develop a 3-factor, 2-level design. Eight combinations of C:N:P ratios were tested on a subset of isolates described in Section 4.2.5 (Table 20) (Plackett and Burman 1946). The trace salts concentration from MME was used. The optimization studies were performed in deep well plates as outlined. The samples were withdrawn from the deep well plates every 3 hours for the first 12 hours and at the end of the 48 hour cultivation period and analysed for the cell growth. Each experiment was performed in triplicate.

Table 20: C:N:P experimental matrix with relative nutrient inputs for the optimisation study

Run #	Factors			Ratio			Inputs [g/200mL]
	Carbon High=4 Low=2	Nitrogen High=0.26 Low=0.065	Phosphorus High=0.0172 Low=0.0043	C	N	P	
1	low	low	low	2	0.065	0.0043	Glucose = 2.16; glycerol = 8.65; citric = 1.29; NH4Cl = 0.696; K2HPO4 = 0.17
2	low	low	high	2	0.065	0.0172	Glucose = 2.16; glycerol = 8.65; citric = 1.29; NH4Cl = 0.696; K2HPO4 = 0.59
3	low	high	low	2	0.26	0.0043	Glucose = 2.16; glycerol = 8.65; citric = 1.29; NH4Cl = 2.78; K2HPO4 = 0.17
4	low	high	high	2	0.26	0.0172	Glucose = 2.16; glycerol = 8.65; citric = 1.29; NH4Cl = 2.78; K2HPO4 = 0.59
5	high	low	low	4	0.065	0.0043	Glucose = 4.33; glycerol = 17.31; citric = 2.59; NH4Cl = 0.696; K2HPO4 = 0.17
6	high	low	high	4	0.065	0.0172	Glucose = 4.33; glycerol = 17.31; citric = 2.59; NH4Cl = 0.696; K2HPO4 = 0.59
7	high	high	low	4	0.26	0.0043	Glucose = 4.33; glycerol = 17.31; citric = 2.59; NH4Cl = 2.78; K2HPO4 = 0.17
8	high	high	high	4	0.26	0.0172	Glucose = 4.33; glycerol = 17.31; citric = 2.59; NH4Cl = 2.78; K2HPO4 = 0.59

4.3.8.3 Optimization studies

Preliminary optimization of the C:N:P ratio and carbon concentration was carried out in deep well plates using Isolate 1, as described above. The micro-reactor wells, labelled 1 to 3, are characterised by decreasing N content, while rows A – C have increasing P content. Column D contained the blanks: no C substrate was added to Well A4; no N substrate to Well B4; no P content to Well C4.

From observation of comparative growth in the micro-reactors (Figure 30); Figure 31), it seems that there is a certain nutrient threshold where the bacterium moves from growth in suspension to growth at the air-liquid interface, forming a puffy mat. This may be due to increasing carbon concentration or increasing N, the latter being more pronounced. Column 1 has a C:N ratio of 5:1 and Column 2 10:1, both showing good surface biofilm growth. Column 3, at a C:N ratio of 50:1, does not show strong biofilm growth. With respect to the P concentration, an increasing phosphate concentration led to a stress response, indicated by a red colour and the distinctive acrylate smell. This was most pronounced at 2 mol C /L. It remains to be investigated whether these observations are only due to the high substrate concentrations, or characteristics of the C:N:P requirements of the organism.

Figure 30: Growth of *Bacillus* Isolate 1 in microwell containing 1 mol C/ L

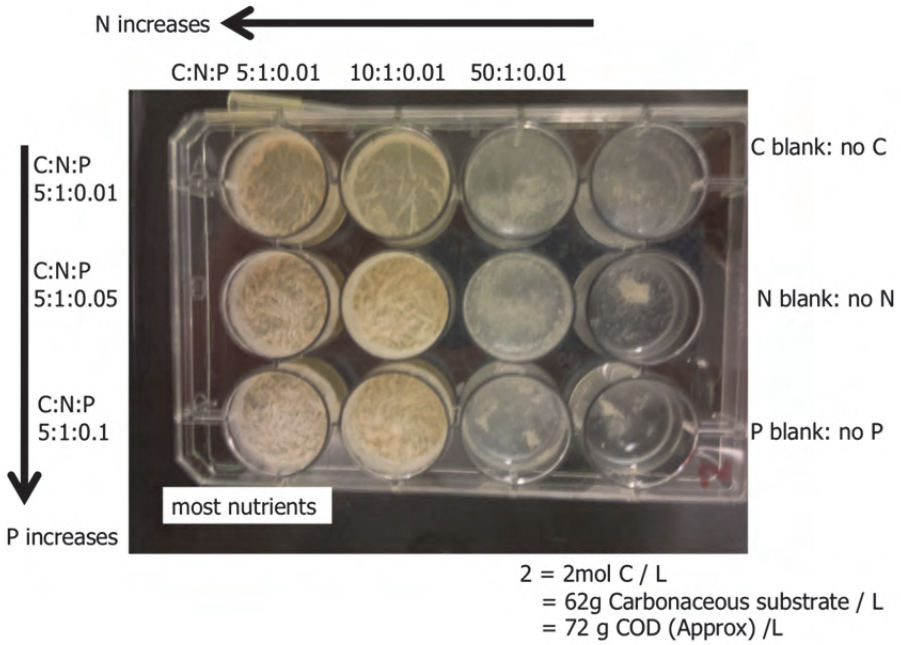


Figure 31: Growth of *Bacillus* Isolate 1 in microwell containing 2 mol C/ L

From initial observations in the micro-reactor experiments, the thick mat biofilm structure and a clear layer directly beneath the mat indicates promising PGA location and potential to colonise a mesh structure which provides an air-liquid interface.

4.3.8.4 Media optimization by factorial design

The glucose modified medium E (MME) was optimised for optimal carbon, nitrogen and phosphorus ratios for high growth rate and biomass production, using a two-level factorial design with carbon factors of 2 and 4, nitrogen of 0.065 and 0.26, and phosphorus of 0.0043 and 0.0172. Summaries of the results for the optimisation experiments for Isolate 1, 8 and 10 (described in Section4.3.7) are shown in Table 21, Table 22 and Table 23.

For isolate 1, the highest OD_{600} (8.15) and maximum specific growth rate (0.194 h^{-1}) were achieved using a C:N:P ratio of 30.7:1:0.065 (2:0.065:0.0043) and 61.5:1:0.065 (4:0.065:0.0043) respectively. An example of an optimisation run is shown in Figure 32.

In the case of isolate 8, the highest OD value was noted for a C:N:P ratio of 7.7:1:0.016 (2:0.26:0.0043), with an OD_{600} values of 11.90. The highest maximum specific growth rate (0.38 h^{-1}) was achieved with a C:N:P ratio of 30.7:1:0.26 (2:0.065:0.0172). For isolate 10, the highest OD_{600} (15.61) and maximum specific growth rate (0.330 h^{-1}) were achieved using a C:N:P ratio of 30.7:1:0.065 (2:0.065:0.0043). The citric acid, glycerol and glucose utilisation rates are shown (Table 22, Table 23 and Table 21). The data gathered to date suggest that optimal growth may occur at lower N and P levels; however this needs to be considered in terms of PGA analysis too.

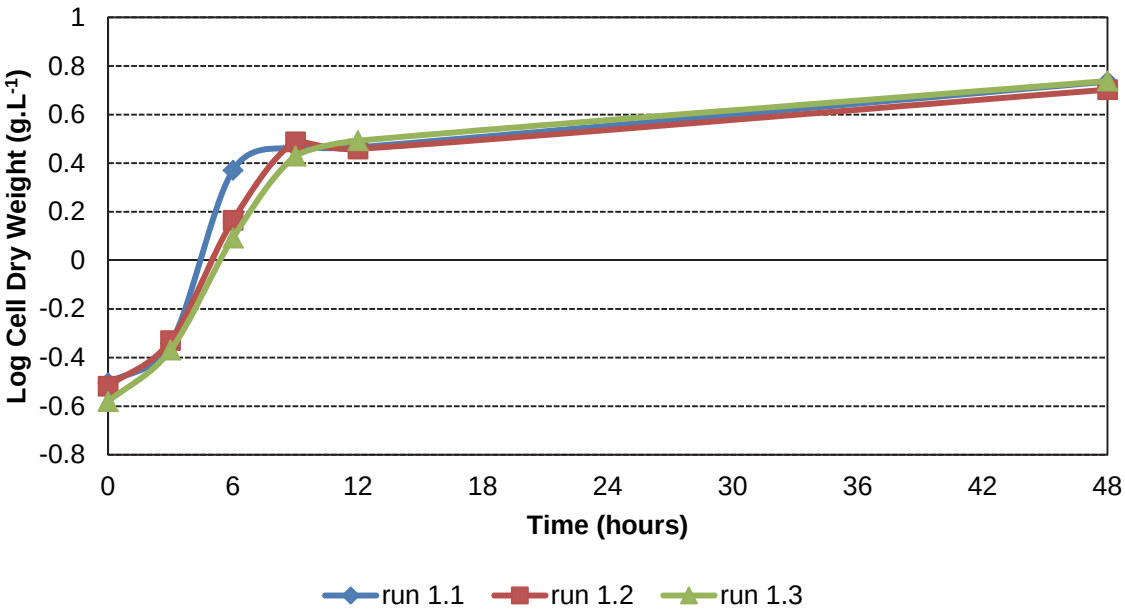


Figure 32: Growth curve for Isolate 1, Run 1 medium optimisation experiment

Table 21: Optimisation study results for Isolate 1 after growth for 48 hours in the various media in micro titre deep well plates. The averages of triplicate experiments are shown.

Run	OD_{600}	μmax (hr^{-1})	Citric acid %	Glycerol %	Glucose %	[γ-PGA] ($g.L^{-1}$)
1	8.15 ± 0.21	0.185 ± 0.03	72.16	72.60	94.27	OS
2	7.73 ± 0.57	0.200 ± 0.04	ND	ND	ND	OS
3	7.24 ± 0.42	0.04 ± 0.01	66.72	67.67	66.19	OS
4	7.93 ± 0.08	0.188 ± 0.00	ND	ND	ND	OS
5	6.11 ± 0.17	0.194 ± 0.06	57.66	58.00	62.00	OS
6	5.90 ± 0.19	0.071 ± 0.00	ND	ND	ND	OS
7	4.78 ± 0.33	0.106 ± 0.02	63.56	67.51	69.00	OS
8	6.35 ± 0.25	0.080 ± 0.00	ND	ND	ND	OS
MME	3.21 ± 0.05	0.110 ± 0.02	11.10	3.79	95.52	OS

OS – outstanding
ND – Not determined
MME – values for isolate in standard MME medium prior to medium optimisation.

Table 22: Optimisation study results for Isolate 8 after growth for 48 hours in the various media in micro titre deep well plates.

The averages of triplicate experiments are shown.

Run	OD_{600}	μmax (hr^{-1})	Citric acid %	Glycerol %	Glucose %	[γ-PGA] ($g.L^{-1}$)
1	9.74 ± 0.27	0.338 ± 0.04	0	0	0	OS
2	9.07 ± 0.24	0.380 ± 0.02	0	0	0	OS
3	11.90 ± 1.56	0.245 ± 0.02	0	60.27	78.96	OS
4	9.69 ± 0.62	0.285 ± 0.01	0	66.26	73.15	OS
5	7.62 ± 0.50	0.318 ± 0.03	ND	ND	0	OS
6	7.05 ± 0.29	0.321 ± 0.02	0	0	100	OS
7	0.03 ± 0.01	0	0	0	0	OS
8	7.58 ± 0.18	0.232 ± 0.00	0	0	0	OS
MME	4.11 ± 0.15	0.149 ± 0.01	0	3.98	53.07	OS

OS – outstanding

ND – Not determined

MME – values for isolate in standard MME medium prior to medium optimisation.

Table 23: Optimisation study results for Isolate 10 after growth for 48 hours in the various media in micro titre deep well plates. The averages of triplicate experiments are shown.

Run	OD_{600}	μmax (hr^{-1})	Citric acid %	Glycerol %	Glucose %	[γ-PGA] ($g.L^{-1}$)
1	15.61 ± 1.05	0.330 ± 0.00	0	0	0	OS
2	14.16 ± 0.25	0.305 ± 0.00	0	0	0	OS
3	11.90 ± 1.56	0.220 ± 0.02	ND	ND	ND	OS
4	3.61 ± 0.62	0.270 ± 0.02	0	0	100.00	OS
5	9.64 ± 0.15	0.292 ± 0.00	0	0	100.00	OS
6	10.45 ± 1.14	0.288 ± 0.01	0	0	100.00	OS
7	0.02 ± 0.00	0	0	0	0	OS
8	0.21 ± 0.01	0.187 ± 0.03	0	0	0	OS
MME	**4.23** ± 0.08	**0.182** ± 0.00	**10.60**	**8.68**	**94.85**	OS

OS – outstanding

ND – Not determined

MME – values for isolate in standard MME medium prior to medium optimisation.

4.3.9 PGA production

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

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

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

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

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

γ-PGA concentration was determined after 48 hours cultivation in the deep well plates, using both MME and ME, as shown in. The values ranged between 35.36 $g.L^{-1}$ and 91.71 $g.L^{-1}$. The highest values were reported in MME for isolate 1 (91.71 $g.L^{-1}$) and isolate 10 (90.89 $g.L^{-1}$). Isolate 12 produced the lowest amount of γ-PGA (35.26 $g.L^{-1}$ in ME and 39.39 $g.L^{-1}$ in MME). γ-PGA was determined from shake flask cultivations of this isolate.

Table 24: γ-PGA concentration ($g.L^{-1}$) of isolates 1, 6, 7, 8, 10 and 12 in deep well plates after 48 hours containing ME or MME: The result from a single measurement is shown.

Isolate	MME	ME
1	91.71	80.99
6	84.87	79.52
7	75.66	77.42
8	77.57	83.42
10	90.89	77.88
12[#]	39.39	35.26

[#] Note – γ-PGA was determined from shake flask cultivations for isolate 12

4.4 Biofilms on synthetic media

This experiment was used to investigate growth of a biofilm on synthetic wastewater, and if this biofilm resembles the expected form.

4.4.1 Experiments on agar plates

Table 12 shows the bacterial strains which were isolated from sewage mixed with activated sludge. Two isolates showed promising extracellular polymer production. Figure 33 shows Isolate 1 growing on an agar plate, illustrating the soft, dry, mucoid colonies. On some agar plates, this isolate grew in a highly mucoid, transparent, clear, sticky biofilm. Figure 34 shows the biofilm on an agar plate illustrating two forms of the biofilm. The colonies on the left are dry to the touch and soft, while the right is viscous, clear and sticky. After a day or more this clear, sticky biofilm became similar to the biofilm on the right.

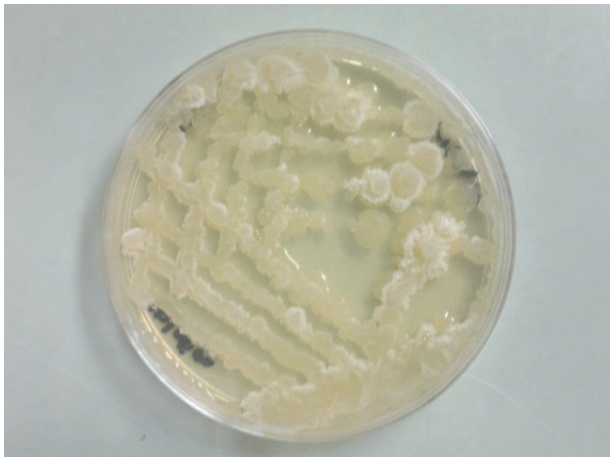


Figure 33: Isolate 1 on an agar plate, illustrating soft, dry, mucoid colonies.

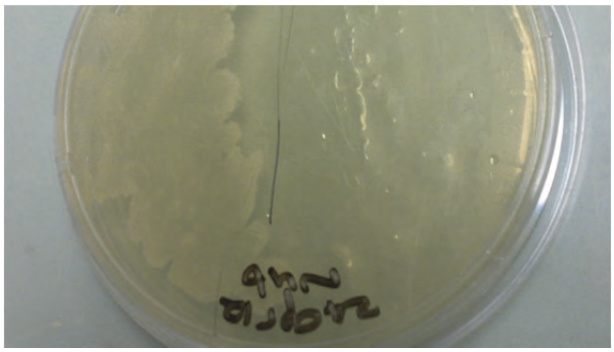


Figure 34: Isolate 1, on an agar plate, illustrating clear sticky biofilm (right).

4.4.2 Microreactor experiments

Microreactor experiments are a quick, qualitative, low-resolution way to explore growth requirements for microorganisms. In an optimized system these microreactor experiments can give as much quantitative information as shake flasks (Betts & Baganz 2006), but in the observations below, only rudimentary growth was examined with emphasis on the potential for biofilm formation. The reactors were not optimized for gas transfer or adequate mixing.

The microreactor wells, labelled 1-4 in increasing C ratio (decreasing N content) and A-C in increasing P ratio (increasing P content) (as indicated in Table 26) were normalized to C content (g/L concentrations shown in Table 27 (1 mol C / L) and Table 28 (2 mol C / L)), to allow comparable biomass formation. Column 4 (rightmost column) contained the blanks. Well A4 contained no added C substrate. Well B4 contain no added N substrate, and Well C4 contained no added P content.

Table 25 shows substrate concentration and composition using well A1 as example. The composition has been adapted from Medium E, rather arbitrarily from work done by Birrer et al and others (Birrer et al.1995).

Table 25: Substrates in well A1 (CNP: 5:1:0.01) as example of substrate concentration and composition

Substrate	**g/mol**	**mol/L**	**mol C/L**	**g/L**	**g COD/ g**	**g COD / L**
Glycerol	92	0.295	0.886	27.2	1.217	33
Citric acid.H_2O	210	0.019	0.114	4	0.635 (without H_2O)	2.5
Total C substrate		**0.314**	**1**	**31.2**		**35**
$(NH_4)_2SO_4$	132	0.2	-	13.2	0	0
K_2HPO_4	174					
$NaH_2PO_4.12H_2O$	358					
Total P		0.002	-	0.348	0	0

Table 26: CNP ratio of Microwell experiments

N=1			**1**			**2**			**3**			**4 (blanks)**		
			C:N=5 low			C=10 medium			C=50 high			C=0	N=0	P=0
			C	N	P	C	N	P	C	N	P	**C**	N	P
A	P low	0.01	5	1	0.01	10	1	0.01	50	1	0.01	**0**	1	0.5
B	P medium	0.05	5	1	0.05	10	1	0.05	50	1	0.05	10	**0**	0.5
C	P high	0.1	5	1	0.1	10	1	0.1	50	1	0.1	10	1	**0**

Table 27: Experiments for 1 mol/L carbon giving g/L substrate concentrations

C g/L	N g/L NH_4S	P g/L K_2HPO_4	**1**			**2**			**3**			**4 (blanks)**		
			C:N=5 low			C:N= medium			C:N= high			C=0	N=0	P=0
			C	N	P	C	N	P	C	N	P	**C**	N	P
A	P:N low	0.01	31	13.2	0.35	31	6.61	0.35	31	1.32	0.35	**0**	6.61	1.74
B	P:N medium	0.05	31	13.2	1.74	31	6.61	1.74	31	1.32	1.74	31	**0**	1.74
C	P:N high	0.1	31	13.2	3.48	31	6.61	3.48	31	1.32	3.48	31	6.61	**0**

Table 28: Experiments for 2 mol/L carbon giving g/L substrate concentrations

C g/L	N g/L NH_4S	P g/L K_2HPO_4	**1**			**2**			**3**			**4 (blanks)**		
			C:N= low			C:N= medium			C:N= high			C=0	N=0	P=0
			C	N	P	C	N	P	C	N	P	**C**	N	P
A	P:N low	0.01	62	26.42	0.70	62	13.2	0.35	62	2.64	0.070	**0**	13.2	3.48
B	P:N medium	0.05	62	26.4	3.48	62	13.2	1.74	62	2.64	0.348	62	**0**	3.48
C	P:N high	0.1	62	26.4	6.97	62	13.2	3.48	62	2.64	0.697	62	13.214	**0**

Isolate 1 was a *Bacillus* species isolated from the Mitchell's Plain Wastewater Treatment Plant. It showed adequate growth and EPS (possibly PGA) production in culture media, but preferred to grow at the air-liquid interface (Figure 35).

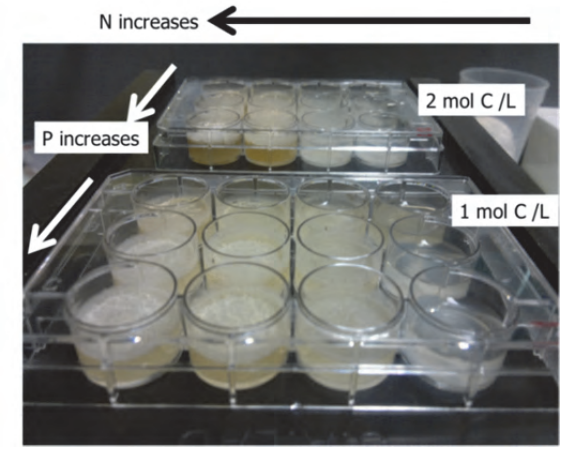


Figure 35: CNP ratio showing increasing N and increasing P ratio, and illustrating the *Bacillus* growth at the air-liquid interface.

From Figure 36 and Figure 37, it is apparent that there is a certain nutrient threshold where the bacterium moves from preferring to grow in suspension to growing at the air-liquid interface, forming a puffy mat. This could be due to increasing carbon concentration, or increasing N as supported by Figure 37. Row 1 has a C:N ratio of 5:1, and row 2 10:1. Row 3, at a ratio of 50:1 does not show strong biofilm growth.

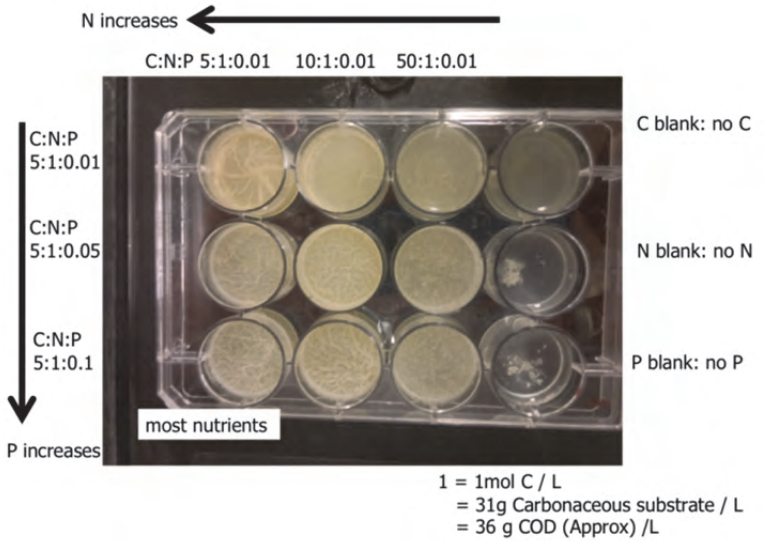


Figure 36: Growth of *Bacillus* in Microwell containing 1 mol C/ L

Increasing P concentration resulted in culture stress, as indicated by a red colour and the distinctive acrylate smell. This is less obvious, but still present, at the lower concentration of 1 mol C /L. These observations show that phosphate clearly plays some role in the *Bacillus* metabolism, but whether it is desired (involving P accumulation through PHA perhaps) or undesirable (for example being too P sensitive) still requires investigation. These experiments are also at high substrate concentration, as the microwells are too small to allow batch experiments at low concentration for adequate biomass accumulation. It remains to be investigated if

these are artefacts of the high substrate concentrations only, or real characteristics of the CNP requirements of the organism.

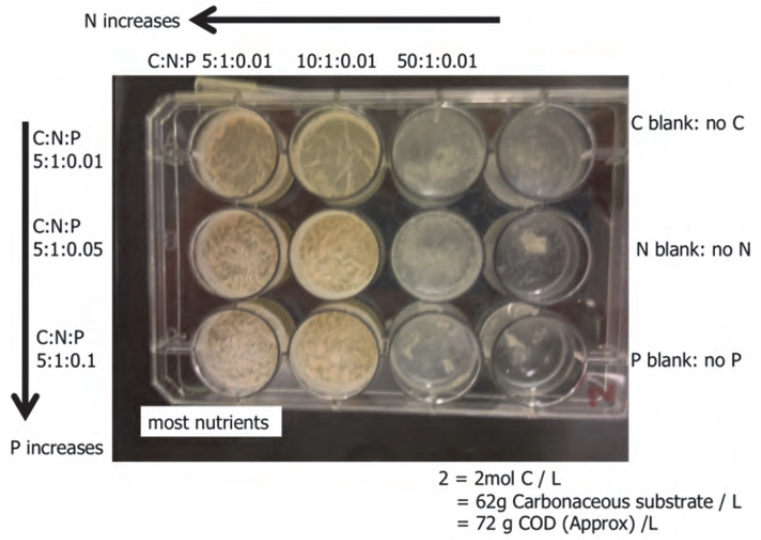


Figure 37: Growth of *Bacillus* in Microwell plates containing 2 mol C/ L and exploring effect of different C:N:P ratios

Further illustration of the biofilm forming nature of Isolate 1 at interfaces is shown in Figure 38 to Figure 42. In conclusion, this *Bacillus*, isolated from a wastewater treatment works, illustrates the growth form sought , which is promising in the reactor configurations we selected to investigate PGA production at low substrate concentrations. Further study should include exploring where PGA production is located. From initial observations from the microreactor experiments, the thick mat biofilm structure and a clear layer directly beneath the mat indicated promising PGA location. Presence of PGA was not confirmed quantitatively in this experiment.

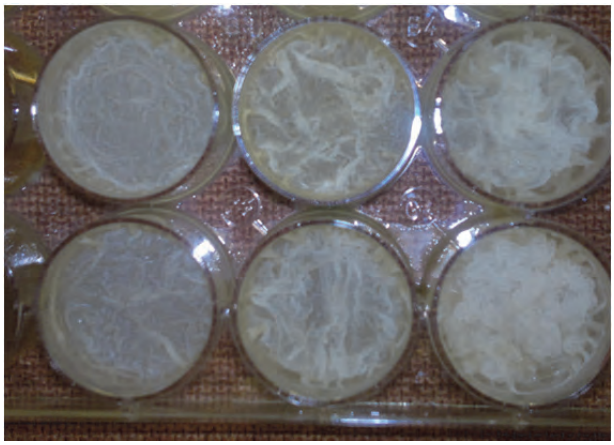


Figure 38: Close-up of biofilm structure of strain 1 in Microwell plates.

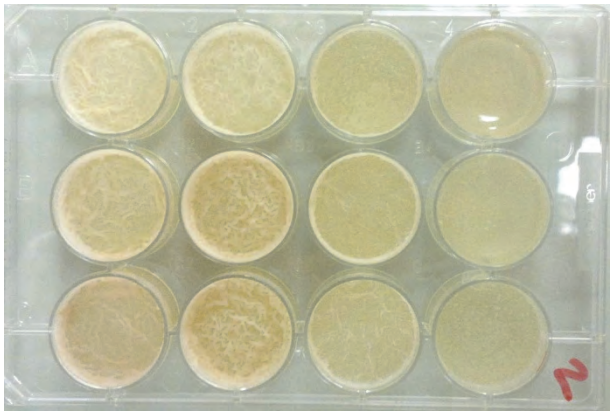


Figure 39: Growth of *Bacillus* in Microwell containing 2 mol C/ L

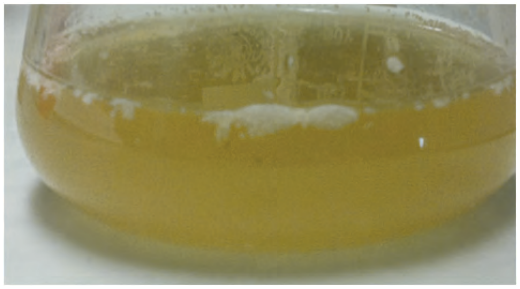


Figure 40: Growth of biofilm at air-liquid interface with mild agitation.

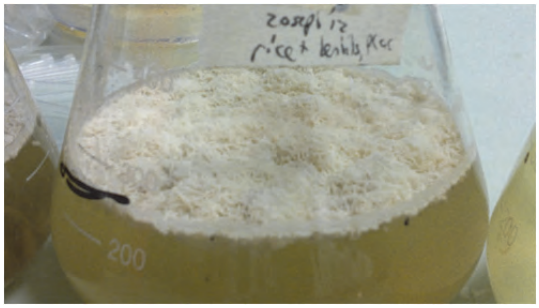


Figure 41: Growth of biofilm at air-liquid interface in a standing culture.

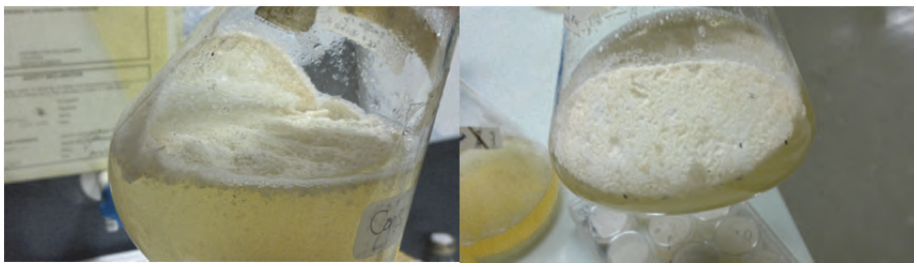


Figure 42: Integrity of biofilm after approximately 30 hours of growth.

5 Domestic Wastewater

The focus of this project is domestic wastewater at a municipal WWTW in the Western Cape, as a raw material for bioproduction. For comparison, related wastewaters are highlighted as well, to illustrate the potential of this approach in other areas.

5.1 Non-sewered areas in South Africa

Large areas of South Africa, and globally, are inadequately serviced in terms of sanitation, which presents a huge opportunity for novel approaches to be implemented without the constraints that retrofitting can present. Carden et al. (2008) conducted greywater sampling at 39 informal settlement sites in six of the nine provinces in South Africa, and data relevant to this project is shown in Table 29.

Table 29: Comparison of greywater Quality Results (from Carden et al. 2006 and references therein)

Variable	Carden et al. (2006)	Eriksson et al. (2002)	Kallerfelt and Nordberg (2004)	Pollution Research Group (2005)	Stephenson et al. (2006)
pH (no unit)	3.3-10.9	5.0-8.7	6.1-7.0	5.8-6.3	-
Conductivity (mS/m)	28-1 763	32-2 000	83-132	144-148	-
PO_4-P	0.7-769	0.6-68	14.8-56.2	11	0.3-18.9
COD	32-11 451	13-549	530-3 520	1 135	999-1 625
Suspended Solids	-	6.4-330	69.0-1 420	-	265.2-1 261
Fats, Oil & Grease (FOG)	8-4 650	3.1-12	-	-	-
TKN	0.6-488.0	2.1-31.5	-	24-30	-
NH_4-N	0.2-44.7	0.03-25.4	-	20	-
Sodium	96-1 700	29-230	-	-	-

Values are quoted in mg/L unless stated otherwise

The absence of hot water in some of these sites caused an increased use in chemicals to achieve the same levels of cleaning, leading to higher phosphates and other chemicals in the greywater than expected, as shown in Table 30. Greywater in high-density informal settlements are pathogenic and highly saline, more similar to

blackwater, and should be considered a sanitation issue rather than a drainage one (Carden et al. 2008). The added physical infrastructural difficulty of access calls for small or mobile novel integrated solutions combined with a long-term operation and maintenance (O & M) solution. Aspects that could suit a wastewater biorefinery approach.

