Toward Maximal Polyhydroxyalkanoate Production from Dairy Manure: Controlling and Optimizing Biosynthesis with Implications for Design

A Thesis

Presented in Partial Fulfillment of the Requirements for the

Degree of Master of Science

with a

Major in Chemical Engineering

in the

College of Graduate Studies

University of Idaho

by

Benjamin S. Carleton

Major Professor: Erik R. Coats, P.E., Ph.D.

Committee Members: Wudneh Admassu, Ph.D.; James Moberly, Ph.D.

Department Administrator: D. Eric Aston, Ph.D.

May 2016

AUTHORIZATION TO SUBMIT THESIS

This thesis of Benjamin S. Carleton, submitted for the degree of Master of Science with a Major in Chemical Engineering and titled "Toward Maximal Polyhydroxyalkanoate Production from Dairy Manure: Controlling and Optimizing Biosynthesis with Implications for Design," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: _____ Date: _____
Erik R. Coats, P.E., Ph.D.

Committee Members: _____ Date: _____
Wudneh Admassu, Ph.D.

_____ Date: _____
James Moberly, Ph.D.

Department Administrator: _____ Date: _____
D. Eric Aston, Ph.D.

ABSTRACT

Polyhydroxyalkanoates (PHAs) are biodegradable, biologically produced plastics with the potential to replace conventional, fossil fuel derived plastics. PHAs can be synthesized from otherwise undesired organic-rich waste streams, yielding a valuable commodity while achieving greater sustainability. To be economically successful, however, the PHA production process must be optimized and have design guidelines established. In this work, using fermented dairy manure as a model substrate, a 12-month evaluation assessed the long-term enrichment of a mixed microbial consortia dominant in PHA producers. Process control challenges led to mixed results, but a maximum intracellular PHA content of 64.7% (total solids) or 74.8% (volatile solids) was obtained. In addition, PHA production employing continuous substrate addition mode as opposed to traditional fed-batch improved operations and process control. Intracellular PHA content was strongly correlated with PHA yield on substrate, a previously overlooked metric, forging a clearer path to process optimization and full scale design.

ACKNOWLEDGEMENTS

This work was made possible by all of my colleagues and mentors who supported and counseled me throughout my entire academic career. In the first place I would like to acknowledge my major professor, Dr. Erik R. Coats, P.E., for the support he has provided – both as an employee in his laboratory and as a graduate student. I would also like to recognize the other members of my committee, Dr. Wudneh Admassu and Dr. James Moberly, and the entire faculty in the Material Science and Chemical Engineering Department. Among colleagues, I would like to first thank Cindi Brinkman for her friendship and lessons in laboratory work. Also, this work would not be complete without my fellow graduate students, namely Derek Probst and Nicholas Guho, or the entire staff of undergraduate assistants who worked behind the scenes to keep my experiments and the entire laboratory operational. To all these men and women I extend my heartfelt gratitude.

DEDICATION

For my wife Christina and my daughter Julianna

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE AND ABBREVIATIONS

ADF	Aerobic dynamic feeding	C_j	Concentration of species 'j'
ATP	Adenosine triphosphate	K_j	Half saturation of species 'j'
CAC	Citric acid cycle	M_j	Mass of species 'j'
CL	Cycle length	N	Subscript, nitrogen
Cmmol	Unit, carbon millimole	n	Optimal volume multiplier
COD	Chemical oxygen demand	P	Subscript, PHA
CSTR	Continuous stirred tank reactor	Q_k	Volumetric flow in for point 'k'
D.O.	Dissolved oxygen	q_S^{max}	Maximum specific substrate uptake rate
EBPR	Enhanced biological phosphorus removal	r_j	Reaction rate of species 'j'
ED	Entner-Doudoroff	S	Subscript, substrate
FBA	Flux balance analysis	t_k	Time at specified point 'k'
F/F	Feast to famine	V_j	Volume of component 'j'
FID	Flame ion detector	X	Biomass concentration or subscript, biomass
GAO	Glycogen accumulating organism	X_k	Biomass concentration at specified point 'k'
GC	Gas chromatograph	$Y_{X/STO}$	Yield of biomass on storage
hr	Unit, hour	Δt_{div}	Change in time for a "division"
HRT	Hydraulic residence time	δ	P/O ratio
LCA	Life cycle assessment	μ_{max}	Maximum specific growth rate
mg/L	Unit, milligrams per liter	μ_{net}	Net specific growth rate
min	Unit, minute	μ_{net}^k	Net specific growth rate for species 'k'
MS	Mass spectrometry	μ_{net}^{div}	Net specific growth rate for a "division"
NH_3	Ammonia		

NO_3	Nitrate
O_2	Oxygen
OLR	Organic loading rate
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
PAO	Phosphorus accumulating organism
PBS	Poly(butylene succinate)
PE	Polyethylene
PET	Polyethylene terephthalate
PLA	Polylactic acid
PMF	Proton motive force
PHA	Polyhydroxyalkanoate
PHB, P(3HB)	Poly-3-hydroxybutyrate
PHB-coV, P(3HB-co-3HV)	Poly-3-hyrdroxybutyrate-co-3-hydroxyvalerate
PHV, P(3HV)	Poly-3-hydroxyvalerate
PP	Polypropylene
PPP	Pentose phosphate pathway
rpm	Unit, revolutions per minute
SBR	Sequencing batch reactor
SRT	Solids residence time
TCA	Tricarboxylic acid
TS, TSS	Total (suspended) solids
VFA	Volatile fatty acid
VS, VSS	Volatile (suspended) solids
wt%	Unit, weight percent

CHAPTER 1: BACKGROUND

1.1 Resource Recovery

Over the last several decades, concerns about climate change and the depletion of the planet's nonrenewable resources have gradually, yet inevitably, led to societal improvements regarding consumption and associated recovery of resources. Some ideas have gained exceptional popularity in the general public including solar and wind energy, consumption of sustainable or "green" products, and the replacement of fossil fuels with bio-based fuels like ethanol or biodiesel. However, the less publicized disadvantages of these technologies and strategies often limit their applicability, including but not limited to, geographical restrictions (e.g., sunny or windy locations for solar and wind energy) and conflicting competition with arable land for food crops (e.g., corn to ethanol) (Yates and Barlow, 2013). While no avenue should be left unexplored, it is clear more effort needs to be undertaken to reduce net consumption overall, or improve energy yield on that resource consumption, or both. The term "resource recovery" is often used to refer to the latter. In general, resource recovery aims to reduce environmental impact and overall energy use by repurposing waste products for another application. Household recycling, as one example, is only the first step in resource recovery, as the materials must still be processed and converted before the end product is consumed again. Another example, and the focus of this research, is to convert an otherwise undesired liquid waste stream into a high-value marketable product by utilizing naturally occurring microorganisms. In this case, a bioplastic with properties and applications similar to synthetic petro-plastics can be engineered from

highly undesirable substrates like municipal wastewater, industrial effluent, and dairy manure. Such a process has the two-fold effect of eliminating an environmental pollutant and reducing dependency on non-renewable resources (i.e., fossil fuels used to generate plastic). Thus, the motivation for understanding this process is not simply an academic exercise, but a real attempt to realize environmental sustainability.