Table 30: Average water use and quality in different settlements (Carden et al. 2008)

Value	Density (du/ha)	Average water use (L/du.d)	Greywater generation (L/du.d)	NH_4 (mg/L)	TKN (mg/L)	Total P (mg/L)
Lower quartile: Low settlement density						
Minimum value	3	80	225	3	7	2
Mximum value	5	180	675	13	56	112
Mean	4	130	412	5	31	27
Upper quartile: High settlement density						
Minimum value	25	55	1 196	2	43	5
Maximum value	162	140	13 365	22	172	240
Mean	45	88	3 029	9	113	92

5.2 Domestic waste to Landfill

Organic waste can make up 50% of solid waste. In addition, people without access to adequate sanitation may make use of plastic bags and dispose of these in domestic solid waste. These make their way to landfill sites. Landfill leachate, while not forming part of water and sanitation strategies, is similar in composition to municipal wastewater with regards to nutrient content, and compares to industrial waste streams in terms of metal and hazardous chemical content. Landfill leachate is considered the most complex and hazardous waste stream to remediate. A study by Sawaittayothin and Polprasert investigated treating landfill leachate through wetlands (Sawaittayothin and Polprasert 2007). The composition of the landfill is shown in Table 31, and shows that the CPN values are similar to greywater as well as municipal wastewater values, with the additional load of heavy metals and potential presence of toxins and chemicals.

Table 31: Characteristics of landfill leachate used in subsurface-flow constructed wetland experiments (Sawaittayothin and Polprasert 2007)

Parameters	Unit	Range	Average	SD
pH	-	7.7-8.1	8.73	0.3
Conductivity	mS	8.1-8.5	6.61	2.6
Salinity	ppt	4.7-4.9	453	0.3
COD	mg/L	1 820-4 100	2 950	811
BOD	mg/L	370-950	775	212
TKN	mg/L	140-1 260	389	490
Total P	mg/L	19.0-26.8	24.7	3.0
Faecal coliforms	per 100 mL	1 600-4 940	2 236	1 336
Apparent colour	ADMI unit	300-1 000	642	223
Cd	μg/L	0.13-3.00	2.41	1.10
Mn	μg/L	2 500-6 370	4 746	2 008

5.3 Dry sanitation

Human waste is measured in terms of Population Equivalent (PE), which is the global average value for human waste. It can be measured in volume or Biological Oxygen Demand (BOD), before any treatment or dilution, by for example reticulated systems:

1 PE=0.2 m^3 per day

1 PE = 60 g BOD per day

Dry sanitation requires a different approach in terms of bioprocessing (or treatment) than wet systems, because of the much lower water content, higher corresponding substrate concentration and the more solid consistency. Table 32 shows the typical composition of human faeces and urine (Polprasert 2007), while Table 33 shows the variation of the nutrient load in different countries (Henze et al. 2008). While the potential presence of metals and recalcitrant chemicals are reduced in on-site disposal systems, excretion of metabolised pharmaceuticals and personal care products absorbed by the skin can introduce potentially hazardous chemicals into the system. Foreign objects can also be thrown into the system.

Table 32: Composition of human faeces and urine (Polprasert (2007)

Parameter	Faeces	Urine
Quantity (wet) pppd (g)	100-400	1 000-1 310
Quantity (dry) pppd (g)	30-60	50-70
Moisture content (%)	70-85	93-96
Approximate composition (% dry weight)		
Organic matter	88-97	65-85
Nitrogen (N)	5.0-7.0	15-19
Phosphorous (as P_2O_5)	3.0-5.4	2.5-5.0
Potassium (as K_2O)	1.0-2.5	3.0-4.5
Carbon (C)	44-55	11.0-17.0
Calcium (CaO)	4.5	4.5-6.0
C:N ratio	6-10	1
BOD5 content pppd (g)	15-20	10

Table 33: Person load in various countries in kg/cap/yr (2002 data) (Henze et al. 2008).

Parameter	Brazil	Egypt	India	Turkey	US	Denmark	Germany
BOD	20-25	10-15	10-15	10-15	30-35	20-25	20-25
TSS	20-25	10-15		15-25	30-35	30-35	30-35
N total	3-5	3-5		3-5	5-7	5-7	4-6
P total	0.5-1	0.4-0.6		0.4-0.6	0.8-1.2	0.8-1.2	0.7-1.0

(COD roughly twice BOD)

5.4 Composition of Raw Influent at Wastewater Treatment Works

The composition of several wastewater treatment works (WWTW) are shown in Table 34 to Table 38. Table 34 and Table 35 are South African, using data from WWTW in Athlone and Mitchells Plain, Western Cape (from City of Cape Town) and Darvill in KZN (Gaydon 2007). Average values used in industry, based on Swedish values are listed in Table 36. For comparison, data from treatment works in Spain (Colmenarejo et al. 2006) are shown in Table 37. Lopez-Vazquez et al. (2008)

compared microbial populations at full scale enhanced biological phosphorus removal (EBPR) plants in the Netherlands. The average operational values are shown in Table 38.

Table 34: Athlone, Mitchells Plain WWTW, Cape Town, South Africa

Athlone WWTW Raw wastewater			Mitchells Plain WWTW raw wastewater		
	Mean	SD		Mean	SD
SS	351	149	SS	750	360
VSS	304	108	VSS		
StSol	13	4.9	StSol		
COD	880	526	COD	1 465	560
TKN	56	13	TKN	92	45
NH_3	32	7.6	NH_3		
Total P	9.2	2.4	Total P	19	12
ortho P	5.5	1.7	ortho P		
pH	7.25	0.28	pH		-
Conductivity	140	23	Conductivity		
Cl	211	42	Cl		
Alkalinity	275	42	Alkalinity		

(Athlone data 1997-2010, Mitchells Plain data 2008)

Table 35: Influent sewage quality, Darvill, Kwazulu-Natal, South Africa

Parameter	Unit	Feedtank	Darvil Influent	Typical Sewage (Ekama 1984)
Alkalinity	mg/L	284	241	
COD	$CaCO_3$	537	602	500-800
NH_3	mgO_2/L	31	26	
NO_3	mgN/L	0.29	0.54	
pH	-	7.1*	7.1*	
SOG	mg/L	63	-	
SRP	mg/L	7 596	6 426	
SS	mg/L	238	254	270-450
TKN	mgN/L	51	46	35-80
TP	mg/L	11.3	12.2	8-18
* mode value				

Table 36: Estimated revised Swedish values for urine and faeces

Parameter	Urine		Faeces		Toilet paper		Blackwater (Urine + faeces)	
	g/p.d	%	g/p.d	%	g/p.d	%	g/p.d	%
Wet mass	1 500	89.8	140	8.4	24	1.4	1 670	100
Dry mass	58	52.3	30	27.0	23	20.7	111	100
Nitrogen	11	88.0	1.5	12.0	-	0	12.5	100
Phosphorous	1.0	66.7	0.5	33.0	-	0	1.5	100
Potassium	2.7	73.0	1.0	27.0	-	0	3.7	100

Table 37: Characteristics of raw wastewater in six municipal wastewater treatment works in Spain

Parameter	Units	Value
pH	-	7.5 ± 0.3
Conductivity	μS/cm	751 ± 74
TSS	mg/L	194 ± 86
Nitrites	mg/L	0.14 ± 0.07
Nitrates	mg/L	0.83 ± 0.33
Ammonia	mg/L	18.5 ± 7.4
COD	mg/L	477 ± 105
BOD_5	mg/L	225 ± 75
Temperature	^oC	19.3 ± 3.8

Table 38: Example of the sub-fractions in the readily biodegradable COD in raw municipal wastewater, total COD 400 g COD/m^3 (Henze 1992)

	g COD/m^3	N content g N/g COD
Acetic acid	25	0
Higher volatile fatty acids (VFAs)	10	0
Alcohols (ethanol methanol)	5	0
Lower amino acids	10	0.14
Simple carbohydrates	10	0

5.5 Comparison between wastewater composition and bioprocess requirements

To consider wastewater biorefineries, it is useful to compare the well-known bioprocess conditions to the conditions typical of wastewater. This provides a starting point for research, as well as an indication where supplementation may be required. This is most relevant to the organic composition, but the unwanted components like heavy metals, toxic chemicals and presence of other lifeforms must also be considered.

5.5.1 Organic composition

The traditional modelling of wastewater COD composition is a mix of simple chemicals, including volatile fatty acids, simple carbohydrates and amino acids. A hydrolysis term is used to convert the more complex molecules to simpler molecules. An example of the readily biodegradable COD fraction is shown in Table 38. To simulate this in experimental conditions, synthetic wastewater is used. A typical composition is shown in Table 39.

Table 39: COD composition of synthetic wastewater (Cokgor et al. 1998)

Component	Fraction (% COD)
Acetic acid	41
Proopionic acid	17
Ethyl alcohol	8
Glutamic acid	17
Glucose	17

Table 40: Comparison of composition and concentration of the broth required for PGA production and domestic municipal wastewater

	PGA (g/L)	**Carbohydrate substrate g/L**	**NH_4Cl g/L**	**[P] g/L**
PGA broth (Huang et al. 2011)	101.1	80-120	0-7	1.5 -2.3

		PGA (g/L)	**COD g/L**	**TKN g/L**	**Tot P g/L**
Athlone WWTW 1997-2010	Mean	??	0.880	0.056	0.009
	SD		0.527	0.013	0.002
Mitchells Plain WWTW 2008	Mean	??	1.485	0.092	0.019
	SD		0.56	0.045	0.012

COD: Chemical Oxygen Demand, Most organic substrates range 1.3-1.5 g COD / g substrate; TKN: Total Kjeldahl Nitrogen

This composition is based on respirometric analysis of domestic sewage together with textile, dairy, meat processing, tannery and confectionery wastewaters. For ease of comparison, the composition of the broth required for PGA production is compared with the wastewater composition at Athlone WWTW in Table 40 . Take note of the orders of magnitude difference in concentration.

5.5.2 Metals concentration

Metals concentrations in wastewaters are highly dependent on the type and source of the wastewater. Section 4.3.2 discussed the required metals for optimal PGA production using *Bacillus*. Studies on *Bacillus* in the HYBACS wastewater treatment also indicated an increased requirement for Mg (pers. Comm.). Preliminary experimental results in this project support the requirement for these metals, as very poor growth was obtained on medium with low, or no metals present. Dosing may then be required to optimise PGA production, keeping in mind that the effluent must comply with the General Authorisation Standards listed in Table 41.

On the other hand, higher metal concentrations may have a beneficial effect on PGA production, as the PGA may be naturally produced either to sequester and inactivate harmful metals, or to be used to increase the bioavailability of required metals. This is supported by observations by Jackson et al. (2009), where *Bacillus* was a dominant organism in metal bioremediation of the Plankenberg River. PGA produced in sites where metal sequestration through PGA has been achieved, cannot be used in applications where metals are not allowed, for example, soil conditioners. Preliminary studies on metal sequestration are not considered in detail in this project.

Table 41: Limits on Metals in final effluent, general authorisation standards.

Substance / Parameter	**General Limit (mg/L)**	**Special Limit (mg/L)**
Dissolved Arsenic	0.02	0.01
Dissolved Cadmium	0.005	0.001
Dissolved Chromium (VI)	0.05	0.02
Dissolved Copper	0.01	0.002
Dissolved Cyanide	0.02	0.01
Dissolved Iron	0.3	0.3
Dissolved Lead	0.01	0.006
Dissolved Manganese	0.1	0.1
Mercury and its compounds	0.005	0.001
Dissolved Selenium	0.02	0.02
Dissolved Zinc	0.1	0.04
Boron	0.1	0.5

5.6 Productivity potential of wastewater biorefineries

From a basic mass balance point of view, significant amounts of carbon, nitrogen and phosphorous are available in a wastewater stream that could potentially be converted to a product of value. Table 42 shows the tons of materials passing through two WWTW in the City of Cape Town on a daily basis. The City of Cape Town metropolitan area accommodates 3.5 million people and is served by 23 wastewater treatment systems.

Table 42: Productivity potential of wastewater biorefineries (data provided by City of Cape Town)

	ML/Day	**Estimate ton C/ day**	**ton N / day**	**ton P / day**
Athlone Design Capacity	120	70	6	1
Mitchell's Plain Design Capacity	37.5	37	3	0.7

In summary, Chapter 1 illustrated the suitable composition of wastewater for bioprocessing as well the potential mass available. It also posits that there are many unsuitable or unpredictable aspects of the wastewater that need to be considered in the design of the units if they are to function. The next Chapter considers product recovery from less than ideal product streams, and Chapter 9 looks at how to combine unit processes to accommodate the unpredictable or inconvenient aspects of the stream.

6 Current resource recovery processes: effective use of phase change in product recovery

Product recovery effectively relies on extracting the product into or onto a different phase to selectively remove it from the bulk media. An example flowsheet for a conventional bioprocess is shown in Figure 43. For such a conventional bioprocess for the production of a commodity product, 50 to 80% of the cost is associated with the raw material. Using waste material as feedstock has the potential to greatly reduce this cost. However, in the case of wastewater, the dilute and variable nature of the waste stream adds challenge to product recovery and necessitates the use of additional process units, with an associated increase in operating cost. The resulting trade-off can be optimised by careful reactor design to produce a system with overall benefits, both economic and environmental.

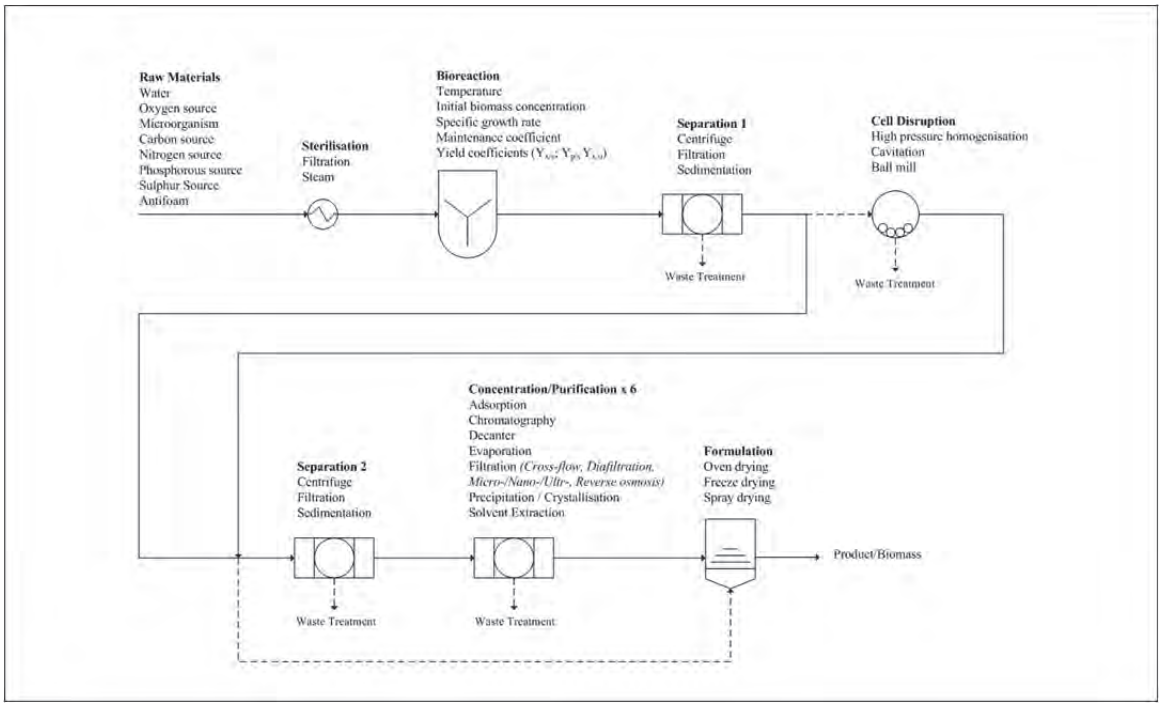


Figure 43: Generic process flow diagram (Harding 2008)

Phase change is an important tool for providing an effective means for concentration of the desired component and ready removal of unwanted components. The following are examples of the coupling of the wastewater treatment with resource recovery, describing the specific phase change employed and the reactor system used. These existing examples inform how wastewater biorefineries can be implemented.

6.1 Liquid to liquid plus gas and solid: Water reclamation and reuse

In wastewater treatment, when resource recovery is considered, it is usually seen in terms of water reuse (Tchobanoglous et al. 2003, Chapter 13). Water reuse is traditionally for land application, either for watering food crops or planted recreational areas. More recently, water reuse is considered in applications where public exposure to the water is limited, for example toilet flushing, industrial water streams, fire protection, and air conditioning. Here a lower quality water may be used. Groundwater recharge is an application where the natural water cycle is mimicked. In drought stricken areas with special polishing steps, wastewater can be purified to drinking water quality. It is important to ensure the wastewater is of domestic origin, contains no hazardous chemicals or heavy metals and complies with effluent standards.

The treatment methods for water reuse are no different in principle to conventional treatment, but may have more stringent criteria. Advanced treatment methodologies may include granular medium filtration, carbon adsorption and reverse osmosis. These systems may be suitable for recovery of products like metals and dyes with minimal plant redesign.

- Product is liquid, extracellular, not biomass associated.
- Separator system: Most systems

6.2 Biochemical conversion: Solids and bio-solids

Most wastewater treatment takes place with associated sludge formation. This is the name given to solids before beneficial use is achieved and may represent the largest, most complex problem an engineer faces in the wastewater treatment field (Tchobanoglous et al. 2003, Chapter 14). Solids and bio-solids is a term increasingly used in place of the former ‘sludge’; this renaming illustrates the changing thinking afforded to solids, starting the conversation of beneficial uses for ‘wastes’. Processes used in solids treatment include:

- Moisture removal: thickening (concentration by physical or chemical means, typically through sedimentation in the presence of absence of flocculation), conditioning, dewatering, drying
- Stabilisation: digestion, composting, incineration
- Products that can be recovered from this process are in the solid fraction, either intracellular or biomass associated.
- Separator system: settling tank systems.

6.3 Liquid to gas: Biogas production

Anaerobic digestion uses oxygen deficient, reactor chambers for the metabolism of complex organics to methane and CO_2. It is currently a competitive wastewater treatment technology due to the costs saved on aeration as well as the reduction in and stabilised nature of the by-product sludges (Henze et al. 2008, Chapter 16).

- Product is an extracellular gas, not biomass associated.
- Separator system: A pressure resistant vessel that limits oxygen supply.

6.4 Solid to liquid: Lipid extraction from sludge (to biodiesel)

Activated sludge processes produce two types of sewage sludge after primary and secondary treatment processes. Primary sludge consists of floating grease and solids, and collected after the primary settlement tank (PST). Secondary sludge is composed mainly of microbial cells and suspended solids, known as activated sludge.

Lipids, the raw material of biodiesel production, is a natural mixture of mono-di- and tri-glycerides, cholesterols, free fatty acids, and lipids cross-linked to other components, for example phospholipids and sphingo-lipids. In wastewater fractions, lipids are mainly found in the FOG (fats, oil and grease) fraction.

Lipid extraction for biodiesel production from municipal sewage sludge poses several challenges, reviewed in Siddiquee & Rohani (2011):

1. Pre-treatment of raw sludge for efficient lipid extraction;
2. Lipid extraction from the sludge;
3. Biodiesel production methods from solid sludge, including catalyst selection;
4. Process economics and safety.

Raw primary sludge and secondary sludge contains 4-5% (w/v) and 1-2% (w/v) solids, respectively. This dilute nature of the sludge is the greatest factor affecting the process. Siddiquee & Rohani consider the use of dried sludge as more feasible.

- Product is liquid, extracellular, not biomass associated
- Separator system: Primary settler tanks, modified downstream processing

6.5 Liquid to solid: Phosphate recovery as struvite

Phosphorous removal is well suited for resource recovery, as the current treatment method involves converting dissolved phosphates into suspended phosphorous which can be retained in a separation process (Henze et al. 2002, p 327). Chemical removal of phosphate takes place in four stages and occurs relatively fast:

1. precipitation
2. coagulation
3. flocculation
4. separation

Phosphate crystallisation into apatite is a relatively slow process. Soil can also bind phosphorous because the same ions present to precipitate the phosphorous are used in wastewater treatment, viz. iron, aluminium and calcium.

- Product is solid, extracellular, not biomass associated.
- Separator system uses settler tanks with chemical dosing, with modified downstream processing.

Biological phosphate removal relies on phosphorous uptake into biomass as an reserve which, under anaerobic conditions, can be used to pick up substrate. Phosphorous accumulating ability is widespread among heterotrophic microorganisms (Henze et al. 2002, p 109), but competition over substrate, particularly low molecular fatty acids, are a threat to the successful operation of the process. This mechanism can be exploited in three ways (Rittmann & McCarty 2001, p 535):

1. Normal phosphorous uptake into biomass
2. Precipitation by metal-salts addition to a microbiological process
3. Enhanced biological phosphorous uptake into biomass.

- Product is solid, intracellular and biomass associated.
- Reactor-separator system is an enhanced biological phosphate removal (EBPR) process

6.6 Liquid to solid: Biohydrometallurgy

Bio-mining is an important example to use to model municipal wastewater treatment for resource recovery. It also involves dilute streams in a complex environment, with economic relevance. The metals can be concentrated via bio-sorption – comparative to floc formation, or by redox reactions, comparative to COD removal. As with wastewater treatment, process sterility cannot be achieved, and positive selection through continuous flow, and washing out slow growing populations, is employed (Rawlings & Johnson 2007).

- Product is solid, extracellular, not biomass associated.
- Reactor-separator systems: heap leaching, similar to trickle towers.

6.7 Liquid to solid: PHA polymer recovery

Polyhydroxyalkanoates (PHA) is related to phosphate accumulation in EBPR (enhanced biological phosphate removal) processes, exploiting the cycling of PHA and polyphosphate accumulation in the microorganisms (Kleerebezem and van Loosdrecht 2007).

- Product is solid, intracellular, biomass associated.
- Reactor-separator system: All EBPR systems; requires cell disruption for recovery of the PHA.

6.8 Liquid to solid: Algal biotechnology

Algal biotechnology is already similar to wastewater treatment, with high dependence on external temperature, dissolved nutrients and light conditions (Winter 1999). Using native species for general products like single cell protein (SCP) is cheaper but yields less valuable product than highly controlled processes producing, for example, carotenoids. The future of algal biotech in wastewater is likely to lie somewhere between these approaches. Most current research involves oil production. Downstream processing of algae has the advantage of the relatively large cell size compared to bacteria, and so can exploit differential centrifugation or settling. The gas vacuole that manages light intensity in the algae can be used to harvest the cells by flotation through control of the light intensity (dim light has potential to lead to cells floating).

- Product (biomass) is solid, (intra)cellular and biomass associated
- Other products, like lipids, are liquid, intracellular and biomass associated
- Excreted products are liquid, extracellular and not biomass associated.
- Reactor systems: Algal bioreactors.

6.9 Liquid to solid: Phosphate and nitrogen recovery into macrophyte biomass: wetlands as reactors

Using photosynthetic plants, or macrophytes, as engineered nutrient removal devices is a biomimicry application, inspired by the way nature manages nutrient removal in water bodies (Benyus 2003). It is relatively new in wastewater treatment, and has much potential in producing products like fibres and non-food agricultural products (Todd et al. 2003). Wetland inoculation and growth rates are different from conventional waste treatment reactors and hence currently difficult to model and predict, but it is a very good conceptual biomass retention example (Kadlec 2009). Wetlands employ heterotrophic bacteria for BOD removal, but have additional design advantages due to the macrophytes involved (Rittmann & McCarty 2001):

1. Reduced light and algal bloom – leaves and stems above the water.

2. Reduced heat transfer – leaves and stems above the water insulate against heat loss.
3. Regenerative biomass retention media – stems and roots in the water column are colonised by biofilms and help accumulate a large bacterial population.
4. Solids separation – stems and roots help to capture colloids.
5. Aeration mechanism – stems and roots may give off oxygen during photosynthesis and stimulate bacterial metabolism. This may stunt plant growth, which may be beneficial (less maintenance) or detrimental (leading to plant death and rotting).

- Product is solid, macrocellular, biomass associated.
- Reactor-separator is a modified natural wetland system

6.10 Conclusion: Shifting from waste treatment to product recovery

The examples listed above mostly use standard reactor design and improvements in product separation, with standard downstream processing (summarised in Table 43 and Figure 44). Little consideration of overall system optimisation is reported.

While these examples show that some product recovery from waste resources is already occurring, these are not widely accepted and frequently not optimal. Often, existing systems have been retrofitted. This carries associated loss of potential revenue due to unoptimised productivity, limiting industry acceptance and return on investment. Carbon based resource recovery methods are better developed, due to the need for energy; however focus is still on water remediation rather than maximising use of organic resources. Development of phosphate recovery systems is gaining momentum as the imminent shortage of phosphorus is realised. Nitrogen recovery has to compete with the well-researched, well implemented nitrogen removal to nitrogen gas. A fundamental shift in reactor design and reaction principles is required to achieve nitrogen product recovery.

The processes used have to contend with the challenge of handling large volumes of liquid and low productivity, motivating the importance of phase separation. Most reactors are separate from the phase separation step, and do not contribute favourably to product removal. The exception is the case of biogas, where the two steps are combined with some challenges like gas entrapment and unfavourable gases co-evolving.

For a brief introduction to case studies of industrial bioproduction, see Waites et al. (2001), Part 3. A thorough discussion on biorefineries is given in Kamm et al. (2006 volume 1-2).

Table 43: Summary of resource recovery processes in wastewater (Synthesis from Tchobanoglous et al. 2003; Grady *et al.* 2011; Polprasert 2007), Rittmann & McCarty 2001)

Process	**Phase**	**Cellular Location**	**Downstream Processing**	**Biomass Associated?**
Water Reclamation & Reuse	liquid	extracellular	polishing pond	no
Biogas	gas	extracellular	gas capture	no
Struvite – chemical precipitation	solid	extracellular	settling / washing	no
Biological P removal	solid	intracellular	biomass waste stream	yes
PHA production	solid	intracellular	settling, cell rupture, purification	yes
Lipids from sludge (chemical recovery)	liquid	extracellular	chemical purification	no
Algal biomass	solid	whole cellular	settling, drying, dewatering	yes
Lipids from algae	liquid	intracellular	chemical purification	yes
Wetlands	solid	whole, macro cellular	various (e.g. fibre extraction, burning)/ none	yes
Bio-mining: bio-sorption	solid	Intracellular or cell surface adsorption	various	yes
Bio-mining: redox reaction	solid	extracellular	settling	no
Bio-solids	solid	whole cellular	settling, dewatering	yes

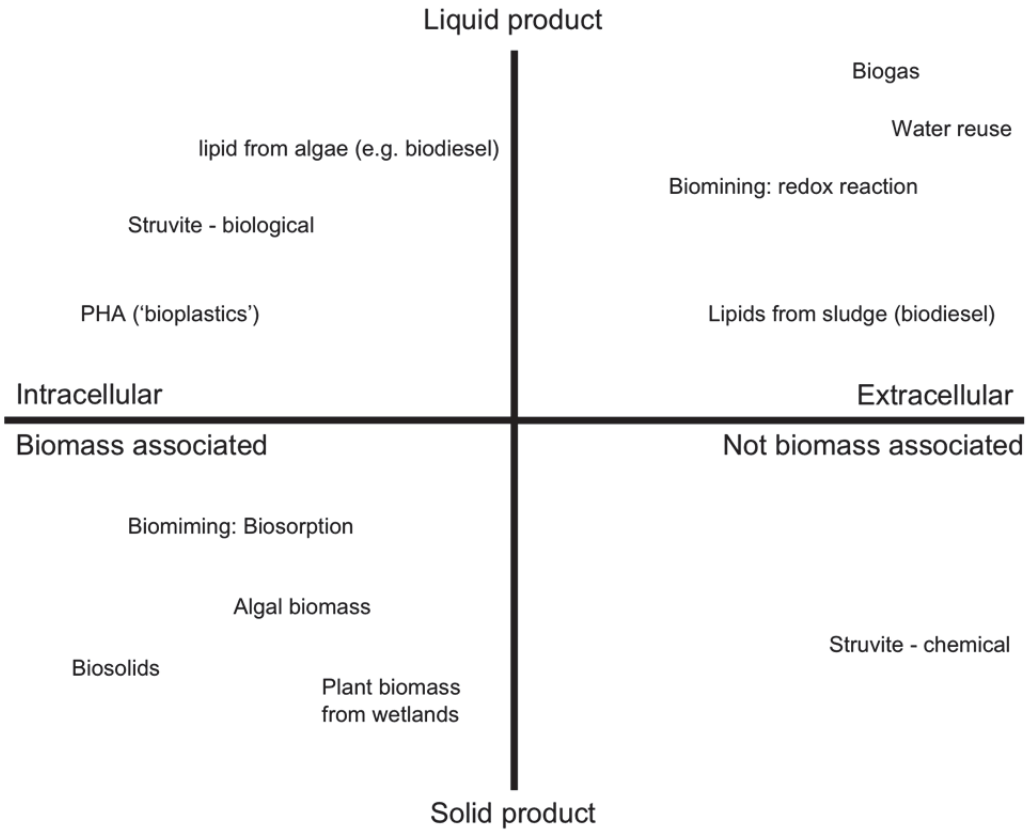


Figure 44: Summary of product recovery from waste resources.

From these examples, it can be seen that there are main two requirements to realise effective production from dilute (waste) streams:

1. *Decouple hydraulic residence time and bio-residence time*: Examples include phase change to separate the product, and biomass retention to increase the apparent biocatalyst concentration.
2. *Design for downstream processing (DSP)*: Reactor design affects the cost of DSP significantly. In dilute waste streams, DSP methods like centrifugation are not cost effective as the volume is too great for energy-intensive conventional separation processes. Such challenges cannot be addressed at DSP level, but needs to be prevented by thorough reactor design.

These requirements are considered alongside reactor design in Chapter 7.

7 Bioreactors for Bioproducts from Wastewaters

This project is focussed on recovery of valuable bioproducts from dilute, sub-optimal streams conventionally considered as waste and unfavourable to bioprocessing. The project acknowledges that waste streams typically have variable quantity and quality, limiting process optimisation. Further, wastewater composition is not optimal for conventional bioproduction.

Reactor design is the main area that can be optimized for bioproduction. Reactor design for wastewater treatment is well developed, with South Africa being a gobal pioneer in biological nutrient removal (e.g. the UCT system, Henze et al. 2008). There are two main challenges with the current approach to reactor design, however. Firstly, optimization of reactors as single units leads to a system where the overall system may not be optimally functional. Secondly, wastewater treatment reactors, specifically, are not designed for product recovery. A systems approach is required to bring about greater functionality, reduction in cost and increased overall productivity (Richardson 2011, Richardson et al. 2012).