1.2 Polyhydroxyalkanoates

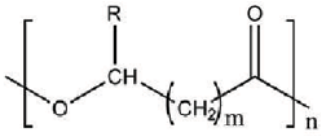
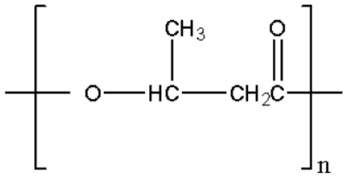
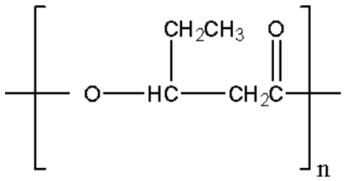
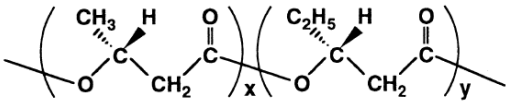
A bioplastic, in general terms, is any polymer that is created in part or in whole from microbial metabolic processes. However, in most cases, the bacteria performing said metabolisms are responsible only for monomer production, and the polymerization is performed chemically and separate from the biological activity (Chen and Patel, 2012; Serafim et al., 2008). Indeed, the monomers needed for "traditional" plastics usually derived from fossil fuels can be harvested biologically to produce well-known materials like polyethylene (PE), polypropylene (PP), and polyethylene terephthalate (PET) (Chen and Patel, 2012). Microbes can also produce acidic monomers like lactic acids and succinic acid that can be polymerized to form polylactic acid (PLA) and poly(butylene succinate) (PBS) (Chen and Patel, 2012). This second class of polymers, along with starch based polymers, have become of particular interest to researchers in recent years due to their novelty and marketable potential as fossil fuel replacements (Yates and Barlow, 2013). A third class of polymers, polyhydroxyalkanoates (PHAs) derived from hydroxyalkanoic acids, has the additional property of being completely polymerized directly by the microbes themselves. Briefly, PHAs function as a carbon and energy storage polymer for many forms of prokaryotic life, synthesized under stressful conditions – similar to internal glycogen

reserves or polyphosphate (Serafim et al., 2008). However, when extracted from the microbial cell, PHA becomes a useful petro-plastic substitute. Since the PHA production process can entirely omit the polymerization step (and associated costs), this form of bioplastic has an economic and engineering advantage over other bioplastics.

1.2.1 PHA Chemical and Material Properties

Polyhydroxyalkanoates are a general class of polymers composed of monomers called hydroxyalkanoates. As the name of the structure implies, each monomer is a carbon chain with an alcohol group on one end and a carboxylic acid on the other. The monomers are dehydrated during polymerization, resulting in the general form for PHA as shown in Table 1. The “R” group and the “m” number of carbons between the two functional groups are arbitrary and specifically define a particular specie of hydroxyalkanoate. It has been estimated that somewhere between 125 and 150 different monomers of PHA exist (Chen and Patel, 2012; Sudesh et al., 2000). In the context of real biological systems, only two monomers are produced with consistent regularity: 3-hydroxybutryate and 3-hyroxyvalerate. In polymer form, these chemicals are abbreviated P(3HB) and P(3HV), or PHB and PHV, respectively. The “3” indicates the alcohol group is on the third carbon, and “butyrate” has 4 total carbons while “valerate” has 5 total carbons. In many cases these two monomers, if both are present, will be polymerized randomly and form the co-polymer P(3HB-co-HV) or PHB-coV (Wei et al., 2014).

Table 1: Names and structures of common PHAs

Polymer Name	Abbr.	Chemical Structure	Reference
Polyhydroxyalkanoate (general form)	PHA		(Chen and Patel, 2012)
Poly(3-hydroxybutyrate)	PHB, P(3HB)		(Beserker79, 2005)
Poly(3-hydroxyvalerate)	PHV, P(3HV)		(Beserker79, 2005)
Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)	PHB-coV, P(3HB-co-3HV)		(Sudesh et al., 2000)

Physical properties of PHA are heavily dependent on the composition and relative abundance of monomers. For example, pure PHB is known to be very brittle and difficult to anneal, while pure PHV is known to have low tensile strength and difficult to set; however, the co-polymer PHB-coV, within a certain range, exhibits attractive mechanical properties similar to synthetic plastics. Table 2 shows the comparison of common PHAs to common synthetic plastics, and Figure 1 shows the specific effect of 3HV content on the mechanical properties of PHB-coV.

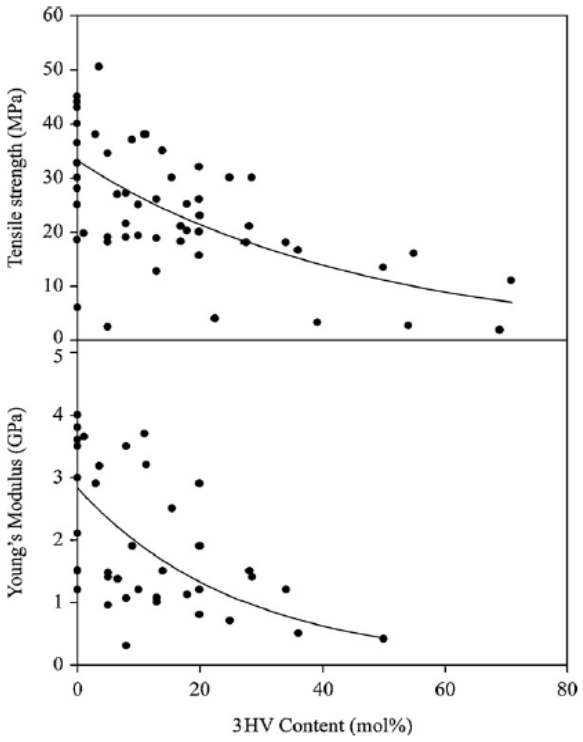


Figure 1: Effect of 3HV content on properties for P(3HB-co-HV) (Chen and Patel, 2012)

Table 2: Properties of common PHAs and synthetic plastics (Sudesh et al., 2000)

Sample	Melting temperature (°C)	Glass-transition temperature (°C)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation to break (%)
P(3HB)	180	4	3.5	40	5
P(3HB-*co*-20 mol% 3HV)	145	−1	0.8	20	50
P(3HB-*co*-6 mol% 3HA)[a]	133	−8	0.2	17	680
Polypropylene	176	−10	1.7	38	400
Low-density polyethylene	130	−30	0.2	10	620

[a] 3HA units: 3-hydroxydecanoate (3 mol%), 3-hydroxydodecanoate (3 mol%), 3-hydroxyoctanoate (<1 mol%), 3-hydroxy-*cis*-5-dodecenoate (<1 mol%).

Ideally, the composition of PHAs produced from biological systems will be such that they can directly compete with synthetic plastics. Bacteria can convert many forms of simple carbon substrates to PHA, but the most direct precursor is a suite of compounds called volatile fatty acids (VFAs). VFAs are short chain carboxylic acids or their conjugate salts, including acetate (2 carbons), propionate (3 carbons), and butyrate (4 carbons). In general, the parity of carbons will determine the parity of the final PHA composition; in other words, even-numbered VFAs will result in PHB and odd-numbered VFAs will result in PHV. If a mixture of VFAs is present, some evidence suggests (Dias et al., 2008), the co-polymer will result in a ratio similar to the ratio of even-to-odd carbon molecules. Therefore, in order to have ideal physiochemical properties, the feedstock should have a mixture of VFAs with heterogeneous carbon parity.

1.2.2 PHA Biodegradability

An additional useful feature of PHAs is their biodegradability. Since the function of PHA is as a biological storage compound to be used for consumption during non-stress conditions, it follows that many bacteria can secrete enzymes capable of breaking down the compound (Madison and Huisman, 1999). Thus, another advantage of PHA over other

bioplastics is the fact that, since bacteria are responsible for polymerization, they can also depolymerize easily. PLAs, PBSs, and starch polymers are also biodegradable, but less so due to the fact that the polymerization step is performed synthetically. In the case of PP, PE, or PET production, even though the monomers can be produced biologically, the polymer is not biodegradable since few, if any, bacteria possess the enzymes capable of breaking the chemical structure (Chen and Patel, 2012). A demonstration of PHA biodegradability is illustrated in Figure 2 after 10 weeks of treatment in an anaerobic digester.

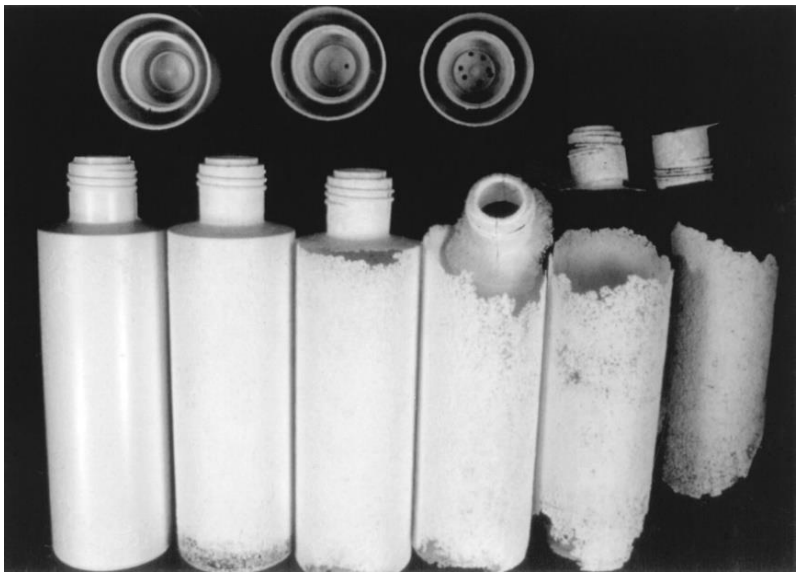


Figure 2: Treatment of PHA at 0, 2, 4, 6, 8, and 10 weeks. (Madison and Huisman, 1999)

The advantages of a biodegradable polymer synthesized from waste carbon for environmental conservation are obvious. Biodegradable materials do not indefinitely pollute the land, and they usually derive their materials from a renewable cycle. For example, PHAs derive their existence from organic carbon compounds, which derive from animal and plant waste. Plants obtain their carbon source from carbon dioxide fixation, which is where PHA will return once degradation is complete. In contrast, synthetic plastics

made from fossil fuels are unidirectional and are derived from deep underground excavation, resulting in a net increase of carbon dioxide to the atmosphere and plastic waste on the surface of the planet. Of course, this simplification does not account for other effects, like the fossil fuel based energy input to produce PHAs in the first place, but it nevertheless points in the right direction.

1.3 Strategies for PHA Production

P(3HB) was the first PHA polymer to be discovered, isolated in the 1920s by the Frenchman Lemoigne (Laycock et al., 2013). However, it was not until the 1970s when it was first observed in activated sludge for municipal wastewater facilities that its potential began to be realized (Serafim et al., 2008). An accumulation response can be induced by depriving the microbes of an essential growth condition (e.g., oxygen, ammonia, or trace minerals) or by exposure to a carbon oversupply. Likewise, the biomass culture can be either a pure, PHA-capable species or a mixture highly enriched for PHA accumulators resulting from imposed environmental pressure. Three techniques in the literature, in order of relevance to this research and thesis, have been developed: anaerobic feeding (oxygen deprivation, mixed culture), controlled growth feeding (nutrient deprivation, pure culture), and aerobic dynamic feeding (carbon oversupply, mixed culture); each are discussed below.

1.3.1 Anaerobic Feeding

The first observation of PHA storage under an engineered environment was in the context of enhanced biological phosphorus removal (EBPR). In EBPR, the biomass is dynamically cycled through an anaerobic and aerobic zone, with options for other stages

depending on the objectives of the treatment facility (Oehmen et al., 2007). The anaerobic zone is always the furthest upstream where readily biodegradable carbon, usually in the form of VFAs, is in abundance. However, without an external terminal electron acceptor, ordinary cellular function and growth is inhibited. Thus, the facultative heterotrophs with the greatest chance of survival will be those that are capable of storing VFAs as PHA anaerobically at the expense of a secondary storage molecule. Then, when they are exposed to the aerobic (respirative) environment, all the exogenous readily biodegradable carbon is already depleted and those microbes that have PHA storage can utilize it for growth and to replenish the secondary storage reserves (Serafim et al., 2008). PHA synthesizing microorganisms that utilize only glycogen for secondary storage reserves are called glycogen accumulating organisms (GAOs) and those that utilize glycogen and polyphosphate are called phosphorus accumulating organisms (PAOs). A typical profile of the anaerobic-aerobic cycle is shown in Figure 3 where VFAs are anaerobically consumed and stored as PHA at the expense of glycogen and internal phosphorus and then aerobically reversed.