7.1 Review

Current wastewater treatment reactors are well designed to achieve nutrient removal through biomass retention, but with limited design towards product recovery. These requirements inform the reactor design constraints, which limits the scope for bioprocesses using waste streams in existing plants.

Fortunately, the unit processes for downstream processing (DSP) are fairly well developed and easily adapted to a chosen reactor. From a design perspective, the current practice is to adapt the DSP to fit the reactor, and not to design the reactor to facilitate the downstream process in terms of effort, cost and energy. This review highlights some considerations for improving this approach.

7.1.1 Establishing the reactor environment

All reactors fulfil a function of providing a suitable environment to convert a substrate to a useful product. As such the following factors need to be considered in the generic design of reactors (Shah 1979):

- Physical and chemical nature of feed
- Nature of catalyst and its aging function
- Nature of reaction
- Product sampling and analysis (*and recovery)*
- Isothermality
- Residence-time distribution
- Steady state
- Ease of construction and cost

- Meaningful data analysis
- Flow maldistribution and extraneous mass- and heat-transfer effects.

In bioprocessing and wastewater treatment, the substrate is highly variable in composition and concentration and mostly more dilute than in chemical reactors. Additional factors influencing design of wastewater treatment/bioprocess reactors include the following (Henze 2008):

- Physical substrate characteristics
- Chemical substrate characteristics
- Concentration of substrate
- Concentration of undesired contaminants
- Presence or absence of oxygen
- Efficiency and reliability required
- Climatic conditions
- Number of different biological processes required in overall treatment system.
- Process Controller skill and experience
- Cost of investment, time for construction, operation under different reactor configurations.

7.2 Selection and Design of Bioreactors for Wastewater Treatment

7.2.1 Engineering philosophy in reactor design

The most basic distinction in any reactor concerns the mixing and flow regimes. Reactors can be agitated using a motor-driven impeller, mixed by aeration, oscillation or fluid circulation through pumping. Further, they may be operated with near optimal mixing or under plug flow conditions, or hybrids of these. Reactors can operated in batch, fed-batch or continuous mode. Each type can be set up in stand-alone, series or parallel configurations. The (bio)catalyst inside the reactor can be set up as freely suspended, in granules or as a biofilm. Different technical jargon exists between chemical, civil and environmental disciplines, but the fundamental distinctions remain the same.

There are three main philosophies around reactor design: chemical engineering reactor design, civil engineering reactor design and environmental management.

The most prominent is traditional *chemical engineering* reactor design, based on the main assumption of available and cheap energy and non-aqueous solvents, leading to an approach that uses increased temperature and pressure to facilitate increased rates of production and extraction. The traditional chemical engineering approaches are demonstrated in the petrochemical and mineral processing sectors, and aimed at efficient product recovery. This discipline has contributed much in terms of process engineering knowledge, and new approaches are developing rapidly to meet the

needs of bioprocesses and sustainable process engineering, which is more appropriate to the biorefinery concept.

The *civil engineering* approach to reactor design is based on the biological nutrient removal of wastewater. High cost processes are not considered for this low value "waste", and reactor designs generally are low cost and low maintenance, which is good from a biorefinery perspective. The aqueous biological nature of the streams also necessitates close-to-ambient temperature and pressure. The main feature of civil engineering reactor design that needs to be shifted to align with the biorefinery approach is the focus on waste removal, rather than product recovery (Henze et al. 2008). With the need for waste treatment and low access to capital investment, the approach is more aligned with natural processes, and uses these to meet "a challenge that needs to be kept in check in order to prevent harm to either humans or the environment" (Winter 1999).

The third philosophy is broadly called *environmental management*, or environmental biotechnology. The advantage of this approach is robust and long term thinking, as these systems are designed to maintain themselves without ongoing human intervention over the long term. Examples are treatment wetlands (Kadlec 2009) and nature conservation areas. This philosophy may at times place nature above humanity. The challenges to this approach include the perception that it is in direct contradiction to the high productivity of chemical engineering, is unable to treat wastewater at a high enough rate for medium to high density municipal populations, thus affecting human safety and health, and requires too much land area, thus encroaching on housing developments (Malan 2010). A more positive perspective is that this approach contributes to a systems level view for biorefineries, ensuring that the various stakeholders, and unit processes are all considered.

The type of bioprocess engineering required for a wastewater biorefinery combines aspects of all these philosophies, aiming to reduce overall cost and produce valuable products (Kleerebezem & van Loosdrecht 2007) while restoring natural capital (Hawkens et al. 2010). The most successful approaches to date are applied in some agricultural applications (Polprasert 2007; Pauli 2010). The philosophy pursued in this project could be argued as being 'post-environmentalist' (Hobbs et al. 2013); with humankind and nature in balance, and with humans being an influential, keystone species, learning from nature or the wider environment of which humans are also part (biomimicry), with some aspects of success still being measured in economic terms. In order to achieve this, partnership between chemical, civil and environmental engineering approaches (summarised in Table 44) is required. This is underpinned by a process of design that incorporates nature, biology, society and individual human needs.

The trans-disciplinary requirements in the academic arena may be better managed through transcending the research to enter the public space. When a language is developed to explain biorefinery concepts to the layman, this will also facilitate effective communication between the different disciplines, and speed up appropriate

policy decisions. This is a difficult process, and the inevitable challenges need to be resolved through long-term civic engagement over many years.

Table 44: Differences and similarities between engineering 'philosophies' (assuming effective design and implementation)

Characteristic	**Chemical engineering**	**Civil engineering**	**Environmental management**	**Bioprocess Engineering**
Hierarchy	Profit > Man > Nature	Man > Profit > Nature	Nature > Profit > Man	Nature = Man = Success (subset of which = profit)
concentrations of substrates	varied, up to very High	very Low	varied, mostly very Low	Medium
Tolerated cost of investment	High	Medium	Low	Medium
Tolerated running cost	High	Low	Low	Medium
Expected value of Products	High	Low	Low	Low-High (focus in this project on high)
Energy requirements	High	Medium	Low	Low-Medium
Water requirements	High	Low/negative	Low/negative*	Negative*
Environmental impact	High	Low	Negative*	Negative*

* Negative = Restorative impact, negative footprint.

7.2.1.1 Chemical Engineering: Reactor types

Most of current bioprocess engineering knowledge is based on chemical engineering. Table 45 gives a summary of the basic designs employed in the field (Fogler 2005). Industry predominantly makes use of tried and tested designs with minimal modification required by the specific processes to maximise robustness, which means that these designs may not be fully compatible with the specific constraints required for wastewater biorefineries.

A different way to approach large scale production is by a modular series of small reactors. Micro-reactors avoid the challenges presented by scale up through what is called 'number-up', or modular addition of small reactors to achieve scale (Hessel et al. 2004, Hessel et al. 2005). Because of the specific mass transfer challenges that living organisms require, bioprocessing does not benefit as much as chemical processing from economy of scale, hence the modular approach could be beneficial.

Table 45: Basic Chemical Engineering Reactor Designs

Type of Reactor	**Description**
Continuous Stirred Tank Reactor (CSTR)	Tank, can be used in various modes – batch, fed-batch or continuous, in single, several in series, parallel and combination of these.
Tubular Reactor	Tube shaped reactors for gas phase reactions requiring turbulent flow
Packed Bed	pipes, reactive distillation columns
Membrane reactors	(only good for lower temperatures)
Differential reactors	Used in research and analysis
Microreactors	Lab scale and smaller reactor sizes employed in modular fashion

7.2.1.2 Civil Engineering: Reactor types

Wastewater Treatment historically falls in the realm of Civil Engineering and as such is considered mostly with regards to the physical construction of the macro environment, with the biological component being a serendipitous component as illustrated in (Tchobanoglous et al. 2003). Reaction engineering from this perspective serves to provide a necessary service, rather than the most productive and profitable approach. Winter (1999) noted that an outcome of this has been the tendency to provide wastewater treatment to meet the lowest standard enforced by legislation, rather than the best achievable standard or returning the water to its original quality.

Biological understanding has advanced significantly, but is mostly implemented through retrofitting existing plants. Wastewater treatment is for the most part not concerned with skills and capital intensive product recovery. Further, it aims to reduce sludge production by converting most nutrients in the water to gaseous products (Table 46, Tchobanoglous et al. 2003). The attempt to minimise sludge and save costs led to developments in biological nutrient removal instead of chemical dosing, opening up the research arena for bioproduction. Winter (1999) has produced an informative work on the history of wastewater treatment.

Table 46: Major biological treatment processes used for wastewater treatment (adapted from Tchobanoglous et al. 2003)

Type	Common name	Use
Aerobic processes		
Suspended growth	Activated-sludge process Aerated lagoons Aerobic digestion	Carbonaceous BOD removal, nitrification Carbonaceous BOD removal, nitrification Stabilisation, Carbonaceous BOD removal
Attached growth	Trickling filters Rotating biological contactors Packed-bed reactors	Carbonaceous BOD removal, nitrification Carbonaceous BOD removal, nitrification Carbonaceous BOD removal, nitrification
Hybrid suspended and attached growth	Trickling filter / activated sludge	Carbonaceous BOD removal, nitrification
Anoxic processes		
Suspended growth		Denitrification
Attached growth		Denitrification
Anaerobic processes		
Suspended growth	Anaerobic contact processes Anaerobic digestion	Carbonaceous BOD removal Stabilisation, solids destruction, pathogen kill
Attached growth	Anaerobic packed and fluidized bed	Carbonaceous BOD removal, waste stabilisation, denitrification
Sludge Blanket	Upflow anaerobic sludge blanket (UASB)	Carbonaceous BOD removal, especially high-strength wastes
Hybrid	Upflow sludge blanket / attached growth	Carbonaceous BOD removal
Combined aerobic, anoxic and anaerobic processes		
Suspended growth	Single- or multistage processes, various proprietary processes	Carbonaceous BOD removal, nitrification, denitrification, and phosphorous removal
Hybrid	Single- or multistage processes with packing for attached growth	Carbonaceous BOD removal, nitrification, denitrification, and phosphorous removal
Lagoon processes		
Aerobic lagoons	Aerobic lagoons	Carbonaceous BOD removal
Maturation (tertiary) lagoons	Maturation (tertiary) lagoons	Carbonaceous BOD removal, nitrification
Facultative lagoons	Facultative lagoons	Carbonaceous BOD removal
Anaerobic lagoons	Anaerobic lagoons	Carbonaceous BOD removal, waste stabilisation

Table 47: Reactor configurations used in the Wastewater Treatment industry

Type	Example
Suspended growth, Stirred tank	Activated Sludge Process
Attached growth	Trickling filters
	Rotating Biological Contactors
Combined Aerobic Treatment Processes (similar reactor design with some changes hold for Anaerobic Processes)	Trickling Filter / Solids Contact, and Trickling filter / Activated Sludge Process
	Activated Bio-filter and Bio-filter Activated Sludge Process
	Series Trickling Filter – Activated Sludge Process
	Activated Sludge with Fixed Film Packing (internal suspended packing and internal fixed packing)
	Submerged Attached Growth Processes (Downflow and Upflow, and Fluidised Bed Bioreactors (FBBR))
	Attached Growth Denitrification Processes (Downflow and Upflow Packed-Bed, Fluidized-Bed Reactors, Submerged Rotating Biological Contactors and Attached Growth Processes)

7.2.1.3 Environmental/Ecological Engineering: Reactor types

Environmental Engineering can include a very wide range of applications, both with biological application (often called Environmental Biotechnology) and abiotic applications. The potential for wastewater treatment through wetlands is often considered as part of Civil Engineering for low density populations and polishing ponds (final stages of wastewater treatment) (Rittmann & McCarthy 2001). Constructed, or treatment wetlands are currently restricted to secondary treatment for small communities, add-ons to aging or overloaded conventional secondary plants, add-ons to lagoons, and tertiary and higher treatment of compliant secondary discharges (Kadlec 2009). Requirements of wetlands for wastewater treatment are the wetland species' ability to grow in waterlogged conditions and with extremes of various soil and water parameters, including oxygen content (often anaerobic with presence of H_2S), pH, toxic wastewater constituents and salinity (Winter 1999, Chapter 12). Treatment wetlands can be divided into three types: Free Water Surface (FWS), Horizontal Subsurface Flow (HSSF) and Vertical Flow (VF) (Kadlec 2009). The active components, or 'bioreactors' are mostly considered to be the plants, or macrophytes combined with the soil and wetland construction, but the ecosystem of bacteria, fungi and higher animals play an important role in the efficiency of the system (Todd et al. 2003).

Other environmental reactor types include soil-based systems. Soil adsorption is implemented through drip and sprinkler irrigation systems which compare to the trickling filters systems used in wastewater treatment. Land treatment can be divided into slow-rate, rapid-infiltration and overland-flow systems. Subsurface

wastewater infiltration is another method of treatment. The underlying principle in all these systems is a combination of physical adsorption and passive biological treatment mechanisms, with the use of natural forces like gravity. These are sometimes supplemented by mechanical interventions (WEF Manual of Practice 2010).

These systems all typically require fewer operational personnel, consume less energy and produce less sludge than conventional systems, but they do require significant land area in their current designs and applications (WEF Manual of Practice 2010). The field of ecological engineering is growing fast with increasing application in bioremediation (Rittmann & McCarty 2001).

The value that these types of reactors bring to the wastewater biorefinery is by adding another means of biomass retention, through larger 'cells' – macrophytes or higher order plants. The nutrient and heavy metal storage potential of wetlands mostly occurs through the associated micro-organisms in the roots of the plants. Rhizodeposition, the passage of carbon to the soil also occurs through the plant-bacterial-fungal partnership (Winter 1999, Chapter 12). Plants play a role in the oxygenation of the soil and the microbial communities around their roots, approximating a well thought-out bioreactor design addressing the challenge of oxygen supply. The interaction between the macrophytes, micro-organisms and the soil approximates the solid substrate fermentation (SSF) discussed in Section 7.3.3.2 as an example of growth on a combination of inert and degradable supports.

7.2.1.4 Bioprocess Engineering: Reactor types

Aerobic bioprocess reactors typically work with dilute media, ambient temperature and pressure, and maintain adequate concentrations of oxygen at the micro-scale (as opposed to an adequate average oxygen concentration). Despite the similarity of these requirements to wastewater treatment, bioprocess engineering has followed an approach similar to chemical engineering, but with reactors utilising a comparatively low substrate concentration, and where needed, carrying special emphasis on cell immobilisation to increase biomass retention. Some differences between chemical and bioreactors are highlighted inTable 48. Kleerebezem & van Loosdrecht (2007) suggest the differences explaining why bioprocess engineering has followed chemical engineering thinking more than the environmental / civil engineering thinking since its inception around the 1950s. These are summarised in Table 49. Most notably the required level of control and sterility are very different.

Table 48: Some differences between chemical engineering reactors and bioreactors

Chemical engineering reactor	**Typical Bioreactor**	**Implication for bioreactor engineering**
Simple reaction mixture	Complexity of reaction mixture	Affects downstream processing & purification, can affect catalytic functionality (catalyst 'poisoning' or feedback inhibition)
High concentration of reactants and products	Low concentration of reactants and products	Inefficient mass and heat transfer
Increase of product with decrease of substrate	Increase in biomass simultaneously with progress of biochemical transformation	Affects downstream processing & purification, non-linear productivity optimisation
Catalyst needs to be added to the system, could have limited catalytic life span	Microorganisms synthesise their own catalysts (enzymes) – 'regeneration' of catalyst	In a well-designed system the progress can be self-seeding / self-organising
Extreme reaction conditions	Mild reaction conditions (temperature, pH)	Potential to be a safer process, demanding less energy. Establishing a cooling gradient may be a challenge
Products are often stable	Products are often labile	Affects downstream processing & purification significantly
Chemical solvents used	Aqueous phase is used predominantly	Constrained to the physical characteristics of water
Sterility is irrelevant	Mostly operate under sterile conditions	Significant energy and skill required in preparation and running of reactor.

Table 49: Historic differences between environmental and industrial biotechnology (Kleerebezem and van Loosdrecht 2007)

Variable	**Environmental biotechnology**	**Industrial biotechnology**
History	Wastewater treatment	Product formation
Basis	Catabolism	Anabolism
Biomass	Mixed culture (sludge)	Specific strains of microorganisms
Process type	Continuous	(Fed) batch
Process models	Lumped black box models	Omics-based metabolic network models
Process Objectives	Minimize effluent substrate concentrations	Maximize productivity
Substrates	Mixed substrates (waste)	Pure and well-defined substrates
Process establishment	Ecological selection by process operation	Specific microorganisms and genetic engineering

7.2.2 General Bioreactor Design Factors

Bioprocess reactors are three phase reactors i.e. gas-liquid-solid reactors (Shah 1979). Some examples of general three-phase reactors are listed in Table 50 (Asenjo & Merchuk 1995; Fogler 2005; Ritmann 2001; Whitaker & Cassano 1989; Winter 1999). The biomass represents the solid phase. Where the raw material is in the solid phase, two solid phases may occur. Microbial cell aggregates, such as flocs and biofilms, offer certain advantages over suspended single cells in downstream processing by facilitating cell-liquid separation by sedimentation or filtration. However, they may offer challenges in terms of mass transfer. The purpose of bioprocessing is to achieve the adaptation of biological methods of production to large-scale industrial use by:

- obtaining the best biological catalyst
- creating the best possible environment for catalytic conversion of the raw material to desired product in the bioreactor, and
- recovering and purifying the products in the most economical way

Table 50: Types of gas-liquid-solid reactors (Shah 1979)

Gas-liquid-solid reactors	Differential reactor
	Fixed-bed reactor
	Stirred-batch reactor
	Continuously-stirred-tank reactor
	Straight-through transport reactor
	Recirculating transport reactor
	External recycle reactor
	Rotating-basket continuously-stirred-tank reactor
	Segmented-bed reactor
	String-of-spheres reactor
Gas-solid reactors which can easily be adapted to three-phase systems	Ball-mill reactor
	Fluidized-bed reactor with agitator
	Stirred reactor with catalyst impregnated on the walls or placed in an annular basket
	Reactor with catalyst placed in a stationary cylindrical basket
	Internal recirculation reactor
	Micro-reactor
	Single-porous-pellet pulse reactor
	Chromatographic-column reactor
Gas-liquid absorbers which may find suitable applications in gas-liquid-solid reaction systems	Laminar-jet absorber
	Wetted-wall column absorber
	Rotary-drum absorber
	Disk column absorber
	Single-sphere absorber
	Gradientless contactor

Reactor operation is affected by five main areas which, in turn, affect the design of bioreactors and wastewater treatment plants. They are introduced below, but not discussed in detail. For more information, the reader is referred to the references (Doran 2002, Henze 2002, Henze et al. 2008, Shuler & Kargi 1992, Rittmann & McCarty 2001, Tchobanoglous et al. 2001, Waites 2001). For controlled bioprocesses, additional characteristics and special requirements of bioreactors are listed inTable 52.

1. Biomass retention
    - Suspended growth, also called Suspended floc, Dispersed growth, Slurry.
    - Biofilms, also called Heterogeneous systems, Fixed-film, Attached growth, Immobilized cell reactors.
        - Packed-bed
        - Fluidized-bed
        - Rotating biological contactor (RBC)
2. Reactor flow regime (also see Table 51)
    - Stirred (batch or continuous)
        - Stirred mechanically
        - Stirring by aeration
    - Plug Flow
3. Mixing
    - Aeration
    - Recycle loops
4. Flow rate
    - Residence time
    - Turbulence and shear
    - Downstream processing requirements and load capacity
5. Reactor arrangements to increase residence time
    - Recycle of settled cells
    - Recycle after settling
    - Recycle before settling
    - Reactors in series
    - Reactors in parallel

Table 51: Basic Summary of Bioprocess Reactors

Type	Description
Stirred tank	Basic tank, continuous, batch or fed-batch, with mechanically moving agitators or impellors
Bubble column	Relies on gas sparging for agitation
Airlift reactor	Relies on gas sparging for agitation, with patterns of liquid flow being more defined than bubble columns, due to physical separation of up- and down-flowing streams
Packed bed	Reactors packed with immobilised or particulate biocatalysts
Fluidised bed	Packed bed with high upflow rate, causing bed expansion – increasing agitation and preventing channelling or clogging
Trickle Bed	Downflow variation of packed bed. Air may be introduced at the base.
Loop reactor	Mixing and liquid circulation are induced by the motion of an injected gas, by mechanical pump of a combination of the two

Table 52: Typical Bioreactor Requirements

Requirements	Reason	Typical implication
Aseptic operation	Avoid contamination	High energy needed for sterilisation
Temperature control	Living organisms have narrow tolerance (typically 25-45 ˚C)	Challenging temperature control
pH control	Living organisms have narrow tolerance (typically pH 5-9)	Challenging control and expensive chemicals required
Ability to obtain a representative sample aseptically	Adequate, representative analysis	Challenging reactor design (especially with combined sterilisation related pressure requirements)
Adequate shear control	Mixing is needed to provide a homogeneous environment, to increase the oxygen transfer rate, improve nutrient provision as well as improve the dispersion of product that could cause feedback inhibition, but stirring also results in organism stress, especially in shear sensitive organisms	Shear control is traded off with poor mass and oxygen transfer and foaming
Adequate mass transfer	Living organisms need a constant micro supply of oxygen and nutrients, not levels that are adequate on average.	Challenging mass transfer
Prevention of cell damage by high shear environment	Stirring biomass results in organism stress, especially in shear sensitive organisms	Challenging mixing regime

7.3 Factors to consider in reactor design for wastewater biorefineries

The reactor designs and philosophies outlined in Section 7.2 inform the reactor designs available for wastewater biorefineries to a large extent. The particular concern, for the dual objective of producing a valuable product while improving the wastestream used to produce it, is outlined here.

7.3.1 Very low concentration of valuable product

One significant challenge of bioprocesses is the dilute nature of the medium, with both substrates and products typically present at very low concentration, typically less than 5% of the total dissolved solids present. When using dilute waste streams like municipal wastewater, which can be a thousand fold more dilute, this aspect is even more challenging. The apparent biocatalyst concentration must be increased to enhance process intensity over the current approach of huge dilute vats of water. This has major implications for mass and energy transfer needs. Aeration and heat transfer in dilute media is inefficient and energy intensive. Using biomass retention, these requirements can be better managed.

The same approach holds for the product. For cost and energy efficient downstream processes, the localised product in an accessible location with high apparent concentration is preferred. Many processes currently use standard reactor setups, and optimize the downstream processing in isolation. Reactor design has scope to facilitate DSP and can have a greater impact on overall process optimization (Richardson 2011, Richardson et al. 2012). The entire process needs integrated optimization, cognisant of the performance at the level of unit operation, process operation and systems operation (including aspects outside of the process).

7.3.2 Aeration

Oxygen supply is one of the main challenges in bioprocessing, because oxygen is sparingly soluble in water. In high volume, low concentration processes typical of bioprocessing in general, the energy for aeration is the biggest burden in terms of economics, energy and the environment. The oxygenation is not just stoichiometrically decisive, but frequently also governs the reaction rate. Aeration in biofilms presents a special challenge due to the additional barrier that the thickness of the biofilm layer poses to oxygen entering the deeper biomass. In wastewater treatment, aeration can be up to 70% of the operating costs.

Aeration can be used as mixing device and, with biofilms, the shear associated with aggressive airflow can also be used for the sloughing of biomass as a rudimentary type of downstream processing. Types of aeration include (Henze et al. 2002):

- Separate aeration of the flow of recycle
- Aeration in the supportive medium itself
- Aeration of the biofilm

Flotation is included here as a multifunctional aerating and separation process. Flotation as applied to wastewater treatment is confined to the use of air as the flotation agent. Table 53 shows common ways of aerating wastewater treatment reactors (Tchobonoglous et al. 2003).

Table 53: Descriptions of commonly used devices for wastewater aeration (modified from Tchobonoglous et al. 2003)

Classification	**Description**	**Use or application**
Submerged		
Diffused air Fine – bubble (fine-pore)	Bubbles generated with ceramic, plastic, or flexible (domes, tubes, disks, plates, or panel configuration)	All types of activated-sludge processes
Diffused air Coarse – bubble (nonporous)	Bubbles generated with orifices, injectors and nozzles, or shear plates	All types of activated-sludge processes, channel and grit chamber aeration, and aerobic digestion
Sparger turbine	Low-speed turbine and compressed-air injection	All types of activated-sludge processes and aerobic digestion
Static tube mixer	Short tubes with internal baffles designed to retain air injected at bottom of tube in contact with liquid	Aerated lagoons and activated-sludge processes
Jet	Compressed air injected into mixed liquor as it is pumped under pressure through jet device	All types of activated-sludge processes, equalisation tank mixing and aeration, and deep tank aeration
Surface		
Low speed turbine aerator	Large-diameter turbine used to expose liquid droplets to the atmosphere	Conventional activated-sludge processes, aerated lagoons, and aerobic digestion
High-speed floating aerator	Small-diameter propeller used to expose liquid droplets to the atmosphere	Aerated lagoons and aerobic digestion
Aspirating	Inclined propeller assembly	Aerated lagoons
Rotor-brush or rotating disk assembly	Blades or disks mounted on a horisontal central shaft are rotated through the liquid. Oxygen is induced into the liquid by the splashing action of the rotor and by exposure of liquid droplets to the atmosphere	Oxidation ditch, channel aeration, and aerated lagoons
Cascade	Wastewater flows over a series of steps in sheet flow	Post-aeration

7.3.3 The need for biomass retention in wastewater treatment

When the substrate concentration in the feed is high (> 10 g COD/L) and rapidly growing organisms (growth rate > 0.1 /h) are used, there is no need for biomass retention from a biomass concentration perspective (Figure 45, Nicolella et al. 2000). In dilute wastewater treatment, biomass retention is advantageous as conversion is limited by the amount of biomass present and retention allows the increase in biomass concentration. This may be applied to the retention of an added or a natural, mixed microbial community. Biomass retention also facilitates the effective decoupling of the hydraulic and solid retention time, required to improve reactor volumetric conversion capacity.

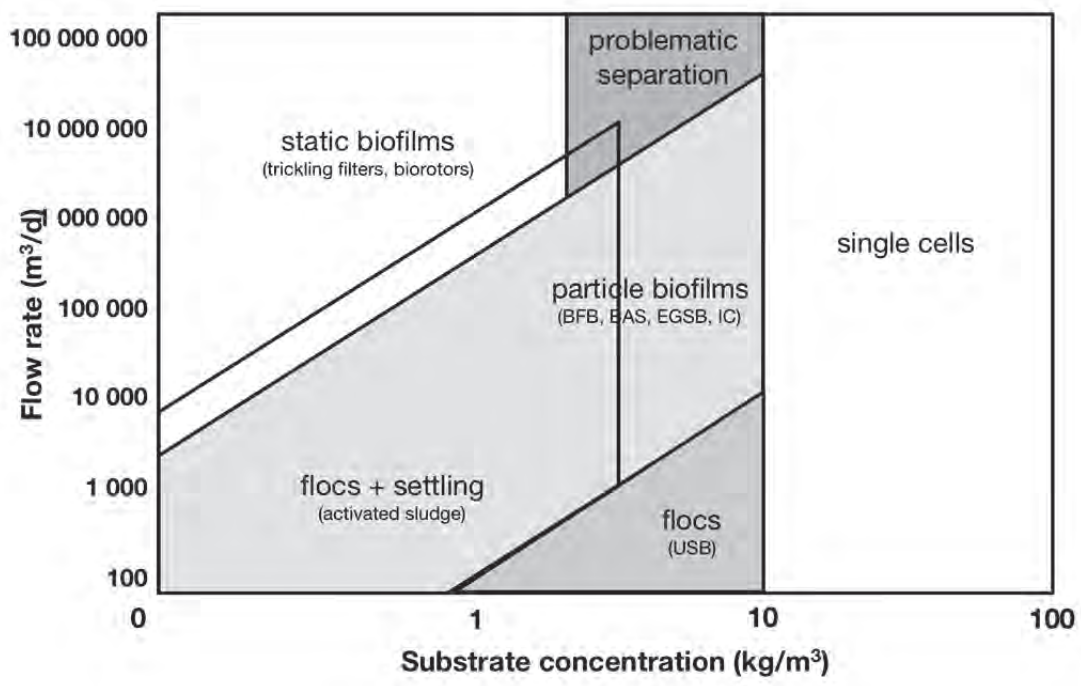


Figure 45: Concentration-flow rate phase diagram for application of floc and biofilm reactors (adapted from Nicolella et al. 2000)

If a bioprocess is designed to produce and isolate a product from a low substrate stream as well as treating the water, retention of the biomass and product recovery are essential. Filters may require less maintenance if the biomass is not suspended, through, for example, a combination of cell immobilisation and filtering, or by including a settling stage. If the product is cell-associated, retention of the biomass forms the first stage of concentration and the retention medium needs to be designed to be suitable to biomass recovery.

If the product is soluble, keeping the biomass separate from the liquid could reduce strain on water purification steps like filters and chromatography resins. Where chemicals are added to precipitate product, biomass can reduce the efficiency and hence may require an increased load. Preventing biomass ingress to the purification

media reduces this matrix effect and contributes to product purity. Biomass retention may make more costly downstream processing technologies more feasible due to lower operating and maintenance costs. Combined design of cell retention for improved productivity and to facilitate DSP gives greater scope to both the macroscopic and microscopic aspects of the retention/growth media.

Biomass retention can be established by recycle loops, immobilization, retaining the biomass in suspended form through selective filters, or a combination of these. Immobilisation relies on the controlled growth of a biofilm or the formation of flocs or aggregates of biomass. Flocs are included as a form of biofilm without solid support in this project to allow inclusion and comparison of the granular sludge with other biofilm techniques. Biofilm reactors can roughly be divided into non-submerged systems like trickling filters and rotating biological contactors, submerged fixed bed reactors and fluidized bed reactors of which the aerobic granular system is a special case. Biofilms growing on membranes are not considered here. The key differences between the types of reactors are the specific surface area of the biofilm attachment medium, mechanisms for removing excess biomass and gas transfer. Types of biomass retention include:

1. Matrix Retention – e.g. biological contactors, biomass support particles (including ceramics, gravels, sand, sponges), trickle bed, some membranes.
2. Particulate biofilms, forming particles like flocs or granules, retained because of their settling velocity, e.g. upflow sludge blanket and aerobic granular sludge or their size;
3. Solid Substrate Fermentation (SSF), including inert and degradable supports.
4. Filters or Membranes, where the membrane is the boundary to prevent biomass leaving the reactor (solid / liquid separation). Can be within reactor or separate unit operations.
5. Flocculate – form particles – retain because of settling velocity, e.g. upflow sludge blanket.
6. Recycle of the biomass phase, following its recovery by settling, filtering or separation; this may also serve a biological function like re-inoculation.
7. Re-inoculation – where recycling is combined with a biological function. If beneficial bacteria can sporulate late in the treatment chain due to low nutrient levels, this recycling loop can activate these spores again under appropriate conditions.
8. Wetlands – macrophytes (plants) as active, regenerative support and nutrient sink (WEF Manual of Practice No FD-16).