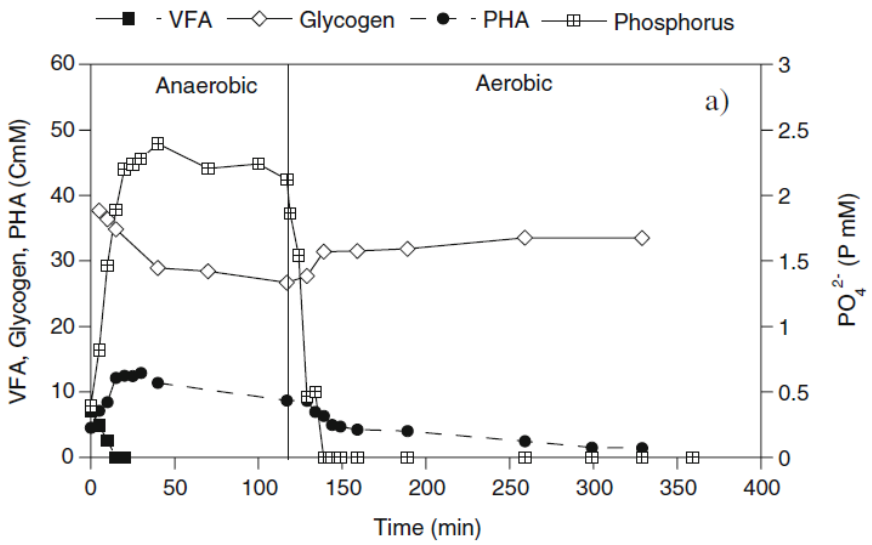


Figure 3: Typical dynamic nutrient cycling in EBPR (Serafim et al., 2008)

Enriching for PAOs is critical for phosphorus removal, but when the objective is excess PHA accumulation, GAOs can obtain higher yields (Oehmen et al., 2007). Some studies such as Bengtsson et al. (2010) have focused specifically on growing GAOs for the purpose of maximizing intracellular PHA. The cyclical process enriches for PHA accumulators, as evidenced by anaerobic storage, but maximum storage is only achieved when a portion of biomass is removed to a separate accumulation reactor and exposed to an extended anaerobic or aerobic feeding period. Anaerobic operation for the accumulation stage is fundamentally limited by the mass of secondary storage compounds and lack of respiration necessary to generate energy and drive the VFA uptake reactions. In contrast, aerobic operation induces PHA accumulation using a carbon oversupply mechanism which is contrary to the oxygen deprivation mechanism the biomass was selected under. Given these two choices, aerobic operation has been more successful but the results have been mediocre, causing a lag in development compared to fully aerobic PHA techniques (discussed below) (Bengtsson et al., 2010; Serafim et al., 2008). Still, assuming the process can be optimized, it would be easy to implement at existing treatment facilities similar to process diagrams proposed by Coats et al. (2007). Research is ongoing to test the viability of this method for PHA accumulation (Appel, 2015; Coats et al., 2011a; Winkler et al., 2011), but the research presented herein will focus on an alternative method for achieving commercial levels of PHA production.

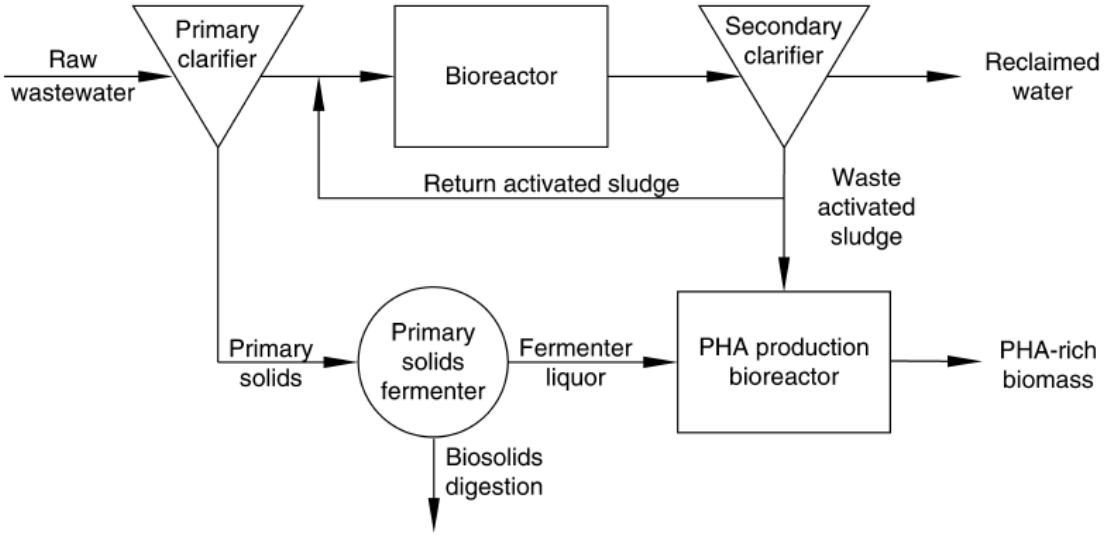


Figure 4: Proposed wastewater facility with PHA production (Coats et al., 2007)

1.3.2 Controlled Growth Feeding

The discovery of PHA in anaerobic feeding for activated sludge led researchers to search for the genera and species that were PHA accumulators. The strategy assumed that a pure culture of a known accumulator could be more easily controlled and coaxed into producing high quantities of PHA not otherwise achievable in a mixed culture (Choi and Lee, 1997; Holmes, 1985; Laycock et al., 2013). The first attempt at commercialization was by W.R. Grace and Co. (Holmes, 1985) in the 1950s and 1960s, but extraction difficulties and impurities led to business failure. Commercialization then ceased until the 1970s when Imperial Chemical Industries Ltd., under the trademark name BIOPOL™, began leveraging new knowledge about extraction and the recently discovered P(3HB-co-3HV) copolymer to produce a biopolymer with more attractive physical properties (Holmes, 1985; Laycock et al., 2013). The discovery of new PHA monomers not only spurred a new capitalist enterprise, but also motivated scientists to discover additional possible monomer units and other microbial species capable of PHA production. Much research was completed by the

end of the 1980s and resulted in the discovery of approximately 125 hydroxyalkanoate monomers and about 250 species of Gram-negative or Gram-positive bacteria capable of producing PHA (Choi and Lee, 1997; Sudesh et al., 2000). While the PHA monomer and microbiology research was of academic interest, from a business perspective, maximizing PHA content while minimizing cost was the driving factor.