Biofilms are generally the first approach in complex resource recovery plants. Apart from biomass retention, the function and requirements of a biofilm can be summarised, in the context of wastewater treatment, as follows (Henze et al. 2002):

- Bacterial attachment onto supporting medium
- Efficient contact between water and biofilm and sludge attached to the carrier

- Controlled growth of the biofilm to prevent clogging
- Adequate oxygen supply to the water for conversion of organic matter.

Apart from biomass retention, the function and requirements of a biofilm in the context of wastewater treatment can be summarised as:

- Bacterial attachment onto supporting medium
- Efficient contact between water, attached biofilm and associated sludge ('interstitial phase') attached to the carrier
- Controlled growth of the biofilm to prevent clogging
- Adequate oxygen supply to the water for conversion of organic matter (Henze et al. 2002).

The main supportive media are stone, ceramics, wood and a variety of plastic designs. The polarity of these media could be exploited to manage separation or selective attachment of preferred organisms or groups of excreted, cell-associated polymeric products. For example, poly-glutamic acid (PGA) is a negatively charged extracellular polymer produced by some *Bacillus* species (Manocha & Margiritis 2010), which could be specifically retained by careful selection of a combination of polar plastic media. Factors to consider in the biofilm include:

1. DO gradient across the biofilm zone
2. Physico-chemical control of microbial aggregates (sloughing, etc.)
3. Bacterial behaviour: mobility at a microscopic level, metabolic requirements and limits (presence of required enzymes), population dynamics
4. Product characteristics: productivity, quality of product, product location
5. Nutrient gradient through the biofilm zone (the thickness of the biofilm)
6. Kinetics and stoichiometry of the metabolic processes.

Biofilm reactors are discussed in more depth in Section 7.4.

7.3.3.1 Filtering (Membranes)

The main role of a filter or membrane is separation, although in some cases the membrane may have (bio)catalytic function as well. The membrane can also improve the bioreactor function directly through, for example, improved mass transfer of gases and a controlled transfer of nutrients. Practical categorisation of membranes is according to the pore size and material composition, which is generally either organic (polymeric) or inorganic (ceramic or metallic). Different configurations of membranes are highlighted in Table 54, in the context of conventional wastewater treatment (Stephenson et al. 2000). Three generic types of membranes exist (Stephenson 2000):

- separation and retention of solids
- bubble-less aeration within bioreactor
- extraction of priority organic pollutants/products from industrial wastewater

Table 54: Advantages and disadvantages of current membrane configurations for conventional wastewater treatment (modified from Stephenson et al. 2000)

Config-uration	**Area: Volume (m^2: m^3)**	**Turbulence promotion**	**Advantages**	**Disadvantages**	**Applications**
Pleated cartridge	800-1000	very poor	Robust construction Compact design Low cost	Easily fouled Cannot be cleaned	Dead end MF
Plate and Frame	400-600	fair	Can be dismantled for cleaning	Complicated design Cannot be back-flushed High cost	ED, UF, RO
Spiral wound	800-1000	poor	Low energy cost Robust and compact Low cost	Not easily cleaned – cannot be back-flushed	RO, UF
Tubular	20-30	very good	Easily mechanically cleaned Tolerant of high TSS	High capital and membrane replacement cost	Cross-flow filtration, high TSS waters
Hollow fibre	5000-4000	very poor	Can be back-flushed Compact design Tolerant of high colloidal levels	Sensitive to pressure shocks	MF, RO

TSS: Total suspended solids. MF: Microfiltration. ED: Electrodialysis. UF: Ultrafiltration. RO: Reverse Osmosis.

One significant advantage of bioproduction processes including membrane-based downstream processing of the entire stream for product recovery, is the reduction of pathogens in the effluent (Stephenson 2000). The processes that may include chromatography, filtration or chemical treatment physically and/or chemically remove big molecules, viruses and microbial cells. Typically this 'tertiary treatment' is driven from a product quality, and hence, profit perspective. The combined specification to include a legislation perspective may introduce much improved compliance. Membrane technologies are currently receiving much interest in the wastewater industry (Table 55), especially for reuse to potable water quality.

Table 55: Existing, typical applications for membrane technologies in wastewater treatment (Tchobanoglous et al. 2003)

Applications	**Description**
Microfiltration and ultrafiltration	
Aerobic biological treatment	Membrane is used to separate the treated wastewater from the active biomass in an activated sludge process. The membrane separation unit can be internal immersed in the bioreactor or external to the bioreactor. Such processes are known as membrane bioreactor (MBR) processes
Anaerobic biological treatment	Membrane is used to separate the treated wastewater from the active biomass in an anaerobic complete-mix reactor
Membrane aeration biological treatment	Plate and frame, tubular and hollow membranes are used to transfer pure oxygen to the biomass attached to the outside of the membrane. Such processes are known as membrane aeration bioreactor (MABR) processes
Pretreatment for effective disinfection	Used to remove residual suspended solids from settled secondary effluent or from the effluent from depth or surface filters to achieve effective disinfection with either chlorine or UV radiation for reuse applications
Pretreatment for nanofiltration and reverse osmosis	Microfilters are used to remove residual colloidal and suspended solids as a pretreatment step for additional processing
Nanofiltration	
Effluent reuse	Used to treat prefiltered effluent (typically with microfiltration) for indirect potable reuse applications such as groundwater injection. Credit is also given for disinfection when using nanofiltration
Wastewater softening	Used to reduce the concentration of multivalent ions contributing to hardness for specific reuse applications
Reverse Osmosis	
Effluent reuse	Used to treat prefiltered effluent (typically with microfiltration) for indirect potable reuse applications such as groundwater injection. Credit is also given for disinfection when using nanofiltration
Effluent dispersal	Reverse osmosis processes have proved capable of removing sizable amounts of selected compounds
Two stage treatment for boiler	Two stages of reverse osmosis are used to produce water suitable for high-pressure boilers

Disposal of Brine from Membranes

With membrane processes for solute separation, a concentrated brine is generated that conventionally needs to be disposed of at cost. Conventional disposal methods of these brines include ocean discharge, surface water discharge, land application, discharge to wastewater collection system, deep well injection, evaporation ponds and controlled thermal evaporation ((Tchobanoglous et al. 2003). This brine can

however be equated to a concentration step in downstream processing. Novel processes could beneficiate these streams, for example the eutectic freeze crystallisation process currently in development at UCT (Randall et al. 2011). Using a cleaner production approach, these brines could be prevented from being produced in the first place or significantly reduced.

Extractive membrane bioreactors

This process refers to an industrial wastewater containing the organic compound to be degraded is passed over one surface of a selectively permeable membrane, while a microbial culture is maintained in an aqueous biomedium at the other surface. The pH and ionic strength, as well as the presence of toxic compounds of the wastewater have little influence on the makeup of the biomedium as the membrane is effectively impermeable to any inorganic or charged species in the wastewater. Thus the biomedium conditions can be controlled to provide optimal growth conditions for the microbial culture in spite of the biologically hostile makeup of the wastewater (Splendiani et al. 2003).

7.3.3.2 Solid substrate fermentation

For the bacterium, a biofilm is a solid surface to which it attaches so as to scavenge nutrients from liquid media moving past it. This is similar to solid substrate fermentation, where micro-organisms grow on moist solid material in the absence or near absence of free water (Pandey et al. 2010). From an engineering perspective, a biofilm reactor has better mass and energy characteristics than a solid substrate reactor. It is, however, important to consider the parallels between biofilm reactors and SSF, for two reasons. Firstly, the comparison can improve understanding of the requirements for successful bioconversion with retained biomass. Secondly, it is expected that with the increased pressure on water demand management, the implementation of dry sanitation options will increase. This would demand research into how to manage the biosolids produced. Understanding dry sanitation as the solid substrate fermentation alternative to wastewater biorefineries will provide a good base in the wider understanding of obtaining value from waste.

Solid state fermentation (SSF) involves the growth of microorganisms on moist solid particles, in situations in which the spaces between the particles contain a continuous gas phase and minimal visible water (Mitchell et al. 2010; Pandey et al. 2008; Rossous 2010). Solid substrate fermentation, the more general term, describes any type of fermentation process that involves solids, including suspension of solid particles in a continuous liquid phase and the reactors highlighted in the previous sections. Most SSF processes use products or byproducts of agriculture, forestry or food processing. Composting is a more specific application of SSF that does not rely on tightly-controlled optimum conditions for the production of a specific product. Mushroom production can also be seen as a type of SSF process (Stamets 2004, 2005). The majority of SSF processes involve filamentous fungi under aerobic conditions. SSF can be relatively resistant to being overtaken by contaminants, and

as such may be more suitable to socio-economic conditions employing relatively unskilled workers. SSF is also generating more interest as a way to use solid waste in order to avoid the environmental impacts that would be caused by its direct disposal. An engineering understanding of SSF is slowly emerging, but the biggest challenge still remains scale-up. Some advantages and disadvantages of SSF over submerged liquid fermentation (SLF), or conventional stirred tank bioreactors, are listed in Table 56 (from Mitchell et al. 2010). These comparisons may change significantly as the relatively lower substrate concentrations prevalent in wastewater treatment works are considered. SSF is of great importance to the wastewater industry as it can open up a myriad of applications for the use of bio-solids.

Bioreactors used in SSF include rotating drum fermenters, tray fermenters, bed systems, column bioreactors and fluidised bed reactors. A basic key for SSF bioreactor selection is included in Figure 46 (adapted from Mitchell et al. 2010). PGA has been produced by Bacillus using SSF with swine manure as the basis of a solid substrate with a 4.5% PGA yield (Chen et al. 2005), and using dairy manure (Yong et al. 2011). *Bacillus* has also been employed in SSF using wastewater sludge for biopesticide production (Zhuang et al. 2011).

Table 56: Advantages and disadvantages of solid-substrate fermentations relative to submerged liquid culture (Mitchell et al. 2010)

Advantages	**Disadvantages**
Potentially provide superior productivity	Slower microbial growth
Low-cost media	Problems with heat build up
Simple technology	Bacterial contamination can be problematic
Low capital costs	Difficulties often encountered on scale up
Reduced energy requirements	Substrate moisture level difficult to control
Low waste-water output	
No problems with foaming	

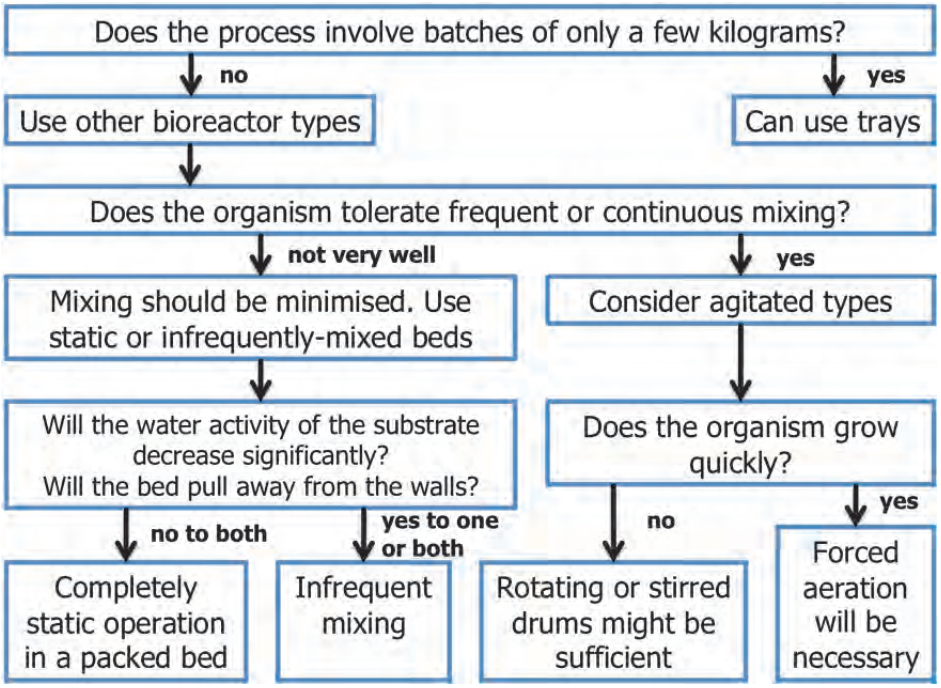


Figure 46: A suggested guideline for SSF bioreactor selection (adapted from Mitchell et al. 2010)

7.4 Reviewing the use of biofilms in wastewater

Biofilm reactors are discussed here as a special case of biomass retention because of their wide-spread use and general acceptance in wastewater treatment.

Wastewater treatment processes are based on the use of three types of microbial aggregates. Static biofilms (e.g. the rotating biological contactor concept discussed in Section 7.4.4), particulate biofilms (e.g. the aerobic granular sludge process discussed in Section 7.4.3) and flocs (e.g. activated sludge processes). The term 'floc' is used to refer to an assemblage of individual cells and micro-colonies occurring under specific reactor conditions or after addition of various agents to the medium (Nicolella et al. 2000, citing Boonaert et al. 1999). There are several design criteria that inform the selection of a specific type of retention system. The impact of the key criteria of flow rate and substrate concentration on reactor selection is summarised in Figure 45 (page 101). For a more detailed discussion the reader is referred to Nicolella et al. (2000). The need for product recovery constrains the selection of reactor designs using these types of aggregates. This informs the final selection of the reactor designs discussed in Sections 7.4.4 and 7.4.3.

7.4.1 Biofilm reactors

Biofilm reactors can reach high cell concentrations of 70 g/L or more, and the cell layers in the biofilm can be highly active contributing to high reactor productivity (Qureshi et al. 2004; WEF Manual of Practice No-35 2010). The biofilm structure

augments long-term activity, facilitating continuous processing. While biofilm reactors have many advantages, they can also fail. As examples, the ability of a biofilm to provide the microbial populations residing in it resistance against toxic chemicals may give them tolerance to survive shock loads, but may also reduce the efficacy of dosing treatments (Rosche et al. 2009). Promoting the dominance of a specific species to improve productivity of a specific product in a mixed culture microbial ecology may be more difficult to achieve than in suspended reactors, because of the more protected environment inside the biofilm (Andersson et al. 2008; Miura et al. 2007).

Active biomass concentrations inside the biofilm are much larger compared to activated sludge systems (Henze et al. 2008). These aspects make it potentially very cost-effective, as well as reducing cleaning and downtime. Rosche et al. (2009) suggest:

> *"Biofilms provide the benefit of a stable environment for the enclosed microbes, which makes them such a prolific and widespread phenomenon in nature. It is therefore not unreasonable to raise the question of whether this biofilm characteristic could be harnessed to develop self-regenerating and long-term stable catalysts for the chemical industry."*

Current wastewater treatment reactors are well designed to achieve nutrient removal through biomass retention, but their design towards product recovery is limited. Biofilm reactors with product recovery need to meet the following requirements:

- Biomass retention through attachment or aggregation of biomass. Solid liquid separation and biomass recycle as a means to retain biomass need to be approached with caution as it may limit product recovery efficiency.
- Good biofilm-water contact, effective mass transport (high specific surface area)
- Growth balanced with detachment (maintenance of active biomass fraction, no clogging)
- An understanding of required supplements: electron donor, electron acceptor, nutrients or alkalinity
- Any other specific considerations toward product recovery: of particular importance to this project

An important consideration is mass transfer over the aggregate-liquid interface. For poorly soluble substrates, the typical penetration depth in the aerobic granular sludge system used to treat wastewater system is shallow (typically less than 1 mm for oxygen (de Kreuk et al. 2007)) and highly dependent on biomass metabolic activity (Beun 2001). This diffusional gradient also means that a growth rate gradient exists within the aggregate, as seen in Figure 47. Mass transport inside a biofilm relies mostly on molecular diffusion, resulting in substrate removal from the biofilm often being mass transport limited. While this can be a drawback, it also allows ecological niches to develop e.g. anoxic regions to aid in nitrogen and phosphate

removal (Mosquerra-Corral et al. 2005). Understanding the interactions between mass transport and substrate conversion processes is necessary to understand the overall performance of biofilm systems, as a particular concern is the possibility of fluctuations in biofilm productivity or product quality over the time course of a continuous process owing to the complex and dynamic nature of biofilms. Process reproducibility is of great industrial importance.

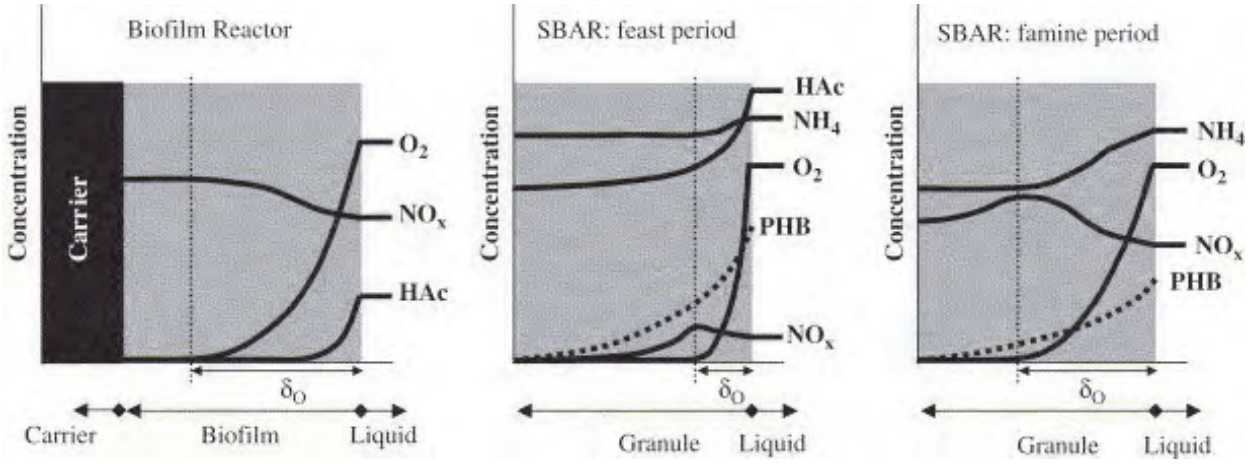


Figure 47: Schematic profile of substrates inside the biomass for a biofilm system and a sequencing batch airlift reactor (SBAR) (feast and famine periods; external mass transfer neglected) = penetration depth of oxygen (μm) (Mosquerra-Corral et al. 2005).

Packed-bed biofilm reactors including trickle-bed and saturated packed beds allow biofilm growth on static surfaces. Aeration can be provided to improve oxygen supply in unsaturated trickle-bed reactors, but presents an even greater challenge in saturated (submerged) beds. With their low surface shear forces, static biofilm reactors are useful in establishing and retaining weakly attached biofilms within reactors, although they provide only a comparably low surface area that is biocatalytically active. Particle-based biofilm technologies, such as airlift and fluidized-bed reactors, are commonly used for biofilms that form on suspended particles or for flocculating biofilms. These reactors have the potential to achieve higher productivity owing to their large specific surface area, effective mixing, decreased boundary layer thickness around the particle and improved mass transfer. This might, however, come at the cost of strong shear forces occurring as a result of harsher hydrodynamic conditions, thus restricting the application of these particle-based biofilm reactors to strongly attached biofilms (Rosche et al. 2009).

Biofilm detachment is one of the most important considerations for biofilm reactors and also influences biomass-associated product recovery directly. Generally, biofilm detachment is attributed to four processes (Van Loosdrecht et al. 1995):

- grazing (the consuming of bacteria from the outer surface of the biofilm by protozoa)

- sloughing (the periodic loss of large patches of biofilm, often destabilised though biofilm growth)
- erosion (the continuous removal of small particles from the surface of the biofilm, primarily caused by liquid shear stress)
- abrasion (analogous to erosion, but caused by collision of particles)

All these, except grazing, can be exploited for product recovery. They all also need to be considered in accounting for product loss. Erosion and abrasion processes are preferred to manage biofilm growth, and are also expected to be the main routes for product recovery. Sloughing destabilises the biofim by resulting in irregular mass distribution and may cause mechanical wear on e.g. rotating biological contactor shafts. The irregularity of the biomass released also affects the fluidization characteristics, and potentially treatment efficiency, of particle based biofilm reactors. Hence, further research on product recovery complements current research to understand better control of erosion and abrasion while minimising sloughing. Losses to grazing need to be factored into yield calculations. The remaining challenge in the engineering of these systems is directed towards the control of biofilm structure and morphology, which depends on, and at the same time affects, the reactor hydrodynamics and mass transfer characteristics.

The two examples of biofilm reactors introduced in Section 7.4.3 and 7.4.5 , the aerobic granular sludge reactor and the hybrid rotating biological contactor, were selected for detailed discussion because the management of biofilm detachment, and hence product recovery, are controllable. The support material of the RBC is relatively planar, while the granular system does not have a carrier, with settling properties that make product recovery possible. In addition, the biofilm zonation in both reactor types (allowing for heterotrophy, nitrification, phosphate removal and denitrification zones intrinsic in the biofilm) is advantageous. Reactor systems employing filter media or fluidised support media were not considered for this project because dealing with the media in the sludge handling is not desired.

7.4.2 Fluidised bed bioreactors: particle based biofilm

The main reactor types used for fluidised, particle-based biofilms are the upflow sludge blanket (USB), biofilm fluidised bed (BFB), expanded granular sludge blanket (EGSB), biofilm airlift suspension (BAS) and internal circulation (IC) reactors.

Lessons learnt from past research on the USB showed that particle size is important, as too small particles force a low velocity which leads to accumulation of suspended solids. The BFB showed that an increased particle size up to about 1 mm in diameter can improve the fluidising velocity which reduces accumulation of inerts, but at larger sizes the conversion rate becomes mass-transfer limited. Therefore control of biofilm thickness becomes important. The airlift reactor (BAS) allows shear by aeration to control biofilm size and structure (Nicolella et al. 2000). This aspect remains one of the challenges in particle-based biofilm research. In addition to these

parameters, product recovery is also important for this project and may be assisted by shear-controlled biomass or polymer removal.

The biofilm structure (density, porosity, roughness, shape) and thickness are important for reactor design as these affect the hydrodynamics, mass transfer and conversion in the reactor. When using this biofilm to produce a product, the impact of the product on these factors have to be investigated.

Particle-supported biofilms have an extra advantage over other biomass retention methods in that they can be easily separated from the bulk fluid. Table 57 lists advantages and disadvantages for particulate biofilm reactors in general. From the disadvantages, it can be seen that many problems can be addressed with better consideration of the carrier. Since the biomass usually has a density lower than the support (typical value for the wet density range around 1040-1100 kg/m^3), increase in biofilm thickness due to biological growth modifies the mixing and fluidisation characteristics, often requiring additional capital expenditure to manage (Nicolella et al. 2000). With the development of the aerobic granular sludge process with complete omission of a carrier support, a different approach to managing particle-based biofilms became possible, and with that more options for product recovery.

Table 57: Advantages and disadvantages of particulate biofilm reactors (general case) (Nicolella et al. 2000)

Advantages	**Disadvantages**
High terminal settling velocity of solids particle-supported: 50 m/h; flocculated sludge: 5 m/h)	Colonisation of carriers and subsequent biofilm formation may result in long start-up times
High reactor biomass concentration particle-supported biomass: 30 kg/ m^3; flocculated sludge: 3 kg/m^3)	Control of biofilm thickness is difficult
High biofilm surface area (3 000 m^2/ m^3, compared with trickling filters at 300 m^2/m^3)	Overgrowth of biofilm leads to elutriation of particles, due to reduced particle density
High biomass concentration and mass transfer area result in high conversion efficiencies (for oxygen, 20 kg/ m^3.d, compared with 3 kg/ m^3.d in activated sludge and trickling filter processes)	Liquid distributors for fluidised systems are costly for large-scale reactors. Further, uniform fluidisation and prevention of clogging must be ensured.
Compact reactor with small area requirements	
High biomass age	

7.4.3 Aerobic granular sludge (AGS) for fluidized systems

Aerobic granular sludge (AGS) is a very new technology, with the first research papers emerging in the late 1990s (de Kreuk et al. 2007a). It was preceded by anaerobic granular sludge, now commonly found in upflow anaerobic sludge blanket (UASB) systems. Aerobic granular sludge emerged as a possible solution to the biggest problems in activated sludge: the large land requirements for settling tanks and the problem of sludge bulking. The granules are defined as a "aggregates of microbial origin, which do not coagulate under reduced hydrodynamic shear ('sludge bulking') and which settle significantly faster than activated sludge flocs" (15 sec vs 20 min) (de Kreuk et al. 2007a). This allows efficient biomass retention, making compact reactors with integrated sludge separation feasible.

The sequentially operated batch reactor (Sequencing Batch Reactor or SBR) is a simple and compact reactor that is fed discontinuously. It is a time-oriented process that can be designed and operated to simulate virtually all conventional continuous-flow activated sludge systems, from contact stabilization to extended aeration, making it a useful approach in a laboratory environment. This aspect, combined with the rapid sedimentation velocity possible with aerobic granules allows product formation and downstream processing to form part of the reactor design at discrete steps (Johnson et al. 2010).

A review on the parameters that are important for the formation of anaerobic and aerobic granule formation has been given by Liu and Tay (2004). The important factors affecting aerobic granulation include:

1. Substrate composition. Granule microstructure and species diversity appear to be related to the type of carbon source.
2. Organic loading rate. Aerobic granules can form across a wide range of organic loading rates, from 2.5-15 kg COD/m^3.day.
3. Hydraulic shear force. High shear force favours the formation of aerobic granules and granule stability. A high shear force stimulates bacteria to secrete more extracellular polysaccharides.
4. Settling time. Settling time acts as a major hydraulic selection pressure on the microbial community (selecting for fast settling bacteria). The formation and characteristics of the granules may be controlled by manipulating the selection pressure.
5. Hydraulic retention time (HRT). The HRT should be short enough to suppress the suspended growth, but long enough for microbial growth and accumulation. A short cycle time (4-6h) stimulates microbial activity and production of cell polysaccharides and also improves cell hydrophobicity. These changes favour the formation of nitrifying granules, but this selective approach may need to be altered slightly for wastewater biorefineries to select for different characteristics.
6. Aerobic starvation. Microorganisms growing in the SBR cycle face periodic fluctuations in the environmental conditions. The aeration period of the

operation actually consists of two phases: a degradation phase where the substrate is depleted, followed by an aerobic starvation phase which selects for microorganisms that can accumulate storage polymer to survive during starvation (de Kreuk et al. 2010; Lin et al. 2010; van Loosdrecht et al. 2010). Microorganisms can change their surface characteristics when they face starvation, becoming more hydrophobic (possibly to aggregate or to sporulate) (Ras et al. 2011).

7. Presence of calcium ion in feed. It has been proposed that Ca2+ binds to negatively charged groups present on bacterial surfaces and extracellular polysaccharides and thus acts as a salt bridge to promote aggregation.
8. Dissolved Oxygen, pH and temperature. These variables seem not to be as decisive in aerobic granulation as they are in anaerobic granulation.
9. Seed sludge. There is some evidence that seed sludge influence the formation and properties of the aerobic granules profoundly. High surface hydrophobicity and low surface density are generally preferred surface properties.
10. Reactor configuration. Completely mixed tank reactors (CMTR) and column reactors give differently shaped granules. The defining parameter is the height to diameter ratio H/D, which should be high enough to improve selection of granules by the difference in settling velocity.
11. Inhibition of aerobic granulation. Free ammonia at concentrations greater than 10 mg/L may inhibit granule formation. The high free ammonia concentration leads to a significant decrease of cell hydrophobicity and extracellular polysaccharide production.

The review by Liu and Tay (2004) further considers the characteristics of the granule. The reader is referred to the original text for a detailed description. A very brief summary is given here, and illustrated in Figure 48. Further references include work by Beun et al. (2002), de Bruin et al. (2004), Etterer (2004) and Li (2009).

- Compared to activated sludge, the granules have the following characteristics:
  - Denser, stronger microbial structure
  - Regular, smooth and round shape, and a clear outer surface
  - Visible as separate entities in the mixed liquor during both mixing and settling phases
  - High biomass retention and excellent settleability
  - Capable of withstanding high flow rates
  - Capable of withstanding high organic loading rates
  - Less vulnerable to toxicity of organic chemicals and heavy metals in wastewater
- Average diameter 0.2-5 mm
- Sludge volume index (SVI) lower than 50 mL/g. Settling velocity of 30-70 m/h. In addition, the SVI of the aerobic granules after 5 minutes should be comparable with that after 30 minutes, termed SVI30 (Anuar et al. 2007)

- Density typically between 1.004 and 1.056 kg/m^3
- Strength measured as having an integrity coefficient greater than 95%
- Hydrophobicity almost twice that of conventional bioflocs
- Specific oxygen utilisation rate (SOUR). The shorter settling time tends to stimulate the respiratory activity of the bacteria significantly. This implies that the microorganisms attempt to regulate their energy metabolism in response to changes in hydraulic selection pressure.
- Storage of granules is possible at low temperatures (4 ˚C). Several factors affect structural integrity and activity, but the granules regain metabolic activity quickly when fed with substrate and oxygen.

Aerobic granulation is significantly faster and easier than anaerobic granulation. So far, it has only been observed in sequencing batch reactors (SBR). Generally, aerobic granular sludge is an exciting technology, but less suitable to streams with high total suspended solids (TSS) and low COD. The two most important parameters in aerobic granular sludge are the shear rate (coupled with aeration) and the COD load. It will be interesting to see how a storage polymer containing nitrogen influences the control of these parameters.

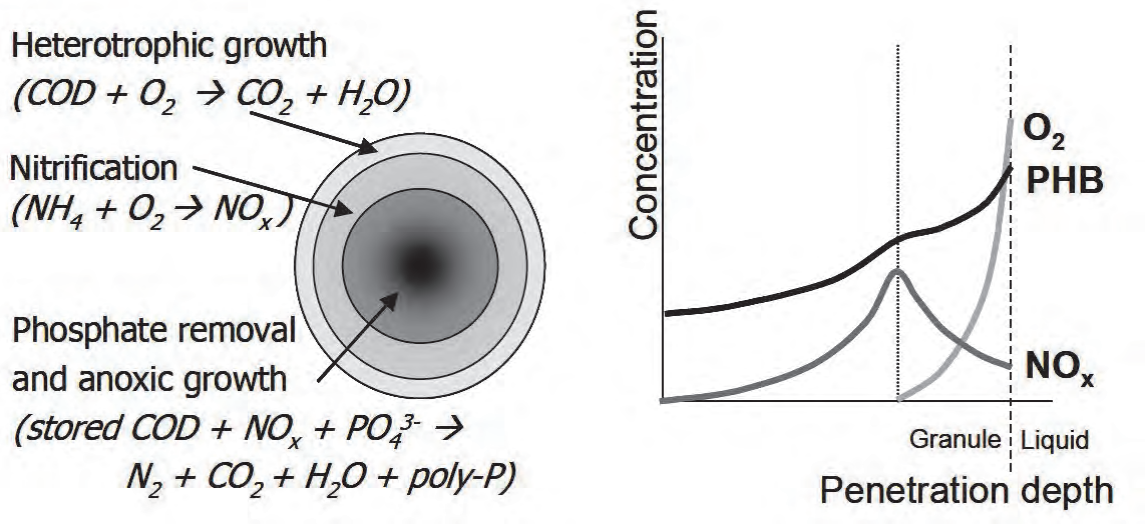


Figure 48 Schematic representation of the layered structure of aerobic granules and of the substrate and electron acceptor concentrations inside the granules during the famine phase (de Kreuk 2006)

7.4.4 Static biofilms: Rotating Biological Contactors

Rotating Biological Contactors (RBC) are non-submerged, attached growth bioreactors, similar to trickling filters, with circular media mounted (approx 3.6 m in diameter for standard units) on a horizontal shaft, partially submerged (typically 40%) and rotated at a speed of one to six revolutions per minute (Grady et al. 2011)

(Figure 49). The media is commonly corrugated plastic media. Benefits and drawbacks of RBCs are listed in Table 58.