A two stage production process has become the industry standard for achieving PHA production through controlled growth feeding (Choi and Lee, 1997). In the first stage, a pure culture known to have high PHA productivity is grown under fully aerobic conditions on an impurity-free substrate – usually glucose or sucrose derived from corn (Serafim et al., 2008). When a critical mass of microbes has been achieved, nutrient limitation is applied (still fully aerobic) which prevents the carbon from flowing to growth and forces it to storage. This step is absolutely essential because, unlike the other techniques, the microbes are not adapted to outcompete flanking populations from environmental pressure. Without it, the pure culture would grow endlessly and would have no incentive to store the carbon. In some cases, residual growth can be allowed but must be tightly controlled (Choi and Lee, 1997; Grousseau et al., 2013). These techniques have been well studied and, as Figure 5 illustrates, can consistently achieve an intracellular content up to 90% PHA by weight (Madison and Huisman, 1999). While this method has some commercial potential, it is not applicable to processing organic-rich waste streams for PHA production.

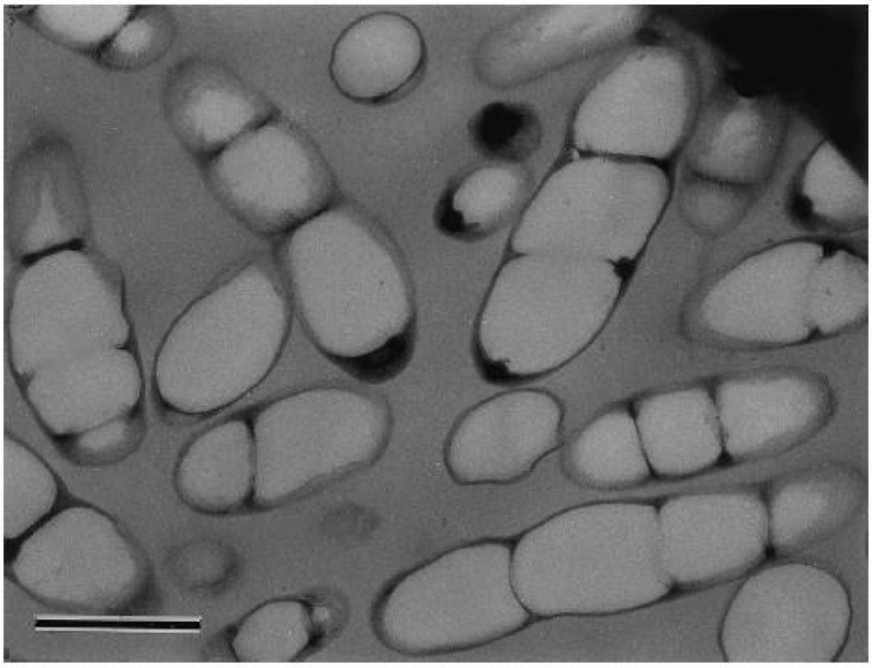


Figure 5: Microbes filled with PHA granules (Sudesh et al., 2000)

1.3.3 Aerobic Dynamic Feeding

Towards the end of the 1990s it became clear that the pure culture method of PHA accumulation was fundamentally limited by its two most expensive line items: namely, the cost of sterilization to sustain a pure culture, and the cost of creating a purified substrate. The next logical question was simply, if PHA is a storage molecule common to many forms of prokaryotes, is it possible to induce a PHA accumulation response from a mixed culture rather than a pure culture? To this end, it was discovered that a well-mixed bioreactor exposed to alternating states of carbon excess and carbon depletion in a fully aerobic environment was capable of selecting for floc-forming, PHA-accumulating biomass and against filamentous bulking organisms (Majone et al., 1996). This cyclical metabolic response of “feast-famine” to exogenous carbon excess and depletion, respectively, has

become known as the engineering tactic called aerobic dynamic feeding, or ADF (Dionisi et al., 2004). The response for PHA storage in anaerobic and controlled growth feeding is driven by growth limitation such as oxygen deprivation or absence of macronutrients like nitrogen or phosphorus, but in the case of ADF, the response is driven simply by an excess of external substrate (Beun et al., 2000). These dynamic conditions were further studied via the development of a generalized PHA model (Majone et al., 1999; vanAalastvanLeeuwen et al., 1997) and continued experimentation that increased the selective pressure for PHA storage (Beun et al., 2002). By the middle of the 2000s, the potential to achieve PHA production by mixed culture, albeit mostly on synthetic feed, had matured into an independent field of study separate from its origin based in activated sludge.

The principle that drives ADF is that more carbon is supplied than the microbes require for growth – the excess amount can then be consumed by accumulators, giving a competitive advantage over non-storing populations. VFAs are necessary in the feedstock because they are the direct pre-cursors to PHA and can be consumed and utilized readily and rapidly (Dias et al., 2006). During the famine period, the populations with PHA storage continue to grow while non-storing fractions are culled out. Figure 6 shows a profile of PHA and VFAs in a typical ADF cycle.

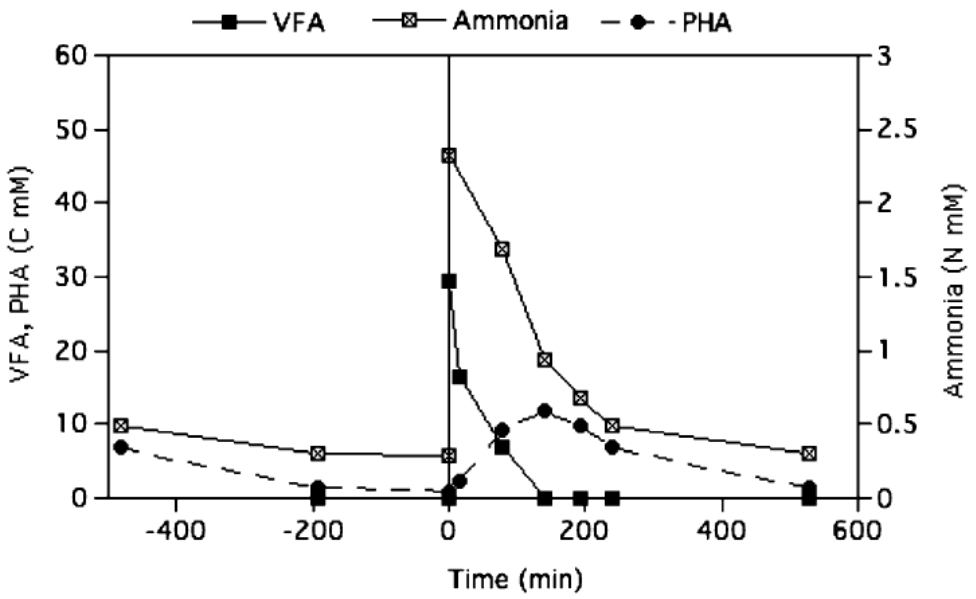


Figure 6: Typical ADF profile (Serafim et al., 2008)