Table 58: RBC benefits and Drawbacks (Grady et al. 2011)

Benefits	Drawbacks
Mechanically simple Simple process, easy to operate Low energy requirements Modular configuration allows easy construction and expansion	Performance susceptible to wastewater characteristics and loadings Limited process flexibility Adequate pre-treatment required

Parameters of RBC's that can be controlled include:

- Carrier media (composition of the disk);
- Rotation speed (aeration and shear);
- Recycling loops;
- Shear;
- Disk diameter.

Conventional RBCs have corrugated disks. The corrugations increase stiffness, increase available surface area, improve mass transfer, and define spacing between individual disks. Standard density media has a surface area of about 115 m^2/m^3.

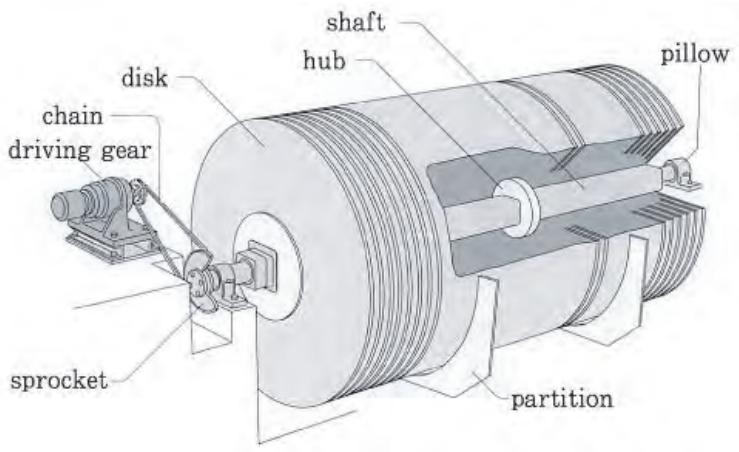


Figure 49: Schematic diagram of a rotating biological contactor (RBC) (source: http://www.thewatertreatments.com/waste-water-treatment-filtration-purify-sepration

The shaft is rotated at constant speed, providing a shear force as well as aeration to the biofilm through the turbulence created. This shear contributes to controlling biofilm thickness as well. For all practical purposes the tank holding the wastewater (contacting the submerged section of the biofilm) can be considered completely mixed and can be modelled as such. Treatment tanks are mostly arranged in series, with an expected population change across the series corresponding to the COD load. The liquid film in the aerated sector is treated as a plug-flow reactor on top of the biofilm.

sewage/rotating-biological-contactor)

The motive force driving a RBC can be mechanical or powered by air drive units. The air drive units increase the oxygen transfer capacity, as well as shear. Typically a flow of 4.2-11.3 m^3/min is used, giving rotational speeds of 1.0-1.4 rpm. Air transfer has many advantages, but can lead to loping, or uneven distribution of biofilm along the circumference. Innovations include a more submerged contactor (70-90% of the disk), which is aerated. A meshed disk design may also reduce this effect due to the mesh acting as anchor for the biomass, but will not eliminate it. Energy input must also be traded against mechanical wear. Apart from shear, biofilm management can be done chemically through oxidation with chlorine or elevating the pH with NaOH. Employing shear in a more specific way includes scouring with blasts of air, changing shear characteristics by reversing the rotational direction, recirculating effluent, and turning off and drying out individual shafts (some recommend weekly to ensure a more active (nitrifying) population (Grady et al. 2011).

7.4.5 Modified mesh-disk Rotating Biological Contactor

Another type of static biofilm reactor is Submerged Fixed Bed Biofilm Reactors (SFFBR). The greater surface density permits a larger biomass per unit volume, while greater void space allows for a higher oxygen and mass transfer to the biofilm, and reduces the clogging risks of the channels of the support media by excessive biofilm growth (Matos et al. 2011; Schlegel & Koeser 2007). Flow resistance through the media is high, and product removal of biomass associated product is virtually impossible unless it accumulates in the sludge, hence this configuration will not be investigated in this project. Modifying a Rotating Biological Contactor (RBC)'s planar disks with a mesh type supported disk is expected to give the biofilm better stability and providing an environment for denitrification. The surface area is estimated to be 2000-3000 m^2/m^3 (Henze et al. 2008, p 494). With a mature biofilm much of this mesh will be occupied, but a significant increase in active biomass is still expected, similar to the AGS process.

This hybrid RBC system uses the rotating biological contactor type as basic system for mixing, but combines the contact surface with a type of SFFBR surface media. This system takes its inspiration from the HYBACS process (HYbrid Bacillus ACtivated Sludge System) (Figure 53, page 128). The rotating disks, instead of being impermeable, consists of a fixed open structured mesh, allowing biofilm to grow inside the mesh as well as on the surface. The rotating disks, instead of being impermeable, consist of a fixed open structured mesh, allowing biofilm to grow inside the mesh as well as on the surface.

This combined strategy allows a safe environment for the slow growing microorganisms responsible for denitrification, increasing denitrification capacity. The integrated biofilm growing on and through the mesh allows for more uniform

biofilm sloughing, reducing strain on the shafts. Biomass associated product recovery can only occur through the biomass on the surface of each disk.

Product recovery can be achieved by rollers or scrubbers fixed onto the disks, shear by aeration, or manual removal of the disks (similar to how honey is harvested).

7.5 Design for Downstream Processing

The fundamental consideration to make a bioprocess from dilute streams viable from both an economic and environmental point of view is in the approach to the downstream processes (DSP) (Harding 2009, Richardson 2011, Richardson et al. 2012). It must be noted that, in these dilute systems, recovery of both the product and the water is essential. The latter may be recycled back to the process or recovered as water of useable quality. In a systems approach, we need to consider the recovery and quality of both streams. There are three main requirements to realise effective product formation from dilute (waste) streams:

1. Decouple hydraulic residence time and biomass residence time. Biomass retention to increase the apparent biocatalyst concentration can also serve as a phase change mechanism to concentrate the product;
2. Ensure adequate nutrient provision to the cells without excessive energy requirement for mass transfer, without compromising the ability to recover the product;
3. Design for downstream processing (DSP). Reactor design and choice of the biological system used affects the cost of DSP significantly. In dilute waste streams many DSP methods are not cost effective, as the combination of volume processed and energy requirement per unit volume is too much. The need for centrifugation, for example, is a challenge that cannot be addressed at DSP level, but needs to be prevented through choice of system and reactor design.

Considerations when separating bioproducts from dilute, complex medium include (S. Harrison, CHE 5070 University of Cape Town course notes):

- The product is usually present at very low concentration, typically less than 5% of the total dissolved solids present
- The solution is complex, with typically less than 10% representing the major product. However a portion of the remaining 90% may include useful co-products for co-extraction
- The product is frequently labile
- The product may have specific characteristics, like charge or differential polarity, to enhance affinity separations
- The solution has a high water content
- The product may be “tunable” – minor changes in solution properties may result in major changes in physical properties

- Biomass-associated product may offer an effective concentrating mechanism, at the expense of cell-rupturing costs.

7.5.1 Options for Downstream Processing of PGA

7.5.1.1 Current Downstream Processing of PGA

DSP and analysis is a persistent challenge in PGA production. The current method of DSP is relatively expensive and time consuming, which may be responsible for the high retail price of pure PGA. PGA is recovered by its precipitation by the addition of four volumes of cold ethanol, followed by centrifugation and gel permeation chromatography (Shih, Van 2001). A recent article exploits the negative charge of PGA through addition of $CuSO_4$ to precipitate the PGA as a replacement for the ethanol precipitation step. Resuspension is achieved by removing the Cu^{2+} ions with a chelating agent like EDTA (Manocha & Margaritis 2010).

Both of these PGA purification methods are not feasible in a wastewater environment without significant modification: Gel permeation chromatography is a viable option if the product can be recovered in a reasonably concentrated form without contaminants that are detrimental to the gel resin. $CuSO_4$ precipitation is not feasible if the Cu^{2+} ions are in contact with the bulk liquid, as the General Authorisation Standard limit for Cu^{2+} ions in effluent is very low (0.01 mg/L). $CuSO_4$ precipitation waste will generate disposal challenges and is not expected to be a viable option.

7.5.1.2 Characteristics of PGA that could be exploited in Downstream Processing

PGA is produced extracellular, but is biomass associated. Where conventional STRs separate extracellular products from the biomass through the shear forces generated in stirring, the low flow rates of treatment works that approach plug flow may allow manipulation of the PGA location in a manner advantageous for DSP options.

PGA is a homo-poly-amino acid. The homogenous nature creates a predictable structure with a homogenous negative charge. The charge through the carboxylic acid side chains creates “tunability” – minor changes in solution properties may result in major changes in PGA’s physical properties. Pure PGA chains form a ‘hairy rod – like structure’, the helical rod-like structure is similar to a protein, and the hairs refer to the side chain carboxylic acid groups (Hammond et al. 2008). It is possible that PGA produced in a mixed microbial culture may be cross linked with sugars, changing its macro structure and charge behaviour, but it is assumed that enough of the characteristic poly-amino acid structure may be conserved to be exploited in product recovery.

The negative charge at neutral pH, and the isoelectric point around pH 4 both are potentially useful characteristics for recovery. Acidifying the broth to pH 4 has two negative implications, however. The first is the use of a large amount of acid and

generating a large amount of acidic wastewater, and the second is the propensity of PGA at pH 4 to coagulate, making resuspension difficult.

Many biomaterials are purified using methods based on differences in polarity or hydrophobicity (like ethanol or ammonium sulphate precipitation) (Doran 1995). Sensitive methods like chromatography are used when protein denaturation needs to be prevented (loss of protein structure and functionality due to disruption of intermolecular forces). PGA is a non-functional protein, which means that many of the separation processes possible for proteinaceous structures can be applied without the usual constraint of denaturation. As seen above with precipitation at pH 4, while this increases processing options, this cannot be applied without limitation.

Because of the negative charge on the side chains of the PGA polymer, sophisticated applications using electricity becomes possible. Electrospinning uses an electrical charge to draw very fine (typically on the micro or nano scale) fibres from a liquid. Electrospinning shares characteristics of both electrospraying and conventional solution dry spinning of fibers. The process does not require the use of coagulation chemistry or high temperatures to produce solid threads from solution. The technique relies on electrical rather than mechanical forces to form fibers. This makes the process particularly suited to the production of fibers using large and complex molecules. Special properties are required of polymer solutions for electrospinning, including the ability to carry electrical charges. While electrospinning is still a young technology, electrospinning PGA has been reported (Lee et al. 2009, Tajima et al. 2011) and it illustrates the possibility of using diverse technologies in waste applications. With further improvement on the high voltage requirement to produce the electrical forces – perhaps by mimicking the spider spinnaret, further cost-effective applications can be developed.

The negative charge could also allow binding to positively charged magnetic particles that can be recovered by applying a magnetic field. This method is called “high gradient magnetic separation” (HGMS).

7.5.1.3 Recovery of product from water

To effect efficient DSP on a large scale in a cost efficient manner, the primary objective of the DSP is to provide the product in a different phase to the bulk material to enable easy recovery of the desired component, while reducing the amounts of unwanted components. Once the product is taken into a different phase, it has to be recovered effectively. Products can be taken into a gas or solid phase. PGA, like most larger biomolecules, will decompose before it reaches the gas phase, so a phase change will have to involve turning the PGA into a solid. Another alternative is to create an emulsion or a gel. This mimics the mucoid form that the PGA forms as part of the biofilm matrix.

PGA is generally accepted to be a product excreted into the bulk solution and not even weakly associated with the biomass. This is certainly the case when PGA is produced in a STR with relatively high shear rates, and high substrate

concentrations. However, at very low substrate concentration the organism needs to keep the excreted PGA close to the biomass to ensure greatest benefit of its intended ecological function. Several biofilm-based approaches allow cultivation at relatively low flow rates and may be used to minimise the separation of the weakly associated polymer from the biomass. In terms of considering PGA recovery, three potential scenarios have to be considered:

1. PGA is excreted into the bulk solution and is not associated with the biomass at all
2. PGA is excreted, but weakly associated with the biomass. This association is easily disrupted
3. PGA is excreted, but strongly associated with the biomass. Significant physical or chemical means needs to be employed to disrupt this association.

7.5.1.4 Scenario 1: PGA secreted into bulk solution

If PGA is excreted into the bulk liquid, the PGA needs to be recovered from a large volume, low concentration environment. Because of the large amounts of liquid being treated, conventional methods of precipitation associated with production routes using high substrate concentrations will not be viable. One option is to separate the PGA from the bulk liquid based on size of the molecule, using ultrafiltration, as is being done on reverse osmosis and membrane bioreactor plants (Stephenson 2000). Here adequate biomass retention is important to minimise wear and clogging on the filter membranes. The water is purified, and the remaining retentate contains the PGA in a more concentrated form. This PGA can then be precipitated, or if the retentate contains a sufficiently high concentration of PGA to form a gel, it may be used directly for a soil conditioner. Alternatively, the PGA can be adsorbed from the bulk solution onto a charge based chromatography column and backwashed occasionally. These methods are energy intensive, but may lead to very pure water as a byproduct.

Flotation is used to separate solid or liquid particles from a liquid phase. Separation is brought about by introducing fine gas (usually air) bubbles into the liquid phase. The bubbles attach to the particulate matter, and the buoyant force of the combined particle and gas bubbles is great enough to cause the particle to rise to the surface (Tchobanoglous et al. 2003). Flotation is preferred over sedimentation when the particles are very small or light. Floated particles are collected by skimmers. The foaming caused by PGA production in high substrate concentration bioprocess can be seen as a natural form of flotation, implying that PGA serves as a ‘chemical additive’ or detergent to improve flotation of particulates. This may mean that PGA will be recovered well using flotation, but also that contaminating particulates would be floated as well. The structure and integrity of the biomass retention plays a role to reduce this contaminating fraction, similar to the retention required to reduce membrane maintenance.

7.5.1.5 Scenario 2: PGA weakly associated with biomass

If PGA is weakly associated to the biomass, gentle disruption may be all that is required to separate the PGA from the biomass. If the procedure used for product recovery is not severe, it may not harmful to the biomass, allowing the biomass fraction to be returned to the reactor after PGA removal to maintain the biocatalyst concentration. Potential recovery methods that can be used in this way include using air flotation, or the force of flowing water.

The assumption here is that PGA is extracellular, and only loosely biomass associated. Because of the low flowrate and hence low general shear rate employed in the rotating biological contactor (RBC), the assumption is that PGA is not disrupted from the biomass and can be harvested as part of the bulk biomass. The bulk biomass-PGA fraction can be separated from the bulk liquid. Compressed air, for example, can then be applied for this purpose, causing localised shear which disrupts this loose association, separating the PGA into the surrounding fluid. Harvesting the PGA in this way would also concentrate the fraction somewhat.

For the hybrid-RBC process, rollers, like the in-situ sludge dryers' belt filter press, could be used to 'scrape' or 'squeeze' the product from the biomass. Roller shafts of e.g. 20 mm diameter can be interspaced between the approx 50 mm wide mesh disks, designed to exert a gentle pressure just enough to apply a shear to dislodge the weakly associated PGA or EPS. The roller would be placed flush to the mesh plate, and slanted at e.g. 45° to the horizontal to promote flowing or floating of the 'scraped' or 'squeezed' PGA-containing fraction.

7.5.1.6 Scenario 3: PGA strongly associated with biomass

One advantage of the Aerobic Granular Sludge (AGS) reactor is the fast settling rate of the granules, which effectively separates the biomass and biomass-associated PGA product into a solid phase. After the granules have settled, a fraction of the biomass can be harvested through a sludge discharge valve following sufficient product accumulation. This harvested fraction can be sheared or otherwise disrupted to release the PGA into the liquid phase in a concentrated form and then treated with ethanol for PGA precipitation and conventional downstream treatment. This is hypothesised to be particularly useful if the PGA is tightly associated to the biomass because of the shear rates employed in the AGS reactor (to maintain robust granules). Care should be taken in this approach to maintain an adequate biocatalyst concentration in the reactor to maintain activity.

While biomass retention is important for reasons outlined in this chapter, it serves different functions depending on where the product is located, and determined whether the biomass itself is recovered or not. This determines reactor selection. Figure 50 is an initial guideline for wastewater biorefinery reactor selection developed through this project.

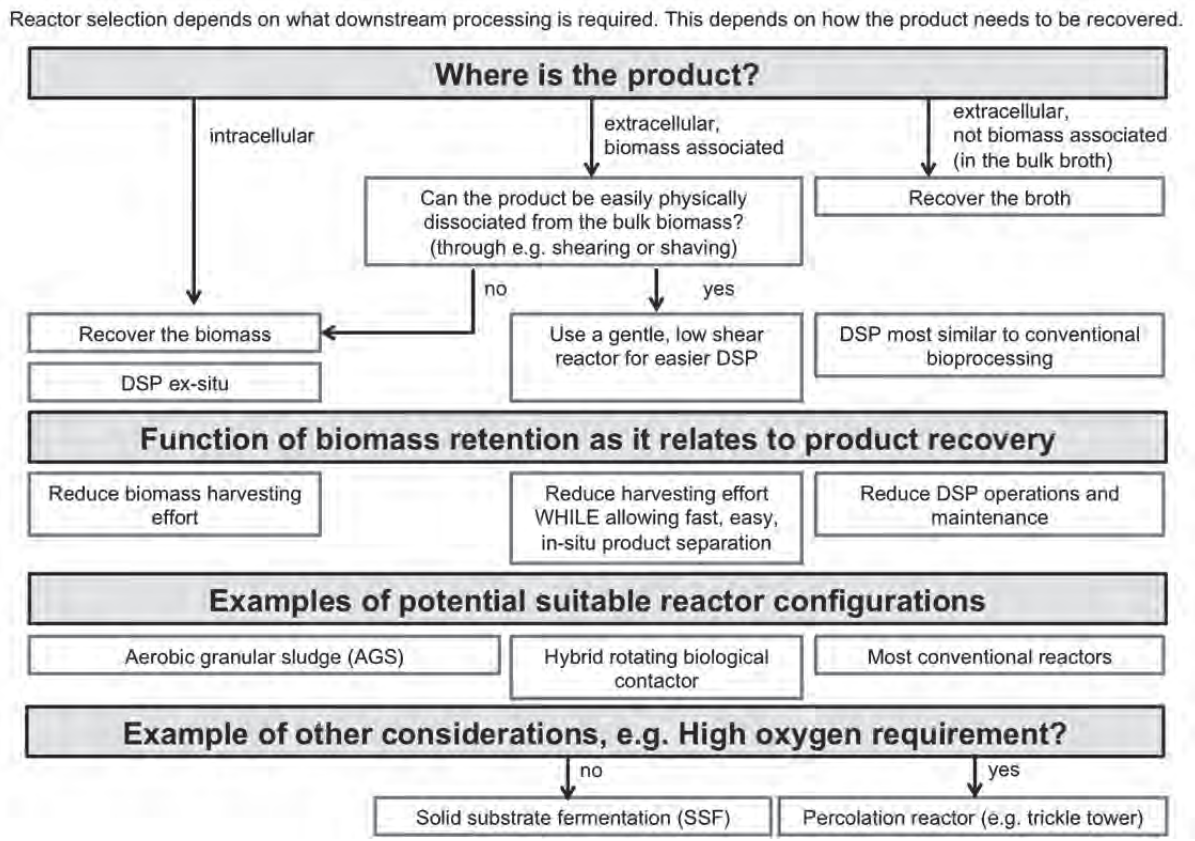


Figure 50: Suggested guideline for wastewater biorefinery reactor selection

7.6 Scale Up: Factors to consider

The challenge of scale-up is to preserve the reaction conditions selected in the process development phase, while operating at a larger scale. However, the process variables are each affected differently by scale. For example, power input per unit volume, and thereby volumetric mass transfer co-efficient, scale as a function of L^3 in a STR (equivalent to volume) where L is the reactor dimension, whereas impeller tip speed scales according to L. Scale-up criteria, i.e. the property selected to be kept the same include geometric similarity (e.g. height to diameter ratio), material similarity (like density, viscosity that can change with pressure changes in e.g. high or deep reactors), or dynamic similarity of the flow fields – keeping the dimensionless numbers prevalent in engineering models, like the Power, Reynolds and Froude number, constant. None, or very few, of these similarities can be conserved on scale up, but depending on the requirements of the cells and the process, some are more critical than others. Roughly 30% of biotech industries use a constant P/V ratio, 30% use constant k_La, (P/V correlates to k_La, these scale in a similar way) 20% use v_{tip} (the tip speed of the stirrer), and the last 20% employ a constant p_{O2} (Picioreanu lecture material, TU Delft). These approaches focus primarily on the stirred tank reactor.

With regard to fluidised bed reactors and rotating biological contactors, reactor design data is less readily available. Hence general opinion is that scale up should be conducted empirically using rigorous pilot-plant scale studies to overcome the lack of confidence in the scale up relationships between laboratory and large scale systems. As indicated by Grady et al. (2011, p843) “consequently, the only safe approach is to use pilot units with full diameter disks”. This caution around the scale up of biological reactor systems pervades the industry for all reactor types, except the STR.

At scale, mass transfer, especially oxygen delivery and carbon dioxide removal, and the related heat transfer are the biggest challenges for scale up. Their relative importance is governed by the available mass and heat transfer surfaces in relation to the mass and heat transfer required. These should already be considered in the design at laboratory level. In the STR, mass transfer limitations typically govern small scale processes with heat transfer taking over on scale up, due to the relative surface:volume ratios. Mass transfer becomes increasingly challenging on using biofilm systems. Overcoming diffusion-controlled external mass transfer resistance through the interstitial layer of the biofilm is the rate limiting step in mass transfer in biofilm reactors. Hence, the specific biofilm area av (in m^2/m^3) becomes important to improve this parameter.

8 Experimental Set-up using Biomass Retention Reactors

8.1.1 Objectives

The objective of the experimental component was to gather qualitative experience on whether the reactors could produce a PGA-containing biofilm, and how the chosen reactors would perform in an on-site, non-sterile environment.

The reactors were selected for this project based on their ability to accumulate a biomass-related bio-product as well as the capacity for controlled product removal in downstream processing. The aerobic granular sludge (AGS) sequencing batch reactor falls in the relatively "higher substrate zone", most suitable to particle biofilms, while the rotating biological contactor (RBC) falls in the "static biofilm zone", most suitable to high flowrates, and lower substrate loading, described in Figure 45 of Section 7.3.

8.1.2 Aerobic Granular Sludge (AGS)

8.1.2.1 Reactor description

As described in Section 7.4.3, the sequentially operated Batch Reactor (Sequencing Batch Reactor or SBR) is a simple and compact reactor that is fed discontinuously.

It is a time-oriented process that can be designed and operated to simulate a conventional continuous-flow activated sludge systems, from contact stabilization to extended aeration, making it a useful approach in a laboratory environment. This aspect, combined with the rapid sedimentation velocity possible with aerobic granules allows product formation and downstream processing to form part of the reactor design at discrete steps (Johnson 2010). The reactor is shown in Figure 51.

The granules are defined as a "aggregate of microbial origin, which do not coagulate under reduced hydrodynamic shear ('sludge bulking') and which settles significantly faster than activated sludge flocs" (15 sec vs 20 min) (de Kreuk et al. 2007a). This allows efficient biomass retention, making compact reactors with integrated sludge separation feasible.

Figure 51: Aerobic Granular Sludge laboratory reactor setup (photo from de Kreuk PhD thesis, TU Delft 2006)

8.1.2.2 Construction of generic AGS reactor

The AGS reactor of 5 litre working volume, shown in Figure 52, was designed and constructed with a focus on achieving a functional, but low cost unit. The following give reactor specifications:

- Container: Acrylic 100 mm inner diameter tube, Perspex covers machined in the UCT Chemical engineering workshop
- Total volume: 7L
- Working volume: 5L
- H/D ration between 5 and 7 (sufficient for granule settling and able to scale to full plant size)
- Inflow: Initially, a fish tank pump was used to pump from a storage container.
- Outflow: GSR Force pilot operated solenoid valve (sold in SA through Festo), ½" 220VAC Brass GSR D43231001. With no current it is closed, opening when current is passed through. No back pressure is required. The outgoing flowrate is limited with a narrow aperture (garden irrigation pipe fitted to the outlet hole).
- Aeration: Fish tank aerator coupled to a sparger. This proved to be insufficient air supply for high substrate concentrations, and was not adequate for mixing.

The system was used to establish an automated sequence of fill-aerate-idle-withdraw. The fill function was achieved with a fishpond pump on a timer switch, drawing fluid from a storage tank. The flow was regulated through a flow switch. Details of construction are listed in Table 59. Withdrawal was achieved through a solenoid connected to a timer switch.

The cycle time was dependent on growth rate and substrate concentration and needed to be optimised on site, by modifying the aeration time appropriately. An example is shown in Table 60. In full-scale plant operation this is incorporated into a continuous process, combining inflow and outflow.

Figure 52: Aerobic Granular Sludge laboratory reactor setup constructed in this project.

Table 59: Equipment for building the AGS reactor

Item	Supplier	Price (incl VAT)	Purpose
Solinoid x 2 012XX ½" 220VAC Brass GSR, D43231002, with modified diaphragm for wastewater application	GSR (via Festo)	R3335.30	Regulate outflow. The diaphragm of an earlier version leaked, I think the surfactants or something that the organisms produce degraded it.
Float Switch	ACDC Express	R459.31	Regulate inflow
Aerator	Aquarium shop in Plumstead	R120	Aeration
Timers	ACDC Express	R120 each	Regulate on/off of flows and aeration
Cylinder	Maizey	R287.25	Reactor container
Covers	UCT ChemEng workshop		Keep dust out, bottom waterproof seal
Nuts, Washers, etc.	Topfast	R255.79	Tighten bottom seal
Tubing	The Cape Town Rubber Co	R467.40	Tubing
Hosing	Hose Solutions	R417.20	Tubing
Taps, plastic fittings	Turf-Aq	R200.87 + R894.47	Manual override, regulate emptying
Extension cord			
Plugs and fittings			
Cupboard	Office Solutions	R700	Housing the reactor

Table 60: AGS Cycle, on a 4 hour frequency.

0:00-0:05	0:05-0:25	0:25-3:57	3:57-3:58	3:58-04:00
Inflow	*Idle*	*Aeration*	*Settle*	*Outflow*
	Bacterial population selection pressure for bacteria able to convert substrate into storage polymer, hypothesize that other organisms requiring oxygen die	Normal aerobic respiration	This should remain very quick to select for fast settling granules, it depends on the height of the reactor, but aim for 30sec/m.	

8.1.3 Hybrid rotating biological contactor (hRBC)

8.1.3.1 Reactor description

The rotating biological contactor, shown in Figure 53 (BluewaterBio design) and Figure 54 (low cost model constructed for this project), was selected because it is a convenient mechanism to allow for biofilm growth. The disks were modified from impermeable disks to an open mesh, to allow the biofilm to grow to a greater depth, creating a greater surface area as well as anoxic and anaerobic zones that are expected to allow for better nutrient removal.

Figure 53: Mesh structure used to inform hybrid design (www.bluewaterbio.com)

RBC's were well studied in the 1900s; however, a few early design problems limited their application. Excess biomass accumulation, shaft breakage, loping of disks caused by unbalanced biomass weight and undesirable biological growths were the main challenges. While these challenges have been resolved on a technical level, the acceptance of RBC's has suffered a blow in industry (Biofilm reactors WEF Manual of Practice, Keyser 2012).

8.1.3.2 Construction of generic RBC

The purpose of the 5L lab-scale rotating mesh-dish biological contactor was to establish if a biofilm can grow on a rotating mesh disk using the micro-organisms selected for this study. If this was possible, it was desirable to test whether this biofilm can be manipulated to produce the mucoid substance of interest. The reactor was not designed to establish the extent of nutrient removal as this was not the main objective of the project.

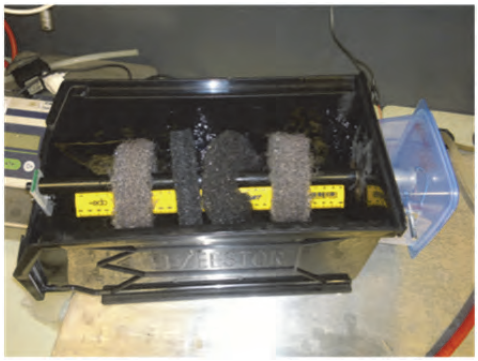


Figure 54: The hybrid rotating biological contactor constructed in this project.

The first motor driving the shaft was variable-speed, to allow for between 1 and 6 rpm rotation, but it was not strong enough. Hence, it was replaced with a motor that rotated at a fixed rate of 1 rpm. The reactor specifications are given below. Further construction details are listed in Table 61.

- Working volume: 5L
- Flowrate: variable, and depends on substrate concentration. Estimate initial flowrate of 1 L/h (24 L/day), but this can only really be tested in the field.

- Container: plastic box (Plastics for Africa, Retreat, Cape Town)
- Shaft: Hippo HDPE rod, black, 20 mm/dia (Maizey, Paarden Island)
- Motor:
    - Version 1: Motor with gearbox 12 V 10 rpm (variable from 6 to 10 rpm) Mantech (this motor struggles with the load)
    - Version 2: Geared 24VDC 0.2A 1 rpm
- Rotational speed: 1 rpm to 6 rpm for laboratory study, 1 rpm for field testing.
- Mesh disks: Cleaning machine disks manually cut to circles.

The plate-like shaft (ordinary stationery ruler) was used to prevent the mesh disks from slipping and not turning along with the shaft. It also introduced some mixing.

Table 61: Equipment for building the hRBC reactor

Item	Supplier	Price (incl VAT)	Purpose
Container	Plastics for Africa	R40.00	Reactor container
Motor	Mantech electronics	R234.84	Drive the shaft at 1 rpm
HDPE rod (black)	Maizey	R58.73	Shaft
Ruler	Checkers	R3.00	Stabilise disks on shaft
Abrasive disks	<cleaning place in Retreat>	R500 for stack of 5	Mesh disks to support biofilm growth
Machining	UCT ChemEng Workshop		Making the pieces fit together

The reactor mixing was adequate, but highly variable across the reactor. It was dependent on the rotational speed, the presence of the ruler, the shape of the mesh disks, and was not directly scalable. The aim of this reactor prototype was to demonstrate production of the PGA-containing material, not to optimise the reactor design.

A variety of mesh materials were shown to work equally well. The cleaning grid was chosen as it was reasonably cheap, easily accessible, planar and homogenous.