The shift in focus from an activated sludge-wastewater treatment phenomenon to specifically PHA accumulation as a resource recovery technology established two important features of ADF. First, in order to maintain the dynamic conditions that favor enrichment of PHA-storing bacteria, reactors are configured almost exclusively as sequencing batch reactors (SBRs). SBRs typically contain within a single cycle length stages for substrate addition, reaction, wasting, settling, and decant. The second feature is the fact that, although activated sludge on municipal wastewater was the original waste stream envisioned, ADF can in principle be performed on any organic carbon rich waste stream. This versatility has vastly enlarged the scope of possible applications, prompting a surge of research interest aimed at optimization.

1.3.4 Life Cycle Assessments

Considering that commercial application is the end goal of bioplastics production research, an economic and environmental evaluation of the PHA recovery processes

compared to fossil-fuel based plastics is necessary. Is the product economically viable and, if not, does it exhibit the potential to be optimized or find value in a niche market? An economic analysis of this sort is referred to as a life cycle assessment (LCA).

The economic LCA question was long ago answered for PHA accumulation via controlled growth feeding. One of the most recent LCAs for solely pure culture production was conducted in 1997 (i.e., nearly 20 years ago), an implicit indication that the process has not had any significant development in nearly two decades (Choi and Lee, 1997). In that paper it was reported that BIOPOL™, produced at that time by Zeneca Bio Products, was selling at then current prices of U.S. $16 per kilogram compared to U.S. $1 per kilogram for polypropylene and polyethylene. Their analysis showed however, that given large enough scale and minor optimization techniques, that price could be reduced to approximately U.S. $5 per kilogram. More importantly, however, the authors conceded that PHA production would not conceivably be competitive with synthetic plastics, but only with other biodegradable bioplastics like polylactide and starch based polymers selling at then-current prices between U.S. $5-12 per kilogram. In 2002, the actual selling price of PHA was nine times higher than synthetic plastics (Serafim et al., 2004). In another study (Gurieff and Lant, 2007), reviewing other LCAs of pure culture, determined that the lowest possible price at the time was U.S. $4.33 per kilogram compared to high density polyethylene at U.S. $1.85 per kilogram - a minor improvement, but for a mature industry,y not attractive enough for general marketability. Thus, the pure culture PHA method has historically, and continues to be, relegated to a specialized niche market.

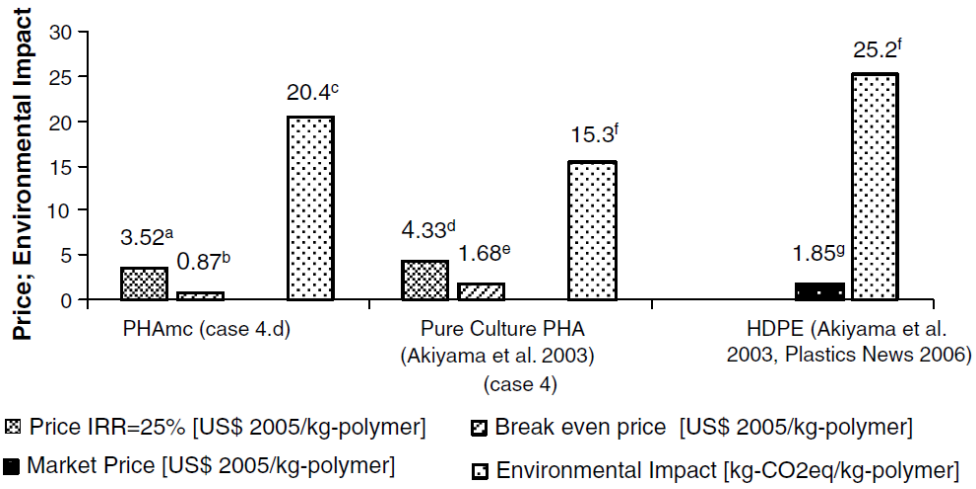


Figure 7: LCA comparing PHA methods to HDPE (Gurieff and Lant, 2007)

The first LCA to consider PHA production from mixed culture applying ADF in a significant way was published in 2007, the main results shown in Figure 7 (Gurieff and Lant, 2007). While a 20% reduction of carbon dioxide emissions could be obtained relative to HDPE, the final product cost, including a 25% rate of return, was still twice that for synthetic plastics. Although this is competitive to other biodegradable plastics, especially pure culture PHA, it is still too high relative to the price of readily available synthetic plastics to be attractive. However, as the authors' noted, these estimates were calculated based on extrapolation of laboratory work and a process/technology that is far from optimized. Avenues for process development were not specified, but optimism remained high that this technology configuration could become economically viable in the future. A more recent LCA that rigorously cover the mixed culture ADF process (Fernandez-Dacosta et al., 2015) did not compare against the pure culture method, and instead focused on the largest barrier to industrialization: extraction of PHA from the biomass. For three different techniques, Case III (see Figure 8) represented the largest cost at a ratio of 1.5 compared to polyethylene terephthalate, and Case I was the lowest cost at a ratio of only 1.1 compared

to PET. According to these authors, the PHA mixed culture process has already improved enough over seven years to be competitive with some synthetic plastics.

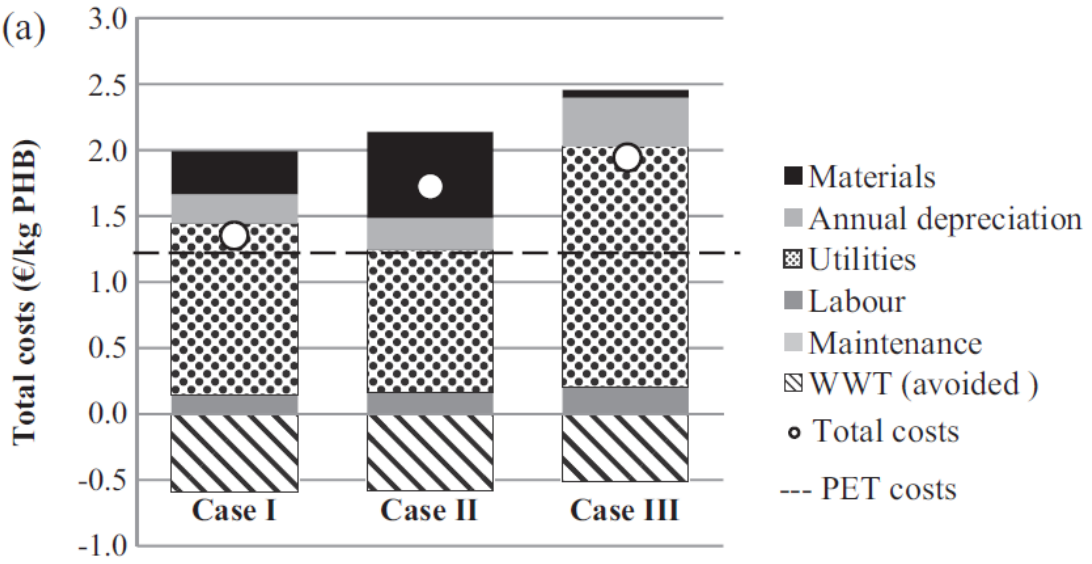


Figure 8: LCA for three extraction techniques (Fernandez-Dacosta et al., 2015)

LCAs can be valuable tools, but their accuracy and reliability, especially for a young industry, should be read with a moderate amount of skepticism (Yates and Barlow, 2013). In particular, extrapolating laboratory scale and results to full scale real systems is notoriously risky. Moreover, different LCAs will draw different conclusions, sometimes even contradictory ones, depending on the assumptions and techniques (Yates and Barlow, 2013). Nevertheless, the qualitative general trend is that PHA production via mixed culture achieved through application of ADF conditions is becoming increasingly attractive.

1.4 Engineering Considerations

From a strictly scientific point of view, each of the above described PHA production strategies has value and are worth further investigation. However, from an engineering

perspective, other factors are high priorities such as potential for commercialization and growth, cost, and ease of operation. From these process configuration choices, ADF represents the highest chance of success and the most deserving of research into process improvement. What follows is an introduction to the experimental and reactor design conventionally used for ADF as well as an engineering analysis of various carbon substrates and a justification for the choice of substrate used in this research.

1.4.1 Three Stage PHA Production using ADF

Volatile fatty acids are building blocks for bacteria to synthesize PHA. At the laboratory scale for ADF investigations, VFAs have often been supplied via a synthetic medium along with ammonium and other minerals required to sustain necessary metabolic processes. While this approach may have some utility at the lab scale to enhance our understanding of ADF, application at full scale for complex waste streams is the ultimate objective. To this end, a three stage process has been nearly universally adopted with slight differences in the operation of the individual stages (Dionisi et al., 2004).

The three stages consist of acidogenic fermentation, culture enrichment, and batch PHA production. A schematic taken from Beccari et al. (2009) is shown in Figure 9 for the specific case of olive oil mill effluents, but the process is almost universally the same regardless of which waste stream is used as a substrate. As described previously, the microbes require readily biodegradable carbon usually in the form of VFAs, although some evidence suggests (Beccari et al., 2009) that other forms of carbon can also be converted to PHA, albeit with lower efficiency. Thus, the purpose of the fermentation stage is to convert

complex, biodegradable carbon and particulate matter in the waste substrate to VFAs. Methanogenic bacteria are prevented from growth (and therefore consuming VFAs in the fermenter) by maintaining high volumetric turnover due to low solids residence time (Albuquerque et al., 2010a; Dionisi et al., 2004). Optimization of acidogenic fermentation has been thoroughly examined in the literature (e.g., (Grady et al., 2011)). Being the first unit in the PHA process, successful fermentation is of paramount importance, but its operation can be considered independently of the remaining stages and will therefore be considered herein simply as a means to an end – namely, the source of readily biodegradable carbon and VFAs.

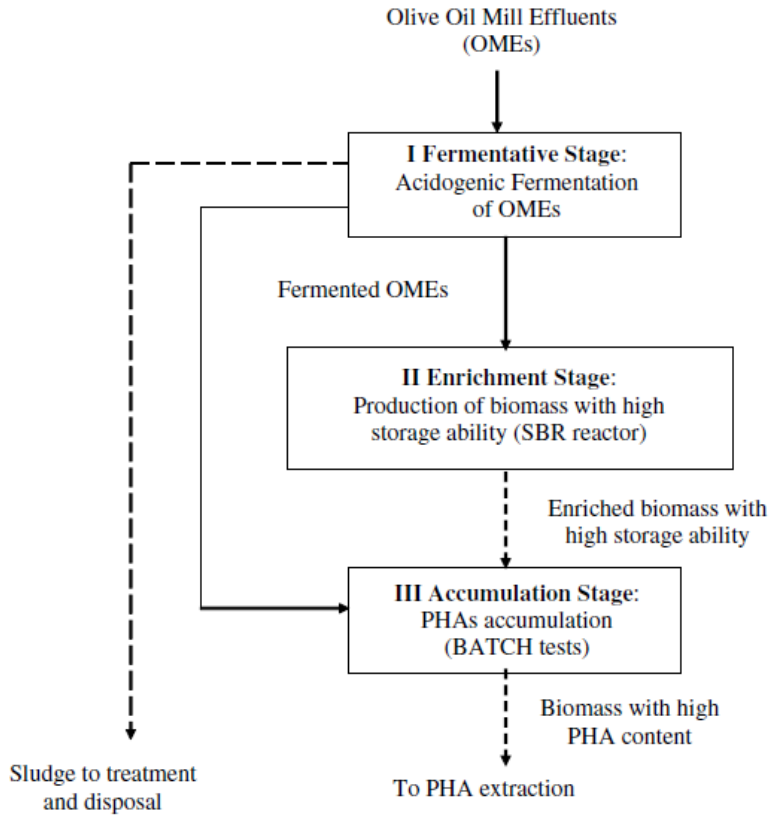


Figure 9: Three-stage process from waste substrate (Beccari et al., 2009)

The second stage in the PHA process is culture enrichment where ADF is applied to provide the selective pressure necessary to produce a highly enriched culture capable of hyper-PHA synthesis. In most cases a sequencing batch reactor with transient feed and decant times is the preferred vessel used to induce the required "feast-famine" response (Beun et al., 2000; Dionisi et al., 2005; Jiang et al., 2011b; Johnson et al., 2009a; Valentino et al., 2015), but in a few rare cases the feast-famine response was produced by utilizing a two stage continuously-fed continuous stirred tank reactor (CSTR) system (Albuquerque et al., 2010a; Marang et al., 2015). In the latter case the process diagram would be modified according to Figure 10 where the "feast" occurs in the first CSTR and the "famine" in the second with the selective pressure controlled by the hydraulic retention time (HRT).