8.1.4 Waste streams considered

Initially the reactors were commissioned in the laboratory, using a synthetic waste stream. Thereafter, they were tested in the field. Athlone wastewater treatment works (City of Cape Town) kindly granted permission to work on-site. Initially the settled sewage, mixed with activated sludge (taken from site 1 in Figure 55 and Figure 56) was chosen for the incoming stream. The chosen bacterium used in this project had been isolated from this stream, and using the stream with this mix of bacteria was thought to hasten the inoculum of the reactor favourably, as it probably contains the bacterial population required for effective wastewater treatment. However, very shortly after reactor start-up the activated sludge completely choked up both reactors. This created anaerobic conditions in the AGS reactor and clogged up the piping to the hRBC, letting that reactor run dry. The reactors were then

moved to the second site, using raw settled sewage (taken from site 2 in Figure 55 and Figure 57).

Figure 55: Location of two test sites at Athone Wastewater Treatment Works.

Figure 56: Composite photographs as an illustration of the first test site's conditions.

Figure 57: Composite photographs of the conditions at the second test site.

Table 62: Composition of wastewater streams at Site 1: Activated sludge (from City of Cape Town Scientific Services)

Athlone Activated Sludge Plant Influent		17 July 2012	24 July 2012	31 July 2012
Total Suspended Solids	mg/L	124	168	148
Settleable Solids	mL/L	<1.0	<1.0	<1.0
COD	mg/L	394	423	395
TKN	mg N/L	45.1	-	-
Ammonia	mg N/L	31.3	31.8	33.4
Total Phosphorous	mg P/L	5.3	-	-
Ortho-Phosphate	mg P/L	3.7	3.6	3.3
pH		7.5	7.5	7.5
Conductivity	mS/m	123	123	121
Cloride	mg/L	157	157	159
Alkalinity	mg CaCO3/L	289	303	312

ACTIVATED SLUDGE

Athlone Activated Sludge Reactor A		17 July 2012	24 July 2012	31 July 2012	7 Aug 2012
Settleable Solids	mL/L	250	280	280	310
Total Suspended Solids	mg/L	4490	4850	4770	4530
Volatile Suspended Solids	mg/L	-	-	-	3660
SVI (Sludge Volume Index)	mL/g	56	58	59	68
DSVI (Diluted SVI)	mL/g	45	41	42	53
pH		6.7	6.7	6.7	6.5

Table 63: Composition of wastewater streams at Site 2: Raw sewage after primary settlement (from City of Cape Town Scientific Services)

Athlone Primary Settling Tank Effluent		17 July 2012	24 July 2012	31 July 2012
Total Suspended Solids	mg/L	152	136	126
COD	mg/L	464	405	402
TKN	mg N/L	47.4	-	-
Ammonia	mg N/L	31.1	33.5	34.2
Total Phosphorous	mg P/L	5.5	-	-
Ortho-Phosphate	mg P/L	3.8	3.8	3.4
pH		7.4	7.5	7.3
Conductivity	mS/m	122	129	122
Cloride	mg/L	158	165	161
Alkalinity	mg CaCO3/L	290	305	303

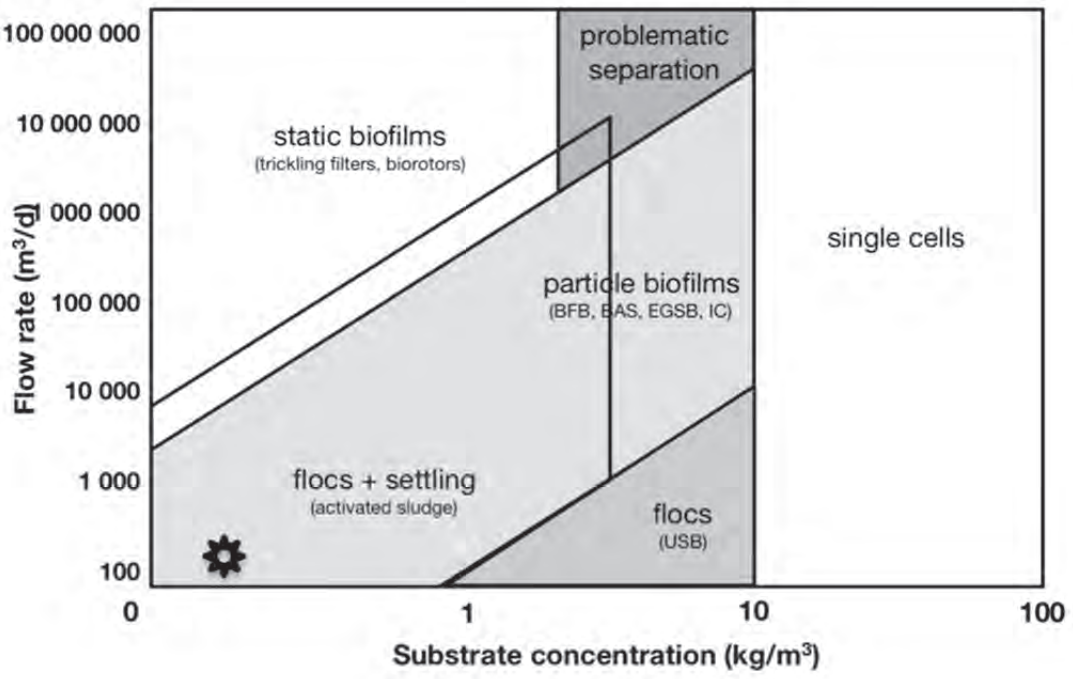


Figure 58: Position of Athlone wastewater in the project reactor setup, indicated by the star symbol, on the concentration-flow rate phase diagram for application of floc and biofilm reactors (adapted from Nicolella et al. 2000) (100 mg/L = 0.1 kg/m^3)

8.2 Experimental Reactor Results

8.2.1 AGS on non-sterile, synthetic media

Following inoculation with biomass harvested from a shake flask shown in Figure 59, the reactor was operated at room temperature to allow establishment of the biomass and induce granulation. No antifoam was used and the reactor was operated under clean, but non-sterile conditions. After a colonisation period, the cyclic operation was established according to Table 60. The reactor is shown in Figure 60 and Figure 61.

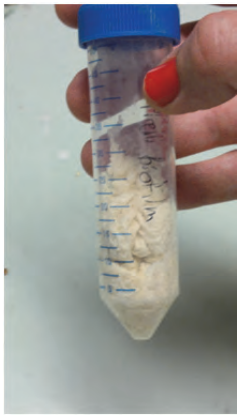


Figure 59: Biofilm harvested from a 100 mL shake flask to be added to reactors containing real wastewater

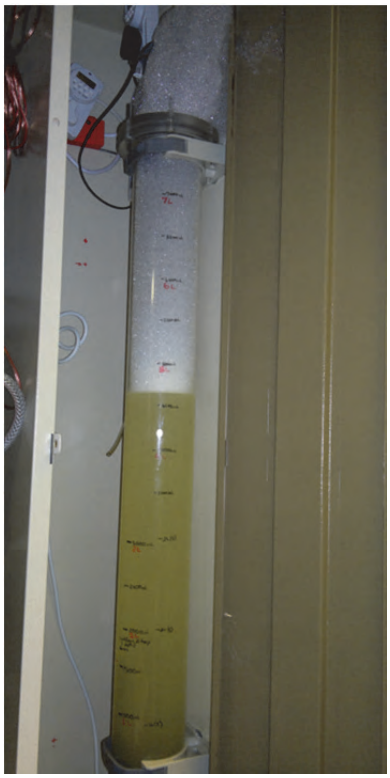


Figure 60 : Inoculated AGS reactor with synthetic media (6 g/L substrate) displaying excessive foaming.

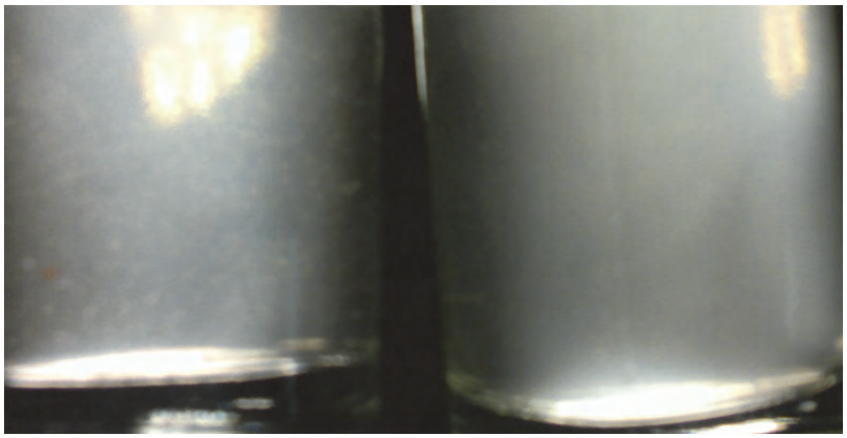


Figure 61: Broth after 24 hours of growth from the AGS (left) and RBC (right). Slight granulation can be observed in the AGS.

8.2.2 RBC on synthetic media

To illustrate that a biofilm can grow on the mesh disk of the RBC, a laboratory scale version was inoculated and operated with a 6 g/L substrate feed, under clean, but non-sterile conditions. There was excessive foaming (Figure 62, Figure 63), indicating a possible production of biosurfactants, enzymes and stress compounds. After careful operation, a tentative biofilm could be observed (Figure 65). At this substrate concentration and the mild disruption of the moving disk, most of the biomass remained in the liquid medium (Figure 64).

Figure 62: Growth of biofilm on mesh of rotating biological contactor.

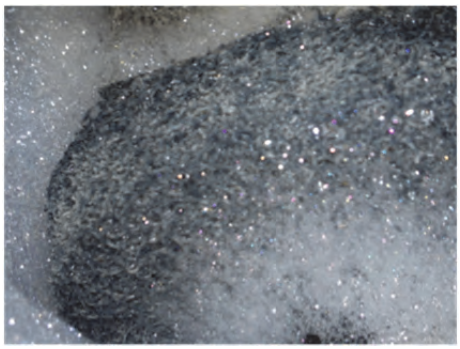


Figure 63: Close-up: Growth of biofilm on mesh of rotating biological contactor.

Figure 64: At substrate concentrations greater than 6 g/L, most of the biomass is still in the liquid medium.

Figure 65: Close-up: Growth of biofilm on mesh of rotating biological contactor with more dilute substrate feed. Note that much of the biomass is still in the liquid phase.

8.2.3 AGS and RCB on activated sludge

On moving the reactors on-site at Athlone WWTP, the reactors were first installed at site 1, using raw sewage mixed with activated sludge. This was expected to inoculate the reactors with the most appropriate bacterial population in the shortest time. The solid load was, however, overwhelming and both reactors were completely incapacitated after 24 hours (Figure 66). The reactors were promptly moved to site 2 where a raw, settled sewage stream was used instead.

Figure 66: The AGS reactor with activated sludge build up at bottom of reactor.

8.2.4 AGS on raw sewage

The initial results of the AGS when operated on raw sewage were encouraging. Granules formed from the sludge that was carried over from site 1 mixed with the raw sewage within 24 hours, as shown in Figure 67. These displayed a mixing pattern that conformed to expectations (Figure 68).

Figure 67: First days after start-up: The AGS reactor with raw sewage and granules looking possible.

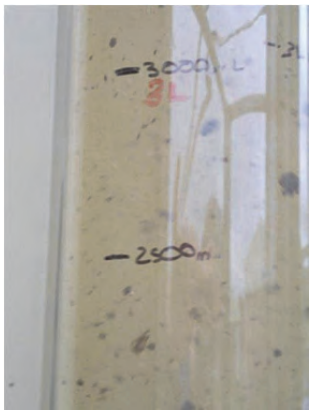


Figure 68: Close up of sludge forming granular particles in the AGS reactor.

It became clear that the mixing in the AGS reactor was not sufficient to prevent a build-up of sludge. Figure 69 shows the settling out of the fine sludge, and Figure 70 and Figure 71 show the reduced, black, anaerobic sludge. A new aerator was installed, but was still not sufficient. Initially the air was supplied through a ceramic disperser that produced smaller bubbles. Later this was removed to produce bigger bubbles with greater velocity. This improved the mixing somewhat, but the sludge still settled out and accumulated at the bottom after a few days.

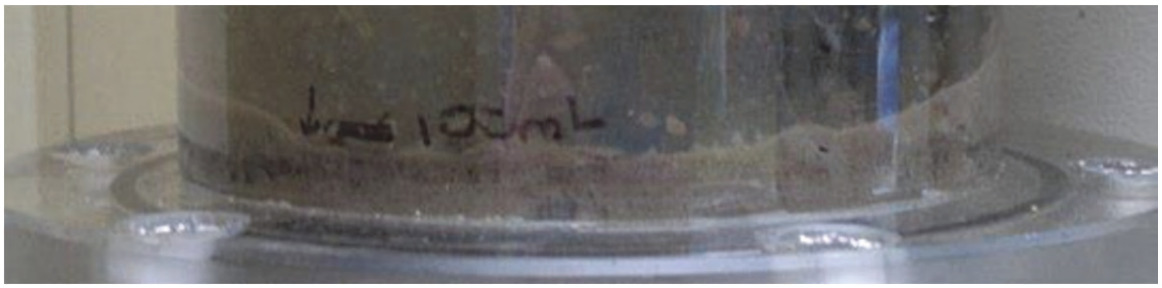


Figure 69: Close up of the settled sludge the AGS reactor.

Figure 70: Accumulation of sludge on top of the flow switch, and on the bottom of the reactor. Note the reduced, black, 'rotten' nature of the sludge.

After a few days of operation there was very fluffy growth on the sides of the reactor (Figure 72). This may be the due to filamentous organisms that plague activated sludge. Aggressive aeration combined with the reduced surface-bulk volume ratio may reduce this problem at scale.

Figure 71: Sample of sludge. No granules visible. Note the reduced, black, 'anaerobic' nature of the sludge.

Figure 72: Very fluffy growth on sides of reactor.

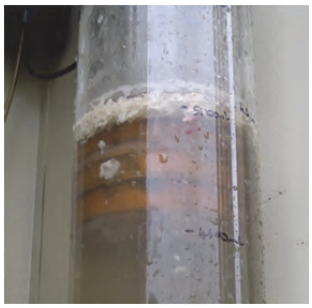


Figure 73: Inoculating the AGS reactor with the biofilm grown in the lab.

The initial granules that formed in the AGS did not remain. In order to inoculate the reactor with the desired organism, biofilm was cultivated in shake flasks in the lab and used to inoculate the reactor on site. The AGS has more promise than the RBC to be successful in this approach as it is a comparatively more controlled environment. Unfortunately, the tendency of the biofilm to seek the air-water interface resulted in the film floating and getting stuck on the floater (of the flow switch) and eventually disintegrated (Figure 73).

8.2.5 RBC on raw settled sewage

The initial aim was to grow an indigenous biofilm to be followed by altering the population dynamics towards the required organism. This worked well initially as shown in Figure 74 and Figure 75. Owing to the operational problems of the pump and supporting pipes quickly becoming clogged, the fluid flow to the RBC was limited, and drying impaired significant growth. In spite of this, the RBC still showed significant promise for biofilm production for bioprocessing, as these challenges can be overcome with adequate design (even at pilot scale) to take advantage of this simple, robust technology. Future designs should have wider diameter piping (at least 12 mm inner diameter), and a holding tank that ensures low-particulate loads entering the pump. Appropriate engineering design principles can be followed, within an achievable budget.

Figure 74: RBC mesh disks after a few days' growth on raw sewage.

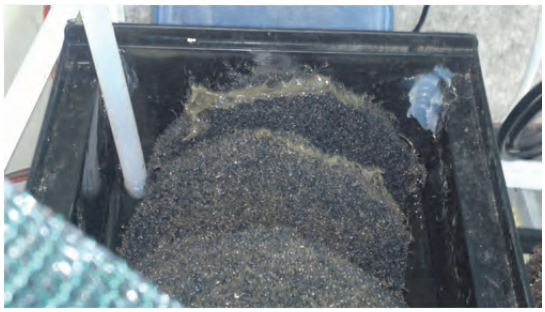


Figure 75: RBC mesh disks after a few weeks' growth on raw sewage. The biofilm visible at the back of the reactor was only observed on one occasion and remained for two days.

As the established biofilm present did not have a mucoid character, biofilm was cultivated in shake flasks in the laboratory and used to inoculate the reactor on site. The biofilm immediately moved to the mesh disk (Figure 76 and Figure 77) but remained for two days at best. Predation by macrofauna is considered as likely as natural die-off of the biofilm.

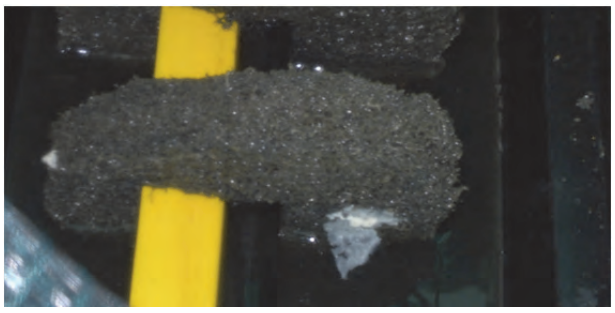


Figure 76: RBC mesh inoculated with biofilm grown in the lab. The biofilm showed a loose affinity to the mesh, but did not remain associated with it, and could easily be dislodged with agitation, as can be seen here.

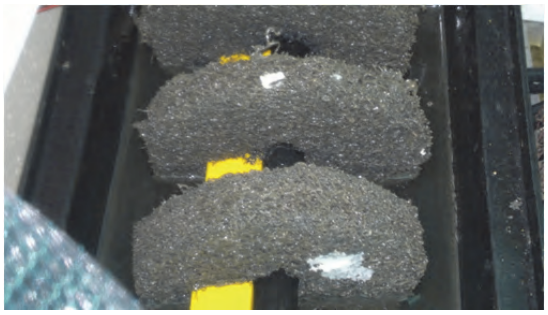


Figure 77: RBC mesh inoculated with biofilm grown in the lab.

The flow to the hRBC was reduced over an unsupervised weekend. This resulted in a very low flow rate through the reactor. Following this, an influx of worms was observed (Figure 78). The worms consumed the biofilm, resulting in poor biofilm structure remaining on the mesh, and this formed a loosely settling sludge as well. This macrofauna phenomena has been observed before (Biofilm Reactors WEF Manual of Practice No.35, p144), and they consider advantages of macrofauna being a reduction of sludge production, improved sludge settleability, and biofilm thickness control. In the context of a wastewater biorefinery, this represents a loss of product.

Figure 78: Worms in the RBC as a result of low flowrates (nuts on the right are 6 mm).

The motor fitted on the rotor shaft with a tight fit, and with operation and slightly uneven biofilm growth on the mesh the shaft slipped, resulting in uneven growth and eventually no rotation (Figure 79). This was a common problem in early RBC designs, but did lead to a distrust of the technology (Biofilm Reactors, WEF). The dry biofilm recovered quickly again if the shaft was rotated to submerge the dry section.

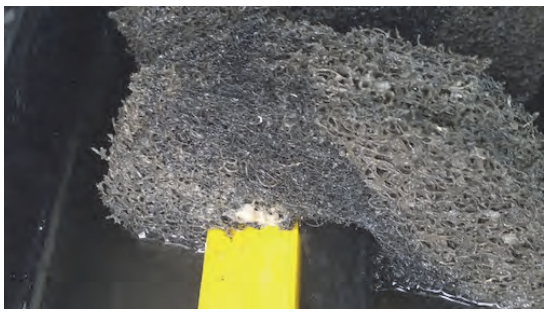


Figure 79: Biofilm growth on mesh causing imbalance on disk – a common problem in earlier RBC designs globally.

8.2.6 Improving oxygen transfer interface: Exploring trickling reactors

As the microbial strain used displayed a strong preference for growing at the air-liquid interface, trickle bed filters were investigated. Both the mesh used for the hRBC and a lightweight expanded clay aggregate (LECA balls) used in hydroponic agriculture were tested. With the trickle bed reactor illustrated in Figure 80, the bacteria still preferred to grow in suspension, at a substrate concentration of 6 g/L. No growth was observed on the LECA balls, while limited growth was observed on the mesh particles.

When the bacteria were grown on the mesh and LECA balls in standing culture (Figure 81) growth was significant on the mesh disks, but the LECA balls still did not illustrate good growth. Similar results were obtained on site with raw wastewater (Figure 82), although some algal growth could be seen on the LECA balls.

Figure 80: A trickle bed reactor with two support media alternatives, inoculated with synthetic media and Isolate 1 ‘: To the left is the mesh used in the RBC, and to the right is a lightweight expanded clay aggregate (‘LECA balls’).

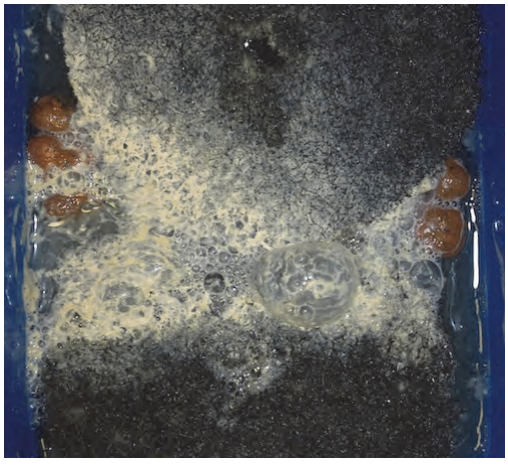


Figure 81: Standing culture to examine biofilm growth on mesh and LECA balls.

Figure 82: Examining the growth on a trickle bed setup with LECA balls and raw sewage.

8.3 General challenges

The challenges on site were mostly due to the particulates in the wastewater clogging up the pump, and the pipes providing feed to the reactor. The challenges did, however, illustrate quite a few potential opportunities to explore for bioproduction, some of which illustrated below. At site 1, where the raw sewage mixed with activated sludge was used, the sludge particles were a problem and hair a major irritation. At Site 2, more fatty substances were present in the feed stream. The fishpond filter became very greasy and this restricted flow in a matter of hours (Figure 83). This filter had to be removed entirely in the ongoing work. This grease could be a source of bioproducts as is, and could be explored as a processing option without any bioconversion.

Figure 83: Fishpond filter foam covered in grease and particulates.

The outer protective casing drew a fair amount of cotton buds, a common bane of the sanitation industry (George 2009) (Figure 84). In addition, there was an accumulation of a white, greasy substance, which could be soap particles, or polystyrene, or normal fatty substances (FOG – fats, oil and grease) (Figure 85).

Figure 84: Fishpond filter accumulating cotton buds – a documented ill in sanitation (George 2009).

Figure 85: Fishpond filter covered in grease and particulates. The white crumbs are either grease or polystyrene particles.

8.4 Reactor-specific challenges

AGS: The flow switch and the timer showed interference, leading to malfunction and maintaining the off position. When the timer was removed, the settling time was greatly reduced as the reactor would refill as soon as the flow switch level dropped to the low level, disturbing the flow and effectively disrupting the idle phase.

AGS: The air flowrate was not sufficiently aggressive to maintain tight granules. This could have been anticipated through de Kreuk's work (2006) on using aggressive aeration, and recycling the air to reduce cost and improve the overall oxygen utilization rate. The laboratory scale reactor used in this project will need significant overhaul to be able to achieve this, however.

RBC: The shaft junction requires modification to prevent the rotor shaft slipping.

8.5 Discussion

The wastewater used in this project was too dilute for efficient biofilm growth, which supports the argument for further water conservation and/or densification in urban sanitation.

The organism used in this project has a strong preference for growing at the air-liquid interface. It is expected that most organisms that produce a large amount of extracellular polymeric substances (EPS) that may be valuable for biopolymer or biosurfactant production, will prefer this interface, as it is the most effective use of their EPS, and effective access to oxygen in the air. In addition the bacteria grew on the plastic mesh, but not on the more polar LECA balls.

Future work is needed to explore reactor design that provides more opportunity for the biofilm to grow at the air-liquid interface, while still having efficient product recovery. Options at this stage are partially submerged fixed bed reactors, trickle tower concepts or similar. Apart from the requirement for adequate product recovery, macrofauna entrapment may also be a problem.

Due to an industry request, biosurfactant production was investigated briefly (Kosaric et al. 1984). Biosurfactant production using fixed film bioreactor processes looks to have huge potential, but it was specifically the downstream processing which uses flotation or other processes that was interesting to consider for bioproduct recovery from wastewater biorefineries.

The presence of macrofauna needs to be considered. A well-functioning treatment works has a diversity of microbial life as well as most components of the ecosystem, so the chemical or biocidal removal of macrofauna is not recommended. These macrofauna can be useful natural predators of pathogens, for example. Process design needs to maintain an adequate physical separation of, for example snails and worms, from the productive bioreactor.

Supplementation of the required metal salts, additional substrate and C:N:P ratio modification was not done, this should be included in future work. Altering the inoculum dose by boosters, which can include spores, was also not investigated. While this is a possible way to increase the desired bacterial population, the cost associated with this method of population control needs to be carefully considered.

The reactor piping needs to be re-designed for broader access that prevents clogging. The AGS reactor needs to have a different air supply. While the on-site experiments are more challenging than laboratory experiments, the learning is far more productive and the real life challenges can be addressed more realistically. The infestation of worms was, in retrospect, an obvious challenge that would never have surfaced in the laboratory.

An easier and more immediate way forward may be to analyse the biofilm in existing wastewater treatment bioreactors for polymer (or muciud characteristics) and biosurfactant presence (i.e. extracellular bioproducts).

9 Wastewater treatment works at the centre of a wastewater biorefinery: Potential synergies

9.1.1 Introduction

The proposed process in this project, to produce PGA-like extracellular polymeric substances from wastewater, is to be seen as a unit process in the context of a complete nutrient removal treatment works, and not expected to be the only treatment process employed. This is illustrated by a possible structure for the wastewater biorefinery given in Figure 86. In order to achieve legal effluent compliance and various other constraints, the various unit processes need to function well together, even if these are managed by different, independent, entities with different objectives.

There are two approaches to wastewater treatment. The first is to treat smaller amounts of more consistent wastewaters close to the site of generation, and the alternative is pooling all waste streams to a common receiving treatment works. In the latter, the treatment works is a receptor for wastes from a range of processes, forming a highly variable environment. The technology used within the wastewater biorefinery needs to handle inhomogeneity in terms of volume, concentration and composition. For example, it must cope in the face of backyard industries that do not classify their waste, process shutdowns causing perturbations of the typical waste stream e.g. the shutdown of metal finishing works over holidays, perturbations caused by deviation from specifications in industries upstream, seasonal or cyclic feed streams etc. Building in buffer capacity and resilient processes and systems is crucial. While a “predict and control” system is desirable to maintain a controlled environment, this is not possible. A “sense and respond” system that can work with dynamic non-equilibrium is preferred. A multi-‘barrier’ biorefinery utilising several unit processes with different strengths and weaknesses can achieve this robustness.

To understand this multi-component biorefinery, the potential synergies are considered around each of the major components: primary settling, bioreactor conversions and polishing units.

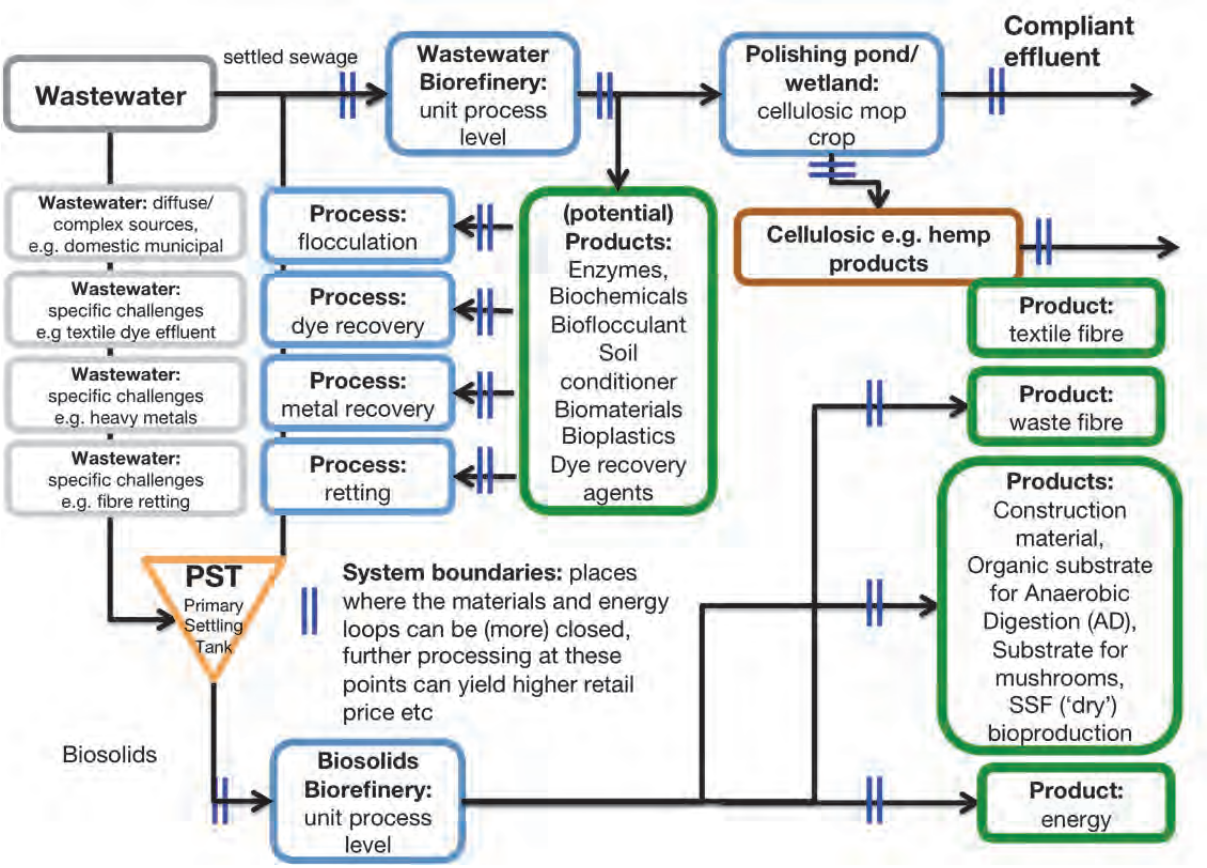


Figure 86: An example of a proposed biorefinery process structure

9.1.2 Potential synergies around the Primary Settling Tank (PST)

The conversion of the COD in waste-water to energy in the form of biogas, discussed in WRC report No. 1732/1/09 (Burton et al. 2009), is consistent with the concept of wastewater biorefineries. The increased temperatures during the biogas production and the periods of controlled anaerobic operation contribute to pathogen removal in the solid fraction. From a holistic perspective, using the entire waste-stream for biogas production has the disadvantage of removing readily biodegradable COD that could be better used for nutrient (e.g. N and P) removal. For the wastewater biorefinery, "waste to energy" is best considered in partnership with the biosolids from the primary settling tank (PST) unit operation, and not the settled sewage liquid stream (Figure 87).

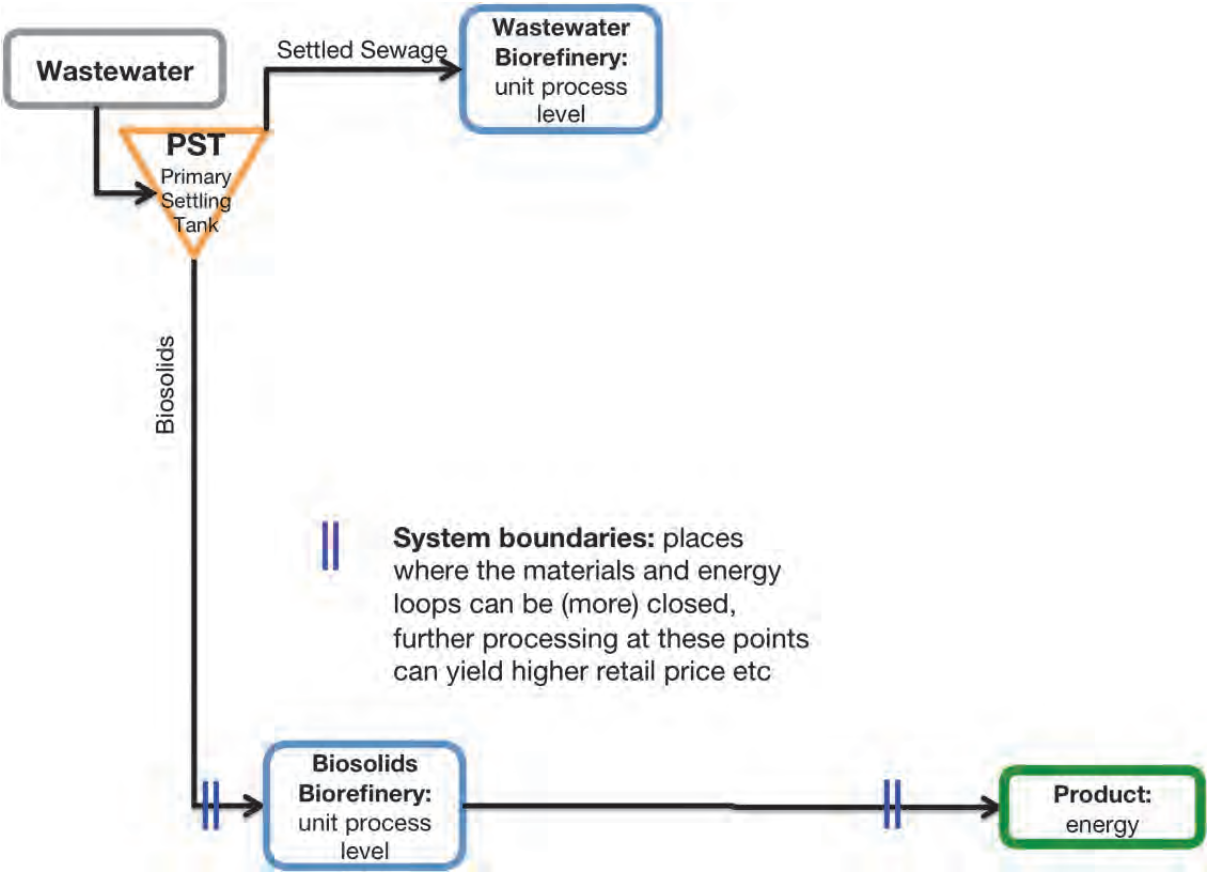


Figure 87 : Well established, but not always economically viable technology, industrial partnership: wastewater to biogas.

The biosolids can be useful for any bioprocess application that requires low water content, benefits from high carbon content and can deal with complex, poorly characterised substrates. Typical examples include biogas formation or composting. Solid Substrate Fermentation is a potential route for waste beneficiation, through, for example the use of mushrooms & other fungi. Composting is an unsophisticated example of this. Use of more sophisticated technologies creates a wider range of products (Figure 88), but introduces higher risk and complexity. In all cases, pathogen removal must be assured. The potential synergies around the primary settling tank are listed in Table 64.

Table 64: Opportunities for synergy – Primary Settling Tank (PST)

	Material flows	Opportunities for synergy
Material inputs	Nutrient rich wastewater containing high organic content biosolids	Combine waste streams with high organic content. High risk of contamination, but a possibility is to use the wastewater to wash landfill waste – increasing organic content of wastewater pre-PST, and producing rinsed landfill waste for further processing.
Material outputs	High moisture biosolids Nutrient rich wastewater with lower particulate content.	Biogas generation using anaerobic digestion (AD), Solid Substrate Fermentation (SSF). Composting.
Utility surplus	Biosolids, nutrient rich water	Nutrient rich water carried through to next unit process.
Utility demand	Adequately sized and designed tank	Retrofitting PST for AD and/or SSF application, appropriate energy source, appropriate mass transport, Can provide energy security on WWTW, low grade heat for adjacent biorefineries

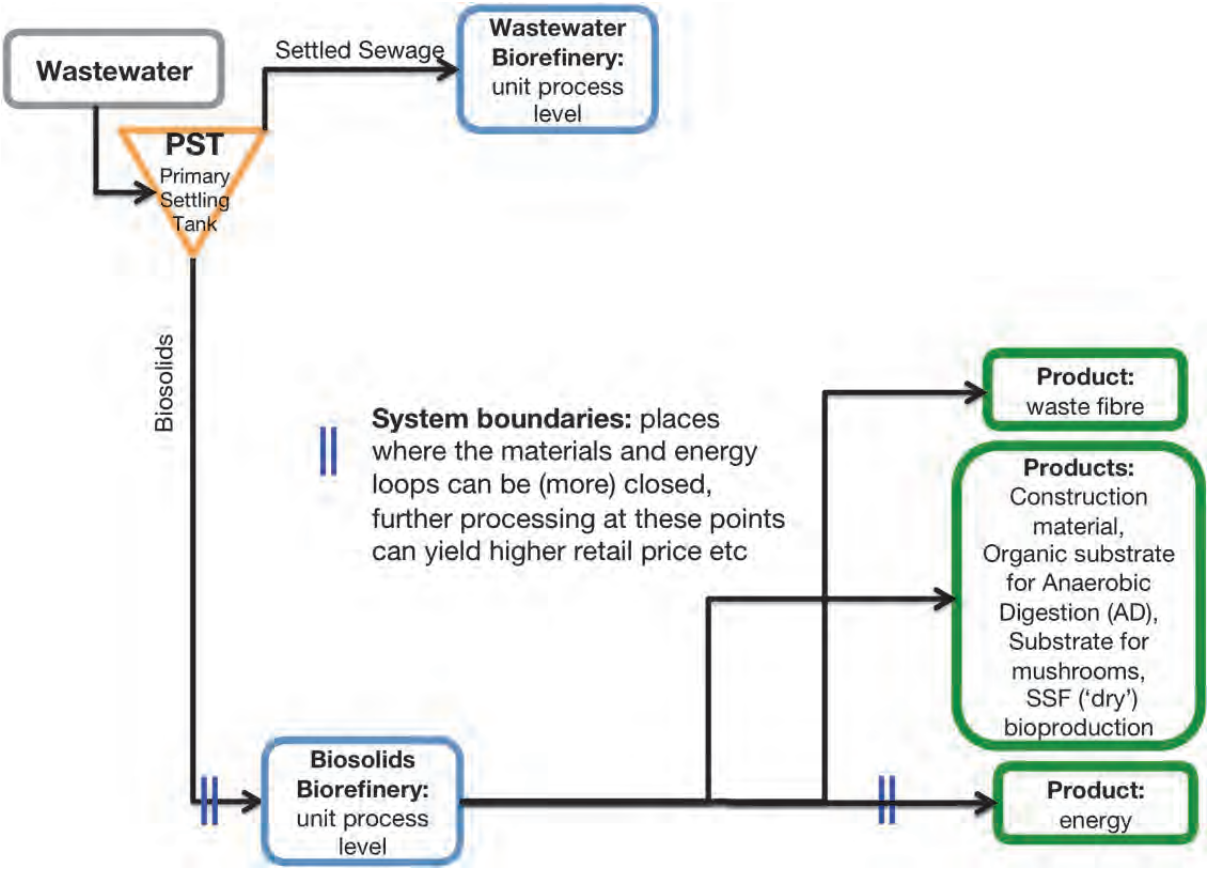


Figure 88 : More sophisticated, higher potential return technologies and partnerships using biosolids.

9.1.3 Potential synergies around the main bioreactors

Nutrient removal occurs in the main bioreactors, offering the opportunity for highly innovative environmental biotechnology. In terms of reactor design, this unit most resembles conventional bioprocessing. Hence it expected that this unit operation, in particular, will see much innovation in products obtained from wastewater (see for example the TEDxBinnenhof talk on youtube by Charlotte van Erp Taalman Kip 'How to turn wastewater into a goldmine'). The process to produce PGA-like extracellular polymeric substances from wastewater, proposed in this project, can be seen as a unit process in the context of a complete nutrient removal treatment works; it is not expected to be the only treatment process employed. In order to meet the output specifications of the treatment works in terms of legal effluent compliance and other constraints, the various unit processes need to function well together, even if managed by different entities with different objectives. This combined optimisation of unit operations contributes to process robustness. In order to achieve this integration across unit operations, bioreactor design is of key importance. It is anticipated that innovation is required in this respect as well, with novel reactor designs being preferred over conventional stirred tank reactors in order to facilitate the relative requirements of hydraulic and biomass retention times, augmentation of the desired microbial ecology and combined design of the production and downstream recovery processes. These concepts are described in Section 1. The concept is illustrated in Figure 89 while the potential to develop synergies is explored in Table 65.

The wastewater biorefinery has potential to be designed to produce products from the wastes treated that can, in turn, improve the running of the plant. Examples include flocculation agents, de-colorants as examples. This concept is illustrated in Figure 90.

Table 65: Opportunities for synergy – Wastewater bioreactor, from point of view of municipality / utility (current situation)

	Material flows	**Opportunities for synergy**
Material inputs	nutrient rich wastewater	
Material outputs	nutrient poor wastewater (aim for effluent compliance)	Effluent compliance harder to achieve – greater flows and more stringent environmental legislation
Utility surplus	gaseous / settled nutrients	Massive opportunity for products that can be produced with retained biomass (focus of this project)
Utility demand	chemicals (lime), compressed air, microbial biomass (biocatalyst),	adequate technology to address increasing wasteflows,

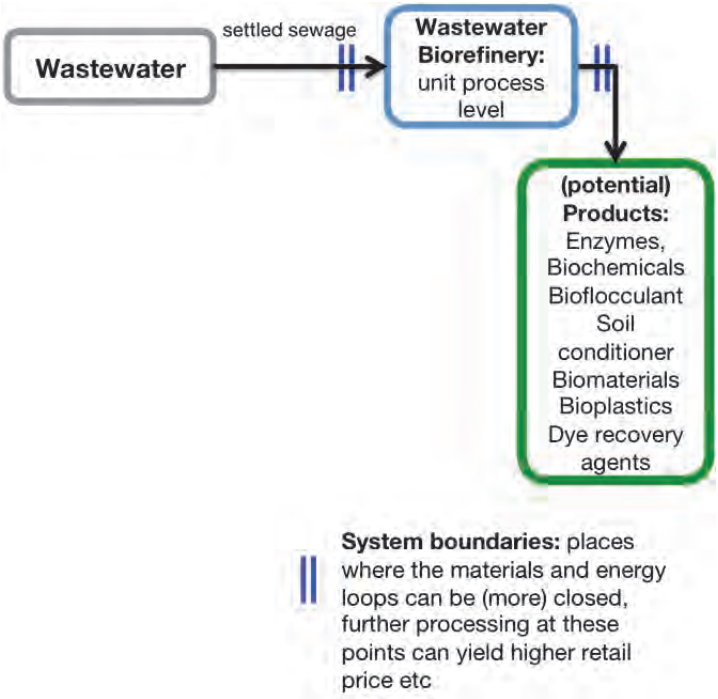


Figure 89: The bioreactor component of the wastewater biorefinery, focussing on the conversion of wastes to products or benign components

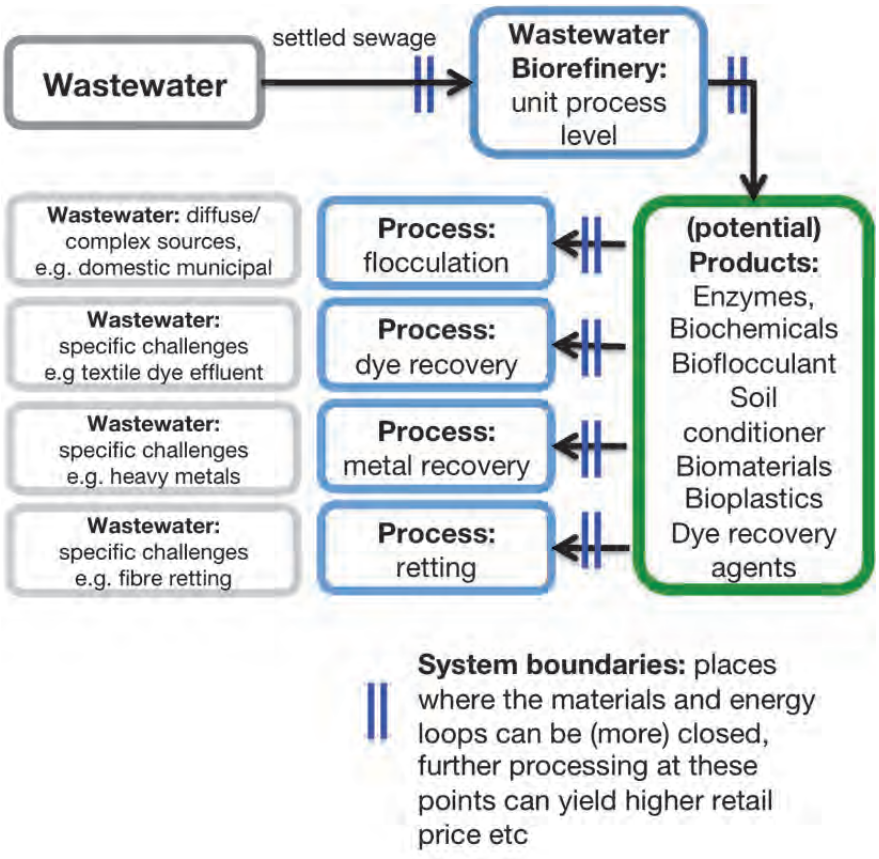


Figure 90: The bioreactor component of the wastewater biorefinery, focussing on the conversion of wastes to products with potential use in the WWTP to enhance its operation

9.1.4 Potential synergies around the polishing pond

In order to be able to design the bioreactor process operations to augment product formation, it is typical for the water stream exiting this section of the integrated process to still contain combined nitrogen and phosphorus as well as residual organics. Wetlands as tertiary treatment options are generating interest as a low maintenance treatment option that provides additional ecosystem services (Turpie and Malan 2010). It must be noted that wetlands are not maintenance free systems. Treatment wetlands as polishing ponds may be included in the waste-water biorefinery to act as a buffer against incomplete nutrient removal and risk management against plant failure in the preceding portion of the integrated processes. This enhances process robustness and the ability to handle surges in waste loads. Such systems are illustrated in Figure 91. In addition, the polishing step may assist in removal of particular nutrients, often N and P, which are not removed stoichiometrically with the carbon load in the bioreactor operations. Such polishing steps may also generate products of value, as illustrated here through the generation of biomass and biofibre products through production of cellulosic mop crops. Alternatively, algal polishing ponds may be used for the production of fertilisers, biomass or lipids for energy, bio-hydrogen and other products.

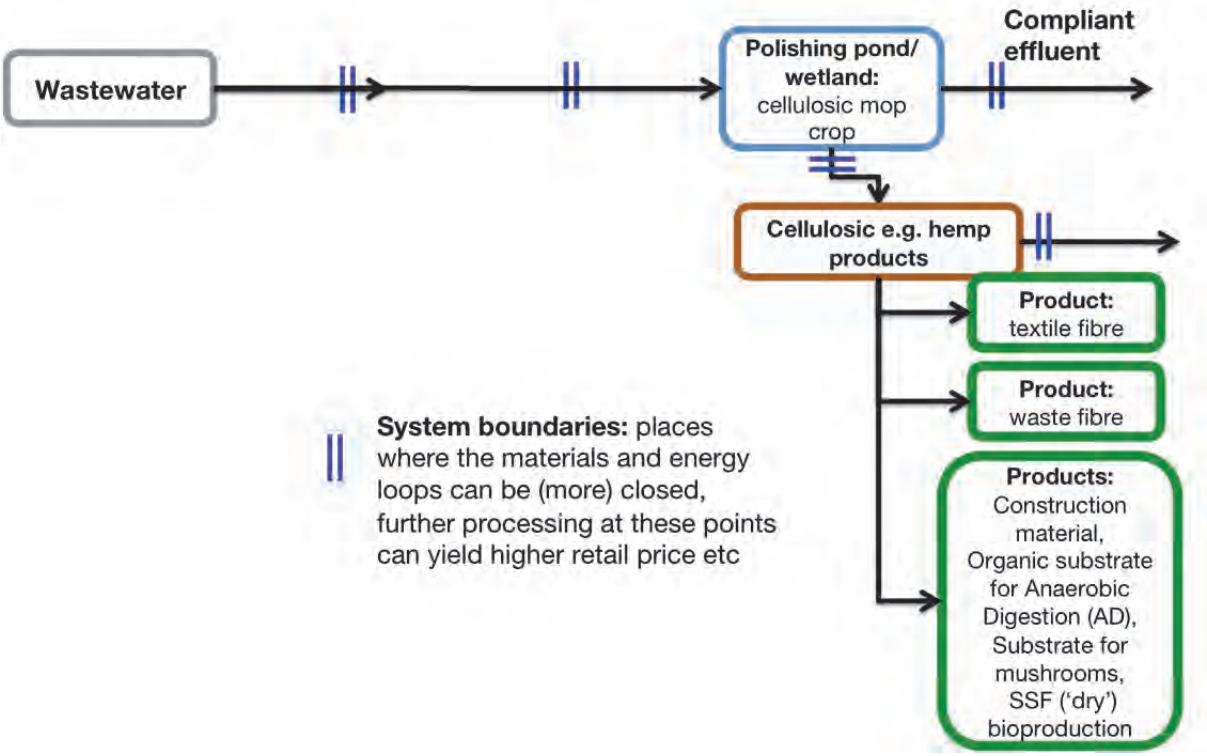


Figure 91: The polishing stage of the wastewater biorefinery, focussing on the removal of low concentration traces of wastes, with potential to combine this with production of products

9.2 Overview

The objectives in wastewater treatment are clearly defined to reduce contaminants in the water to an effluent compliant standard. In bioproduction the objectives are equally well defined as maximising product productivity to maximise return on investment. In the integrated biorefinery, illustrated in Figure 86, and discussed in terms of the main components above, the integration across these process strategies is illustrated.

10 Reflecting on Biorefineries: Stakeholder evaluations

In addition to defining the technical requirements of waste-water biorefineries as a move towards an industrial ecology approach, it is essential to understand the operational, logistic and social factors affecting their realisation. As a preliminary investigation of this, a series of interviews were held. These were supplemented by attending workshops, co-attended by practitioners to evaluate their openness to the concept as well as associated review of the literature.

10.1 Stakeholder interviews

The interviews were directed at practitioners who would be most affected by the legal and economic opportunities and repercussions of these industrial ecosystems as well as people who understand the potential impact on society. They represented an attempt to understand how the wastewater industry and related industries would respond to this concept.

The key question on which the interviews were focused was:

> ***'What are your views around industrial ecosystems with wastewater at the core – in our words, wastewater biorefineries?'***

The interviews were informal and free to take whatever course of discussion emerging. The following were interviewed:

Barry Coetzee	City of Cape Town
Brett Keyser	Stellenbosch Municipality
Anonymous	Customer services director of electricity utility (Feb 2013)
Rethabile 'Thabi' Melamu	PhD Student, University of Cape Town
Ger Bergkamp	International Water Association (IWA)
Ger Pannekoek	Netherlands Water Partnership (NWP)
Paul van Koppen	Netherlands Water Partnership (NWP)
Ulrike Rivett	Dept. Civil Engineering, Univ. Cape Town.
David Schaub-Jones	Sanitation development specialist, SeeSaw

The workshops attended included:

Water workshop, Africa Utility Week, May 2012
Waste management panel, Africa Utility Week, May 2012
Workshops associated with the WISA conference, June 2012
WISA Branch meeting, 20 Feb 2013

10.2 The Findings

10.2.1 The Short Answer: We don't know.

The outcome of the interviews with industry practitioners around this question can be summarized by Barry Coetzee's (City of Cape Town) quotes:

> *"This is a young industry and no-one really knows where it's going yet. Very few are willing to stick their neck out. We're integrating systems that were not integrated previously. This represents a huge risk."*
>
> *"There is an opportunity for waste beneficiation, of viewing waste as a resource. Combined with the increased need for water reuse, and more stringent nutrient removal needs, recycling (of all sorts), already a valuable industry, becomes even more attractive."*

In general all those interviewed felt very positive about this approach, and that the time is right for implementation. The various factors supporting this are outlined below. They do share a note of caution, expressed by Barry Coetzee as follows:

> *"The reality of treatment works is that they are a receptor; this is not a controlled environment. The technology in use needs to cope in the face of e.g. backyard industries that don't classify their waste and just flush it down the drain, shut down of metal finishing works over holidays etc."*

For practitioners to accept the biorefinery concept, assurance around the risk aspects and technical aspects are needed, as well as a clear value offering of the product.

10.2.2 Evolving needs of wastewater treatment creates urgent opportunities

With population growth, economic growth and increasing resource scarcity, the water demand has been increasing exponentially, yet the funds available for treatment are seldom sufficient. In addition, long-term environmental degradation has resulted in the ecosystem services provided by the natural buffers shrinking. This leads to the requirement for more engineered solutions to provide more intensive treatments. External factors like eutrophication, topsoil loss and other diffuse non-point sources make the conventional organic waste management approaches less suitable. At the same time, resources that have previously been mined or manufactured from virgin raw materials must now be sourced from resources of poorer quality, increasing their cost and associated waste burden.

More sophisticated and holistic strategies are therefore needed that work with more dilute and complex resources. By addressing these two linked challenges at the same time, definitive economic, social and environmental advantages are expected (Figure 92). One potential route is to create a more cyclical system, where the wastes are beneficiated and re-introduced into the system as resources.

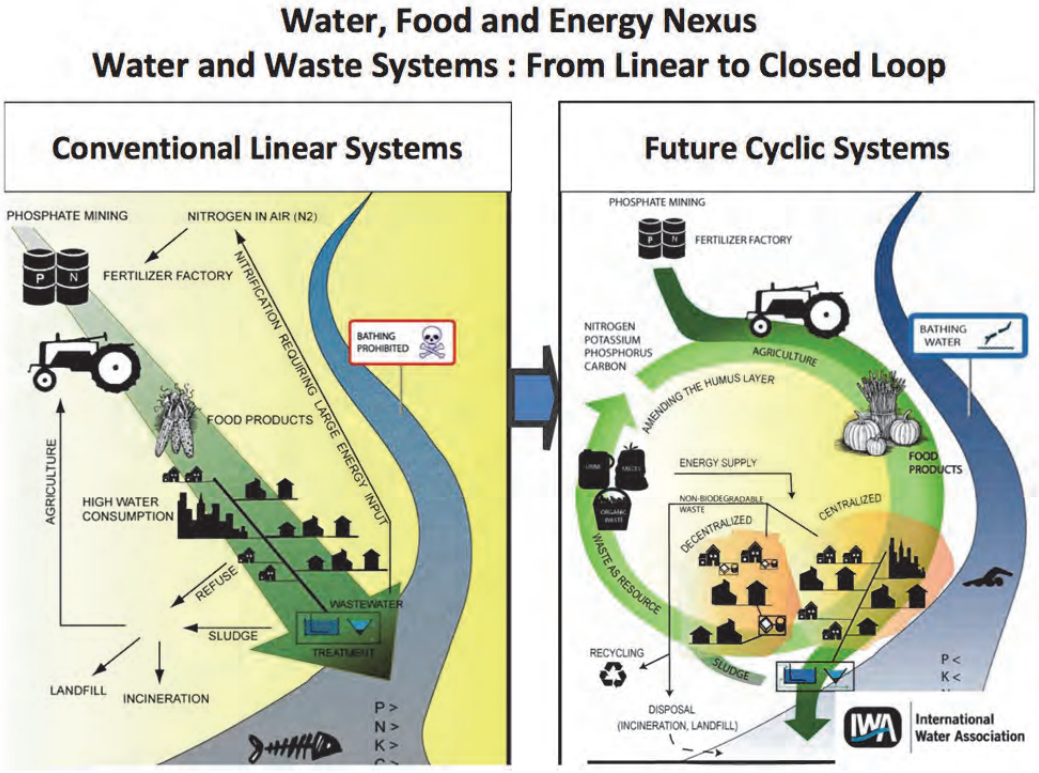


Figure 92: Moving from a linear to a closed loop (Ger Bergkamp, African Utility Week keynote presentation, May 2012).

Ger Bergkamp (African Utility Week 2012) articulated that in order to achieve this cyclic system, water needs to be used in a different way, rather than just reducing volume, requiring:

- Progressive closing of the loop of urban water and treatment – major increases in water use efficiency, energy and nutrient recovery
- A conscious and systematic rethink around water infrastructure, especially the water systems supporting cities
- Joint city and water planning, currently rarely seen anywhere in the world, and
- Opportunity for joint optimization of city needs with the larger scale environment (agriculture, energy, watershed services).

The sustainable natural ecosystem has developed over the ages, starting from an open system as life began on earth in an environment of excess resources. The excess 'wastes' led to a gradual change in the earth's environment, through an era in which limited material inputs as well as energy resulted in limited wastes, to finally develop into the closed system (from a material standpoint) sustained by the input of solar energy (Graedel & Allenby 1995).

Building on this point of view, the resource scarcity and excess 'wastes' that are experienced currently, is leading to another change in the earth's environment. The apparent rapid rate at which this is occurring requires equally fast innovation in our

approach to either reduce resource scarcity or the waste burden, in order to reduce pressure to change or to more easily adapt to the changed system.

10.2.3 Wastes as a critical component in systems approaches

Shannon Royden-Turner (2012) noted, in the context of material balances in informal settlements, that (quote paraphrased to deal with wastes):

> *"[Wastes] are an asset within the city that not only provide affordable [resources] to low-income [entrepreneurs], but also contribute towards the improved environmental sustainability of Cape Town, through the creation of cascading and closed loop material flows, as well as local economic opportunities specifically related to the informal [manufacturing] sector"*

The current applicability of implementing more "circular cities", or a more integrated 'ecosystem' of resource use, is clearly evident as outlined above. Further, the available technologies and favourable paradigms are also better suited to this development than previously. Some of the factors influencing this include:

- The financial crisis of 2008's promoted the use of resource sharing, and cemented the trend of an economy moving away from ownership to services (Botsman & Rogers 2010). The crisis has also provided a renewed focus on job creation, and the political drive towards 'green jobs';
- There is a greater focus on sustainability, and the decentralized approach that promotes this;
- In order to achieve greater decentralisation, a different way of communication is required, along with a flatter hierarchy, greater openness and greater community ownership. The resource sharing, often called the collaborative economy, is developing this structure and paradigm (Botsman & Rogers 2010). Its application is particularly well suited to the principle of inclusivity encouraged in the business environment in South Africa and the strong focus on entrepreneurship;
- The masses of information generated through these more decentralized approaches need to be integrated. This is now possible through developments in the information and communication technologies (ICT); and
- The natural cycle of capitalism is now in a phase favourable to embrace a new technology revolution (Perez 2003) (Figure 93).

Donella Meadows (1999) lists 12 leverage points to intervene in a system, with the power to effect a paradigm shift the most effective one to change a system. While constants, parameters, and numbers (such as subsidies, taxes, and standards) are strong incentives to drive behaviour and can be used to influence a change in the system, they are not capable of changing the mindsets inherent in the behaviour. A big driver influencing mindset, and thereby instigating system changes, is the realization that the size of buffers and other stabilizing stocks, relative to their flows, are decreasing, pushing up raw material prices and waste treatment costs. These

changes include greater awareness of the resources used, greater awareness and action on companies treating their own water, etc. However, the change is currently generated in an *ad hoc* way and largely in isolation to other industry players. At times, it is even done in isolation of other components operated by the same industrial player.

In addition to the direct components of integration, a closed cycle is influenced by the availability of information between systems, transport between systems and the workforce available. The structure of material stocks and flows (such as transport network, population age structures) influence trade, and in South Africa, with the degrading infrastructure, skills shortage and a young population, is a significant driver to change how products are transported, inter-relate and are consumed.

Greater leverage, in terms of stabilising the ecosystem, can be gained from quick response times relative to the rate of system changes, giving competitive advantage to responsive players and placing them in a position to drive the system. Logistics developments globally, and social media in particular have increased the ability to respond and adapt faster. Response to the environment with self-correction is critical. The strength of negative feedback loops, relative to the effect they are correcting against is thus a large factor in changing the system, with the related gain around driving positive feedback loops.

Operation of closed systems is dependent on the quality and trustworthiness of information. The internet and greater information literacy have influenced the structure of information flow (who does and does not have access to information of differing types) which is changing to benefit viable ecosystem economies. Previously, knowledge was asymmetrically distributed. The open source platform and social media platforms are increasing its symmetry, allowing a different set of competitors and different types of players to compete. This changes the operation of the system and its rules (such as incentives, punishment, constraints; implicit or explicitly) and correlates with Perez's (2002) assertion of the lag time in institutional response to technology revolution, discussed later in this document.

The most powerful way to leverage change in a system is to devolve power to add, change, or evolve the components of the system to a wider base *i.e.* to decentralize or allow the system structure to self-organize. Such devolution has potential for chaotic outcome. In order to allow a decentralized system to move in a coherent way, the goal of the system needs to be clearly defined and communicated – in terms of a vision. To enable that vision to permeate the system effectively, a mindset or paradigm need to be embodied, from which the goals, structure, rules, delays, parameters of the system arise. Finally, in order to achieve this, the power to transcend paradigms is required. This is very difficult to achieve, and is an emergent property rather than a designed one, and correlates with "revolutions" – the industrial revolution, and the IT 'tech' revolution being examples of paradigm shifts. The 1960s hippie and feminist movements are other examples of transcendent paradigms. These points to intervene in a system are summarised in Table 66.

Table 66: Leverage points to intervene in a system (Meadows 2012)

Donella Meadows: Leverage points to intervene in a system (from least to most effective)
1. Constants, parameters, numbers (such as subsidies, taxes, standards) 2. The size of buffers and other stabilizing stocks, relative to their flows 3. Structure of material stocks and flows (such as transport network, population age structures) 4. Length of delays, relative to the rate of system changes 5. Strength of negative feedback loops, relative to the effect they are trying to correct against 6. Gain around driving positive feedback loops 7. Structure of information flow (who does and does not have access to what kinds of information) 8. Rules of the system (such as incentives, punishment, constraints) 9. Power to add, change, evolve, or self-organize system structure 10. Goal of the system 11. Mindset or paradigm that the system – its goals, structure, rules, delays, parameters – arises from 12. Power to transcend paradigms

Carlota Perez (2002) argues, in turn, that the prevalent paradigm is inherent in the current technological revolution, and that with technological revolutions come paradigm changes, as shown in Figure 93. Over the last century we have experienced the ages of oil and mass production followed by the age of information technology. She further argues that we are on the brink of a new such technological revolution (Figure 94).

Running out of resources is sensitizing people to the need for the next “revolution”; this needs to have a very cheap input that can be used for a wide variety of things, and create a new infrastructure that deepens and widens markets (Perez 2002). In order for a technology or innovation to be the big bang that starts the next revolution, it needs to propose a solution to the resource scarcity – not to do more with less, but achieve success, or development, in a fundamentally different way. Addressing resource productivity and our use of natural capital are projected to form a key aspect of the next paradigm with biotechnology, biomaterials and nanotechnology as potential players. The role of the integrated handling of waste-water through wastewater biorefineries has potential to contribute to such a paradigm.

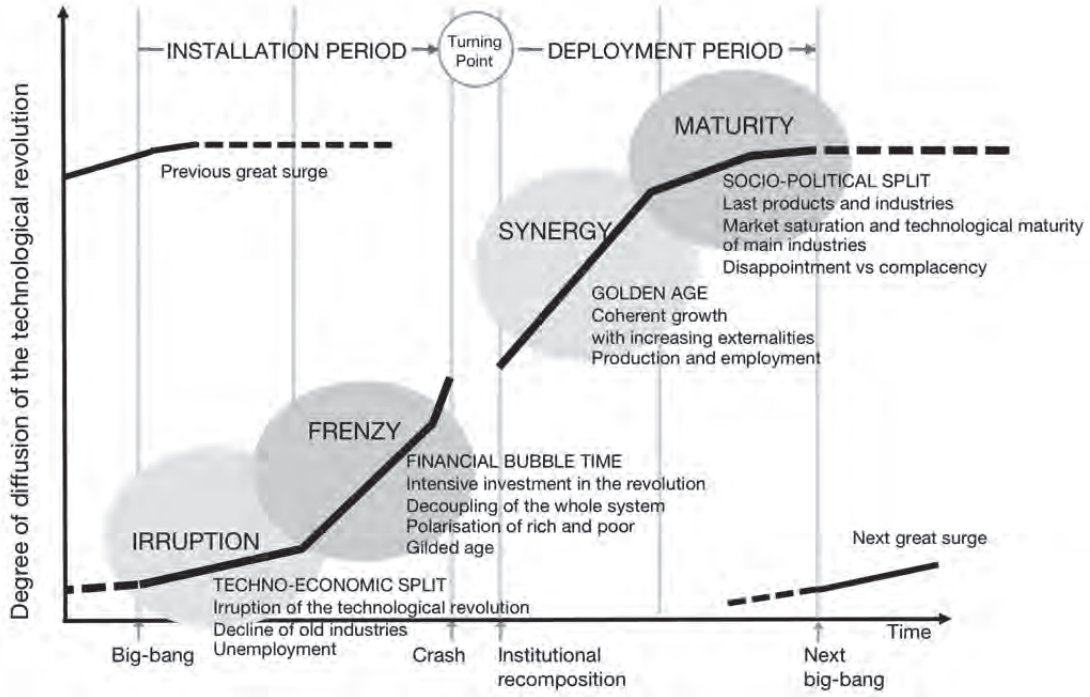


Figure 93: Recurring phases of each great surge in the core industries (adapted from Perez 2002)

Great surge	Technology revolution Core country	Year of big-bang	Big bang *initiating* the revolution	INSTALLATION IRRUPTION	 FRENZY	Turning Point	DEPLOYMENT SYNERGY	 MATURITY
1st	**Industrial revolution** Britain	1771	Arkwright's mill opens in Cromford	1770s and early 1780s	late 1780s and early 1790s	1793 - 97	1796 - 1812	1813 - 1829
2nd	**Age of steam and railways** Britain (spreading to European continent and USA)	1829	Test of the 'Rocket' steam engine for the Liverpool-Manchester railway	1830s	1840s	1848 - 50	1850 - 1857	1857 - 1873
3rd	**Age of steel, electricity and heavy engineering** USA and Germany, overtaking Britain	1875	The Carnegie Bessemer steel plant opens in Pittsburg, Pennsylvania	1875 - 1884	1884 - 1893	1893 - 96	1895 - 1907	1908 – 1918*
4th	**Age of oil, automobile and mass production** USA (Spreading to Europe)	1908	First Model-T comes out of the Ford plant in Detroit, Michigan	1908 – 1920*	1920 - 1929	1929 – 33 Europe 1929 – 43 USA	1943 - 1959	1960 – 1974*
5th	**Age of information and telecommunications** USA (Spreading to Europe and Asia)	1971	The Intel microprocessor is announced in Santa Clara, California	1971 – 1987*	1987 - 2001	2001 - ??	20??	??
6th?	**Age of ecosystems, natural capital** Netherlands?	2008?	Aerobic granular sludge process in Delft, the Netherlands links production and waste management	20??				

big bang — Crash — Institutional recomposition

* Observe phase overlaps between successive surges

Figure 94: Approximate dates of the installation and deployment periods of each great surge of development (adapted from Perez 2002)

Because the industry is new, the risk and associated barrier of entry is high. It is necessary to justify to practitioners that the uncertainty is worth exploring and the investments are worth the risk.

The constraints to establish a wastewater biorefinery include:

- Large pieces of land are often available, but are required for forward planning and plant expansion;
- The proposed biorefinery effectively competes with the normal functioning of the works. A plan is required for the new and conventional components to function side-by-side with scalable results.
- Unit processes need to use entire material flows, not small fractions of the stream; and
- Institutional and legal constraints need to be met (Barry Coetzee 2012).

In order to respond to this uncertainty, it is necessary to provide case studies to demonstrate the approach and provide material for informed debate. Further, the product spectrum produced must address a market need. As indicated above, it is not useful to utilise only a sub-set of the waste stream so the product selected must be matched to the volume of raw material (waste-water) available and its organic content i.e. commodity products are applicable, whereas high and medium value products are not. This has led to the consideration of polymers, plastics, soil additives and energy products, to name a few. The acceptance of the market need of the product may be enhanced by selecting a product of use in water treatment, such as a flocculant (provided the scales are matched).

It is expected that the product spectrum can be diversified to multiple products, provided economy of scale is maintained and the multiple products enhance process robustness. One such product may be energy, however energy products do not cater for the removal of N and P, hence a complementary product is required. This is currently proposed by the separation of the bio-solids for digestion to energy, the organic rich liquid phase for commodity products such as PGA and the polishing step for removal of N, P and trace organics to a third product type (e.g. biofibre, biomaterials, fertilisers).

The re-use of nutrients from waste-water has been largely de-coupled through the centralisation of cities; however it remains essential for the move towards a closed ecosystem. This suggests that WWTP should remain as de-centralised as possible. Further, the scale of the WWTP of small towns may be more appropriate as a testbed for case studies on the application of biorefineries. Looking forward to their roll-out to city treatment works, the concentration of nutrients such as N and P for return to agricultural lands may be required.

10.3 Building new wastewater biorefineries or retro-fit existing treatment works?

In addressing whether wastewater biorefineries should be developed as new greenfields project or retro-fitted into existing complexes using a brownfield approach, the benefits of the latter are widely accepted to outweigh a top-down approach in practice. Co-location of symbiotic plants through policy decision is rare and seldom achieves maximal environmental benefit (van Leeuwen et al. 2003, Ster and Ott 2004). The introduction of incentives, facilitation and promotion instruments provide the most impact towards achieving industrial ecology principles. This is further supported by the infrastructure already available to the site e.g. transport of wastes to the site (Interview with Barry Coetzee, City of Cape Town). Van Berkel (2006) quantifies the impact of facilitation and promotion as four fold more effective than greenfields planning, although every case will have a different impact.

10.4 Thinking wider: What is needed to implement an ecosystem economy

The potential of an ecosystem economy and the use of these principles in handling of wastewaters, allowing the establishment of wastewater biorefinery principles, rely on the establishment of several facilitators. These are discussed below.

10.4.1 Economically viable attractors

> *"It is suggested here that for society to veer strongly in the direction of a new set of technologies, a highly visible 'attractor' needs to appear, symbolizing the whole new potential and capable of sparking the technological and business imagination of a cluster of pioneers. This attractor is not only a technological breakthrough. What makes it so powerful is that it is also cheap or that it makes it clear that business based on the associated innovations will be cost-competitive."* (Perez 2002)

Key elements which are expected to be found in such attractors are:

- The capacity of the technology to contribute to nutrient removal and hence wastewater treatment, while providing robustness against system shocks
- A modular nature enabling ease of application from a biorefinery perspective,
- The ability to concentrate the nutrients into biomass that can easily be recovered and processed into valuable bioproducts, or concentrate the nutrients into readily recoverable products directly.
- The emergence of the 'collaborative economy'
- The ability to contribute across the product spectrum – clean water for re-use, saleable products, energy products – in which products are selected on their demand and a matching of their scale to the resource scale

- The inter-dependence of partners.

There have already been initiatives to drive development of closed systems already set up and it is valuable to address factors limiting their rapid uptake. The best known, ZERI – zero emissions research institute (www.zeri.org), works towards finding valuable products that can be produced from waste. These initiatives have seen limited reach, however. One possible reason for this is that many of the products have niche markets i.e. they are high value, small volume products, for example pharmaceutical or neutraceutical products, which do not absorb the wastes they aim to reduce significantly. The advantage of these products is that their higher value can subsidise lower value bulk products like compost or biogas. This subsidization on its own is not sufficient to affect or reverse the massive scale of production that lead to resource scarcity in the first place. In order to achieve that, production methods need to be developed that use this waste to supplement the demand of the products currently produced, resulting in a tighter materials loop, as well as adapting the logistics and industrial partnerships related to these. Demand side management, and systemic thinkers in planning and management is also needed (von Blottnitz 2011).

10.4.2 Systems analysis

On establishing an integrated biorefinery, the impact on the surrounding environment must also be evaluated. Similarly, inside the biorefinery, each intervention must be evaluated in terms of its effect on units downstream, as well as how it affects the community. The initial beneficiation of the Newlands brewery wastewater can be used as example. The wastewater contained high levels of biodegradable COD, which the brewery was being penalized for because it was over the industrial COD limit. It was thus in the brewery's interest to reduce the COD. The nature of the stream made it suitable for energy recovery. The economic and environmental value of the combined reduction of outgoing waste and the supplementation of the energy requirement was clear (Cohen 2006). However, to ensure robustness in the light of outages, the wastewater stream is still transported to the Athlone wastewater treatment works 14 km away. Full environmental benefit will be attained when the outgoing water stream that meets discharge specification is used as an irrigation water source in the surrounding community, providing value to the community. From the Athlone WWTP perspective, the high organic load, while incurring fees to the source, was beneficial to the treatment works to reduce the Nitrogen and Phosphate nutrients through biological means on the plant.

As in all "energy from waste" scenarios, it is essential to understand the role played by the waste resource diverted to energy or product formation in a holistic manner (Barry Coetzee 2012).

Separation of wastewater streams at source enables better use of waste streams and can be better regulated. Through industrial partnerships, it is possible to translate benefits of source-separation from the benefitting partner back to the

partner who needs to do the separating. Without such a network, there is no incentive for the initial separation, preventing its occurrence. Such separation at source may facilitate treatment at source with potential for significant reduction of environmental burden of transport (Cohen 2006).

The need for the contributors to water treatment and use of water as a resource to work collectively or collaboratively is clear. To facilitate this, the differing operating premises of the municipality and private sector need clear recognition (Coetzee 2012). Municipalities are constrained by budget availability and the absence of "profit-based" income generation. Privately run utilities like Rand Water can work on a more business-like 'profit' base where investment can be offset against feasible returns. A public-private partnership (PPP) can cover the shortfall from the municipality's side, where the municipality has to deliver a compliant effluent, and the industrial partner enables this, but with the freedom of using the recovered nutrients as they see fit. Alternative approaches are possible through scoping of potential, e.g. value from solids waste, and motivating significant changes to waste management systems and infrastructure by considering alternate methods or technologies to introduce minimization. Such an approach may introduce alternatives for management of facilities, new technology, skills and efficiency. Effect on service delivery, sustainability and affordability require consideration. In the arena of the public service provider this can be considered through a scope of work / tender process, provided budget is available.

10.4.3 Local context – decentralisation

In natural ecosystems, there is a substantial recycle component, facilitated by the proximity and functional matching of producers, consumers and decomposers. Distance between producer consumer and decomposer imposes travel costs and energy, thereby reducing the recycle flow (Graedel & Allenby 1995). Producers, consumers and decomposers who are in close proximity to each other (in terms of physical distance, but more importantly in response time) can respond to demand and supply ebbs faster (Harrison 2007). It is these interactions that need to be nurtured for the closing of resource cycles related to water.

In Desrochers and Sautet (2008) the emergence of innovative practices and behaviour is supported, including the development of inter-industry linkages and new combinations of existing technologies and materials ("Jacobs externalities"). They position entrepreneurs as a key component:

> "*The issue is not that regional specialization policies are developed at the expense of spontaneous industrial diversity. Indeed, the two can coexist. Rather, we argue that entrepreneurial activity is at the source of regional development and that theory and evidence seem to indicate that spontaneously developed industrial and economic local diversity typically provide a better substrate for entrepreneurs to innovate."*

A key aspect of decentralisation is the move away from central control which adds the challenge of insuring that regulations are met while at the same time facilitating innovation. Further the exploitation of resources in the wastewater in the geographical area of their generation facilitates the closing of cycles critical for sustainability (Harrison 2007). Interesting aspects influencing this approach are reflected in the following quotes:

> *"[a wastewater biorefinery concept] more focused on facilitation than object creation, on transitioning from consumption to participation. The consumer moves from being a passive receiver to an active participant"* (Botsmans & Rogers 2010).
>
> *"The convergence of social networks, a renewed belief in the importance of community, pressing environmental concerns and cost consciousness are moving us away from top-heavy, centralized and controlled forms of consumerism towards one of sharing, aggregation, openness and cooperation"* (Botsman & Rogers 2011).
>
> *"With the advent of computers, and the internet, large pyramids now appears rigid and clumsy. In its place, the decentralised flexible network structure, with a strategic core and a rapid communication system, has shown its capacity for accommodating much larger and more complex global organizations as well as smaller ones"* (Perez 2002).
>
> *"One of the most interesting stories in social change today is how much creative problem-solving is emerging from citizens scattered far and wide who are taking it upon themselves to fix things and who, in many cases, are outperforming traditional organizations or making systems work better"* (Fidelman 2012)

If stakeholders can be engaged in directly participating in the beneficiation, and appropriate management of their wastes, with appropriate control, the circular economy becomes easier to achieve. At the very least, this can provide a more viable model to contribute to the closing of materials and energy cycles. The technology that enables wastewater biorefineries is a necessary prerequisite, but is not sufficient. In order to promote this, it is necessary to take note and develop the underlying principles that allow individuals to engage in complex behaviour in a coordinated way. Ideally, a wastewater biorefinery can achieve appropriate systems-wide value, simultaneously with appropriate decentralisation.

10.4.4 Knowledge diffusion for small scale and large scale plants

From discussions with Brett Keyser, Stellenbosch Municipality (held at the time of the WISA Branch meeting, 20 February 2013: Wastewater Treatment Plants outside of the Urban Edge: Package plants – The shortcomings, risks and solutions, as seen from a municipal perspective), the potential for "package" wastewater treatment plants smaller than 20 ML/day to contribute to this ecosystem economy through

recovery of valuable products appears significant. His recommendations on adequate wastewater management, presented at the WISA branch meeting (20 Feb 2013), focussed on the treatment plants outside the 'urban edge' (the reticulated areas) and the need for good information flow. These are summarised below:

- Apply Green Drop principles for Package Plants, an example of which is currently being developed through SEWPACKSA (represented at this meeting by Spike Bekker)
- Use web-based databases
- Rewrite bylaws to allow for more appropriate package and 'pocket' sized treatment plants
- Rewrite development policies and guidelines to allow for adequate waste management of new urban developments,
- Apply water conservation and Water Demand Management i.e. consider water holistically
- Use role-plays to find solutions – legalities, development contributions etc. These can include role-plays from other sectors that had similar challenges
- Apply this approach to current problem areas, allowing ongoing learning, rather than only applying to new developments.

It is well-accepted that in provision of services to the community, which accounts for a sector of wastewater treatment, consultation and stakeholder engagement is important. Keyser (2013) and Botsman and Rogers (2011) recognise the need for participatory, action-oriented research to develop how customers can interface with the infrastructure currently involved in service delivery and investigate a route to creating communities engaged in waste management rather than passive observers. This requires utilities to become:

> *"more focused on facilitation than object creation, on transitioning from consumption to participation. The consumer moves from being a passive receiver to an active participant"* (Botsman & Rogers 2011, p188).

While the discussion above has focussed on small scale wastewater treatment works, works of any size are expected to gain from being on a web-based database, both in terms of monitoring the performance and maintenance of these plants as well as obtaining information about them for accreditation, for example (Keyser 2013). There was also agreement that the building of works is less of an issue than the continued involvement in maintenance, and stakeholder engagement once the project is running. Improved knowledge diffusion would not only improve the potential of industrial partnerships to move towards an ecosystem economy by allowing identification of opportunities for combined treatment and product formation, but also allow faster and more effective action to respond when there is a disruption in the system. Van Berkel also concludes that

> *"…facilitation and promotion instruments are approximately four times more effective in achieving resource synergies than planning approaches."* (van Berkel 2006).

The following guidelines should support the strategic design process in order to facilitate access for a larger range of users:

- Enhance local visibility (e.g. interactive information on end-user's local waste)
- Enhance information flow
- Fluidify management (decentralize ownership and responsibility)
- Reduce cognitive cost (make it easy to understand)
- Offer different levels of involvement
- Support collective use (Manzini, Francois 2008)

10.4.5 Communication of benefits

The Netherlands Water Partnership (NWP) conducted an assessment on the sanitation sector in South Africa in order to determine potential investment in strategies and interventions for future cooperation. From this assessment it is clear that the perspective of the main challenges in pro poor sanitation differs from the South African to the Dutch partners (Pannekoek and van Koppen 2012). The Dutch view of sanitation approaches this ecosystem economy and is most concerned about the value chain that would contribute to closing the materials cycle, while the South African counterparts are more concerned about improving infrastructure and governance. A value chain approach requires the knowledge diffusion outlined in Section 10.4.4, but also a clear understanding of the mutual benefits to the different players linked in the chain. Clarity of purpose for all stakeholders is critical, as indicated by Pannekoek and van Koppen (2012):

> *"The long term approach should focus on developing roadmaps for sanitation, developing business cases and action research in innovation pilots".*

For implementing and upscaling sustainable solutions, wastewaters need to be valued as resource-providers. Presently, these are viewed as waste and not as a resource. Future emphasis should be made on sanitation as a contributor to the challenges in the water-energy-food nexus. Additionally, adopting this approach allows job creation, lower costs and economic growth. New concepts and business models have to be developed from the value chain approach in which collection, transport, treatment and reuse are taken into account and cultural aspects respected. Within the existing partnership, implementation of pilot projects across the complete value chain will allow testing of the approach.

Possible interventions related to the long term strategy are (Pannekoek and van Koppen 2012):

- Strengthen existing partnerships, with a strong focus on municipalities;

- Develop longterm roadmaps for sanitation with attention for a variety of possible solutions;
- Develop business models at the meso level (value chain) and micro level (sanitation /collection) by inviting the private sector and unusual suspects such as banks/financial institutions (big companies at the meso level, local companies at the micro level);
- Start pilots:
    - Invest in research on value chain approaches for sanitation;
    - Share knowledge and stimulate interaction between public and private sector and knowledge institutes through the 'success hubs' within the existing partnerships;
    - Use depletion of resources (e.g. Phosphorus) as an incentive to invest in sanitation and combine with other waste streams (e.g. animal manure, solid waste).

To achieve the above, communication is a key step at all levels, from the provision of a sanitation service of appropriate quality through responsible use of water services by the community to the exploitation of the resources within wastewater for product formation. This communication includes the user (community and business), the service provider (municipality, government) and the producer of products with social and/or economic value from waste (potentially the private sector or municipality). The communication requires an understanding of system components and the potential value of new approaches as well as information flow.

Communicating with public utilities to facilitate this activity in return for better infrastructure overall, better service delivery and a better industrial ecosystem can be facilitated by independent industry bodies (like Water Regulators). It needs the cooperation of public utilities and the private sector alike.

It is important to note that certain municipalities like eThekwini in KwaZulu-Natal (South Africa) have already invested in pilots investigating the business case and challenges in implementing projects which deal with the entire sanitation value chain. In addition, the Water Research Commission has funded numerous projects and pilots which have researched aspects of the waste to resource concept, the sanitation value chain and beneficiation of wastewater (www.wrc.org.za).

10.4.6 Regulations and Risks

In the establishment of new approaches, such as the development of waste-water biorefineries towards a closing of anthropological ecosystems, it is typically necessary to adapt guidelines and regulations. Further this typically follows the development, leading to tension during the transition phase. This is explained by Perez (2002):

> *"The irruption of a set of powerful and dynamic new industries accompanied by a facilitating infrastructure will obviously have enormous*

consequences both in the industrial structure and in the preferred direction of investment in that period. But the old organizational models cannot cope with or take full advantage of the new potential. The new possibilities and their requirements also unleash a profound transformation in 'the way of doing things' across the whole economy and beyond. Thus each technological revolution inevitable induces a paradigm shift."

Poorly understood regulatory control may pose a risk to successful implementation of unit processes that can contribute to wastewater biorefineries. For instance, at the Cape Flats WWTP in Cape Town, biogas is generated on site and used to dry sludge to pellets. These pellets can be used as a fuel for generating energy. However, it is difficult to implement income generating secondary industries on state owned operations (e.g. municipal WWTP), because motivating for and realising the additional investment and manpower required from existing budget allocations are challenging (WRC 400/09). From discussions with Brett Keyser (Stellenbosch Municipality) and Barry Coetzee (City of Cape Town), the regulations around these processes are adequate, but the interpretations are not done adequately.

According to the practitioners engaged, in South Africa wastewater biorefineries will likely not function as public private partnerships (PPPs). A common argument for PPPs is that they can help alleviate chronic under-investment in capital-intensive projects. They can serve as a vehicle for the injection of private sector financing while allowing government to maintain their fiscal targets and avoid taking on additional debt (Palmer 2009). In the case of wastewater biorefineries, a clearer definition of the public facility's mandate can be explored and areas that fall outside this can be sold to entrepreneurs. Providing effluent compliant wastewater is a public mandate. Generating value from resources in transition (waste) and the infrastructure around that is a private sector activity. As Barry Coetzee notes, the private sector cannot expect local government to fund what is essentially a private sector activity. Communicating with public utilities to facilitate this activity in return for better infrastructure overall, better service delivery and a better industrial ecosystem can be facilitated by independent industry bodies (like Water Regulators), and needs the cooperation of public utilities, but as it is to further a private activity, the onus is on the private sector to promote this.

While it is not possible for risk always to be quantified, and complex systems such as these always will have a great amount of uncertainty, some aspects need to be better understood. For wastewater biorefineries specifically, pathogenic vector removal needs to be understood and managed.

The draft National Environmental Management Waste Act 59 of 2008 (NEMWA) classifies and regulates waste. For example, biosolids may not be landfilled until they are treated. But currently the solids are applied to farm land in a manner that does not exclude vectors. The implications of this remain unclear and are becoming a growing public health and nuisance risk. As indicated by Barry Coetzee of the City of Cape Town municipality (2012):

“Waste beneficiation is a young industry and no-one really knows where it’s going yet. Very few are willing to stick their neck out. We’re integrating systems that were not integrated previously. This represents a huge risk.”

10.5 Conclusion

The combination of increased resource scarcity, an economic downturn and global environmental degradation and climactic change requires a different approach to resource use. The technologies to enable a more cyclic approach to the use of materials are available, and the paradigm to enable widespread adoption of those technologies seems to be emerging. There is a strong need, however, to approach and develop the social capabilities that need to support the technological approach. This report outlines specific steps to build these capabilities that should be taken if wastewater biorefineries are expected to be part of the next revolution.

11 Concluding Remarks

The Wastewater biorefinery

The concept of industrial metabolism requires optimal resource productivity by ensuring that all resources exploited provide the maximum possible products and services. This approach is a key premise to achieving sustainable systems for our society and minimising the environmental burden associated with anthropogenic activity. In addressing this, it is valuable to view wastes, including wastewater, as a potential raw material resource. This approach can be used through the wastewater biorefinery concept which is centred on two major outcomes: the treatment of wastewater to the desired water quality and the simultaneous production of products of value, as either commodity or energy products.

Unit operations in such a wastewater biorefinery include reactors that can reduce nutrient loads in wastewater in a variety of environments, while producing a range of valuable bioproducts that can potentially sustain jobs, meet commodity needs and fund capacity building through their revenue. This review addresses the requirements of such reactor systems in which microbial bioprocesses are used for the production of commodity polymers while reducing nutrient loads. Because of the non-sterile nature of the wastewater environment, it is best to select micro-organisms for product formation that also fulfil a role in the microbial ecology and culture conditions and product which contribute a selective advantage to the microbial community. Hence, wastewater biorefineries are not suitable for all bioproducts. The overall project considers this application in terms of the model system in which microbial communities enriched for *Bacillus* species are used for the production of poly-glutamic acid.

PGA from a Bacillus-enriched culture as an example product

Poly-glutamic acid (PGA) is hypothesized to fulfil an ecological role to give *Bacillus* (or other species producing PGA) a competitive advantage that can be exploited for bioproduction through microbial community engineering in wastewater biorefineries. The reactor design is crucial to achieve this microbial community engineering. In order to achieve the production of relatively pure, N-rich PGA from wastewater, the following need to be addressed:

- Establishment of *Bacillus* as the dominant organism in the mixed microbial community used for PGA production;
- Determining a reactor configuration suited to retention of biomass while processing large volumes of dilute wastewater;
- Selection of a downstream process train that takes cognisance of the dilute product environment, and intercalates with the reactor system in the optimal manner;

- Selection of a reactor configuration and associated downstream processing unit operations that are appropriate for the production of a low value product in dilute solution.

PGA producing isolates have been selected from 18 isolates collected and screened from the Mitchell's Plain WWTP. These have been assessed in terms of growth, nutrient requirements and PGA production and identified at the genus level. Biokinetic parameters have been determined using synthetic wastewater. These data allowed the selection of a lead group of organisms of which one, a *Bacillus* species, was selected for preliminary reactor work and work using waste streams.

Reactor design, product separation and process integration for the WW biorefinery

Reactor selection and design is a key component of the wastewater biorefinery establishment as it offers potential to exploit the ecological advantage of the selected microbial community, to focus on provision of appropriate conditions under low energy input and to design for product recovery.

Two reactor designs have been highlighted that are proposed to fulfil this function as well as contribute to effective product recovery: the Aerobic Granular Sludge (AGS) process, and the hRBC process.

The AGS process is defined by the characteristics of the sludge particles. The granules are defined as "aggregates of microbial origin, which do not coagulate under reduced hydrodynamic shear ('sludge bulking') and which settle significantly faster than activated sludge flocs" (15 sec vs 20 min) (de Kreuk et al. 2007a).

The hRBC process is a modified Rotating Biological Contactor (RBC) system. RBC's are non-submerged, attached growth bioreactors, similar to trickling filters, with circular media mounted (approx 3.6 m in diameter for standard units) on a horizontal shaft, partially submerged (typically 40%) in the wastewater, and rotated at a speed of one to six revolutions per minute (Grady et al. 2011). The circular support media is modified to form a high surface area mesh disc to support an active biofilm.

These reactors were selected for detailed consideration because the management of biofilm detachment – and hence product recovery – are controllable. The support material of the hRBC is relatively planar, while the granular system does not have a carrier, with settling properties that make product recovery possible. In addition, the biofilm zonation in both reactor types (allowing for heterotrophy, nitrification, phosphate removal and denitrification zones intrinsic in the biofilm) is advantageous.

The AGS process is operated as a Sequencing Batch Reactor (SBR). It is a time-oriented process that can be designed and operated to simulate virtually all conventional continuous-flow activated sludge systems, from contact stabilization to extended aeration, making it a useful approach in a laboratory environment. This

aspect, combined with the rapid sedimentation velocity possible with aerobic granules allows product formation and downstream processing to form part of the reactor design at discrete steps (Johnson 2010).

The hRBC process can accommodate a wider range of substrate loads and is a simpler process to operate than the AGS process. The combination of the meshed internal structure and planar disk macro structure gives product recovery options that complement the AGS process, and the overall operation of the hRBC is thought to promote Bacillus dominance.

PGA is generally accepted to be a product excreted into the bulk solution and not even weakly associated with the biomass. Several biofilm-based approaches allow cultivation at relatively low flow rates and may be used to minimise the separation of the weakly associated polymer from the biomass. In terms of considering PGA recovery, three potential scenarios have to be considered:

1. PGA is excreted into the bulk solution and is not associated with the biomass at all
2. PGA is excreted, but weakly associated with the biomass. This association is easily disrupted
3. PGA is excreted, but strongly associated with the biomass. Significant physical or chemical means needs to be employed to disrupt this association.

The AGS and hRBC processes will have different utility towards PGA product recovery depending on which of these scenarios come into play. Scenario 1 and 2 are more suited to the hRBC, while scenario 3 favours the AGS process.

This work carries significance in arguing for a different approach to waste, and serves to introduce the discussion on how to use significant infrastructural limitations to the advantage of wastewater biorefineries. Challenges in optimising the system, rather than individual unit processes, are highlighted which include the different hegemonies present in the interface between environmental and bioprocess engineering disciplines. This report particularly articulates the aspects to consider when reactors are designed as unit processes in a wastewater biorefinery, and contributes to a better understanding of reactor selection in areas where systems are required to meet the needs of biomass retention, processing of large volumes and integrated product recovery. Further, it argues that product recovery is a key consideration at the initial stage of choosing the treatment system as well as the reactor system. In particular, the importance of producing a product in a different phase to the bulk water system is critical for ready separation.

Towards an evolving paradigm: integrating waste treatment and resource provision

Beyond the consideration of reactor design in terms of the integrated process, the beneficiation of each of the settled solids, liquid nutrient rich settled sewage and

subsequent nutrient poor polishing streams is required. The integration of these process steps can be achieved and is clearly outlined with potential product families for each stage.

The report is concluded with a section in which the potential for implementing wastewater treatment approaches conceived through an industrial ecology framework is discussed. The section introduces the relevance of industrial ecology, natural capitalism and biomimicry to wastewater treatment, identifies the requirements and value of these approaches and seeks to provide a preliminary understanding of barriers to their implementation.

The practitioners in the wastewater sector are positive towards the integration of wastewater treatment and value recovery and the closing of the nutrient cycle. However, consideration for the variable feed to a wastewater biorefinery and necessity of process robustness is highlighted, as is the role of the community and the need for excellent communication of benefits. The need to interrogate the role of the public private partnership is recognised.

The combination of increased resource scarcity, an economic downturn and global environmental degradation and climatic change requires a different approach to resource use. The technologies to enable a more cyclic approach to the use of materials are available, and the paradigm to enable widespread adoption of those technologies is emerging. There is a strong need, however, to approach and develop the social capabilities that need to support the technological approach. This report outlines specific steps to build these capabilities that should be taken if wastewater biorefineries are expected to play a part.

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