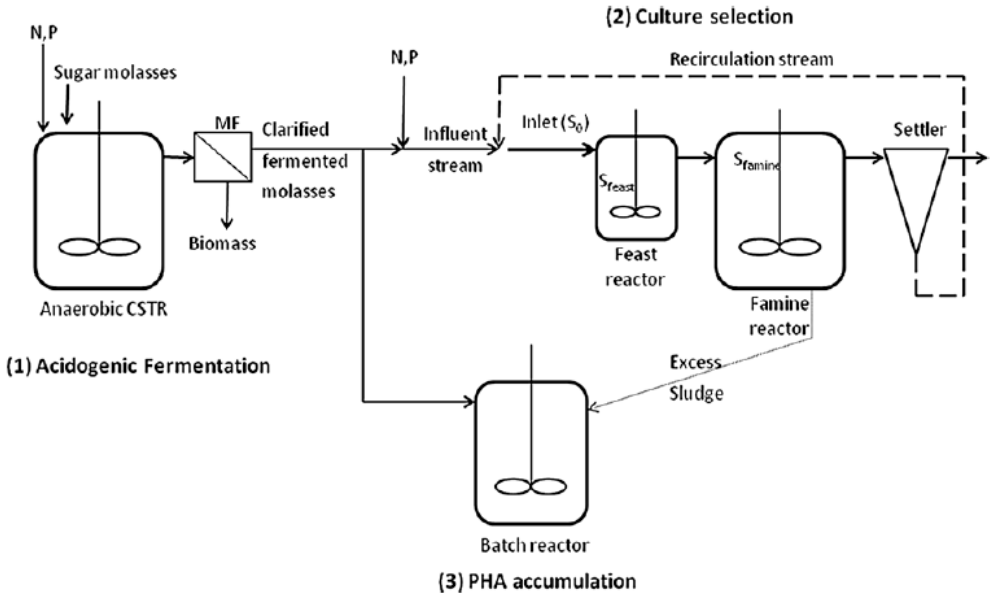


Figure 10: Two-stage CSTR culture enrichment (Albuquerque et al., 2010a)

The third and final stage in the PHA process is a batch accumulation reactor where highly enriched culture (both in fraction of PHA producers and associated kinetics) from stage two, usually the excess or wasted volume, is placed in a separate vessel and fed an

excess of exogenous carbon until maximum PHA reserves are realized. High concentrations of VFAs can be inhibitory on metabolic activity (Serafim et al., 2004), so the carbon is usually supplied as a series of pulses, with the concentration of substrate decreasing to near zero within each pulse (Beun et al., 2000; Dias et al., 2006). As long as substrate and oxygen (or some other terminal electron acceptor – e.g., nitrate) are provided in continual excess, the biomass will continue to accumulate PHA in excess of the intracellular concentrations observed in the culture enrichment stage until some other factor prevents further storage. Once maximal PHA accumulation has been realized, the reaction is terminated and the PHA-rich biomass is removed to an extraction phase (Fernandez-Dacosta et al., 2015) or alternatively, the biomass itself can be used as an end product (Coats et al., 2007).

The advantage of the three stage production process is that each unit can be optimized independently. There have been many successes in the literature in culture enrichment and in obtaining high intracellular PHA yields, yet it is not always clear if the enrichment and production stages were actually operating at optimum. Alternatively, poor performance may be the result of only one poorly operated reactor, but it can be difficult to determine which one. Emphasizing the distinction of and developing criteria for local optimization and may help elucidate which operational scheme is objectively best and strengthen the economic appeal at full scale.

1.4.2 Useful Carbon Substrates

The mixed microbial culture ADF PHA process is appealing not just because it converts a waste stream to a commodity of economic value, but also because it can be

applied to a wide range of byproduct streams. Synthetic medium containing acetate as the only carbon source was the most common feedstock used in early ADF research (Beun et al., 2002; vanAalastvanLeeuwen et al., 1997), which advanced by synthetic medium containing a mixture of organic acids (Dionisi et al., 2004). The use of synthetic substrate was justified to the extent that high levels of control were required for the variables being tested (Serafim et al., 2008) and the introduction of real waste would necessarily include uncontrollable variables and undermine interpretation of the results. This justification is still sometimes used in cases where model verification is required such as in the works of Johnson et al. (2009b) and Mozumder et al. (2014). However, as the ADF process has evolved and process understanding has improved, the focus has necessarily shifted towards its intended target of actual waste products. Some of the substrates used in research include (but are not limited to) municipal primary solids (Coats et al., 2011b), tomato cannery wastewater (Liu et al., 2008), biodiesel (Coats et al., 2007), olive oil mill effluent (Beccari et al., 2009), sugar molasses (Albuquerque et al., 2010a), dairy manure (Guho, 2010), paper mill effluent (Jiang et al., 2012), and whey (Valentino et al., 2015). Each complex waste substrate presents a unique set of advantages and disadvantages, and VFA composition can strongly affect the PHB/PHV balance in the final product. Other problems may include low alkalinity (highly variable pH) and dilute carbon content. For the most part, these challenges are not detrimental to the overall process and each choice is acceptable for research purposes.

1.4.3 Full Scope Regarding Dairy Manure

In the research presented herein, the model substrate used to conduct experimentation on an ADF system was dairy manure. The choice of substrate was motivated by the particularly low monetary value of raw manure, its ready availability in certain locales (i.e., the dairy industry has concentrated operations in certain regions of the U.S., improving the overall economic potential of PHA production from fermented manure), and continued development from previous group research (Coats et al., 2016-under review; Guho, 2010; Hanson et al., 2016-accepted-a; Hanson et al., 2016-accepted-b, c; Watson, 2015; Wei et al., 2014). Moreover, dairy manure is an excellent source of non-dilute, complex carbon which is the first prerequisite for the PHA process. Previous research has shown that manure can generate a sufficient VFA-rich, nutrient-rich effluent (the other prerequisites), which means the results generated herein are applicable to other waste streams. In other words, dairy manure serves as a model substrate that informs the entire PHA process.

The 3-stage PHA process using dairy manure is only one part of the overall strategy envisioned for dairy manure resource recovery. Figure 11 shows the proposed diagram which includes PHA-rich biomass, methane/electricity, lipid-rich algae, and potentially others. Currently the most common practice for manure disposal is land application (either raw, or partially stabilized from a lagoon); translating it into full resource recovery facility could significantly offset costs for farmers and provide environmental benefits.

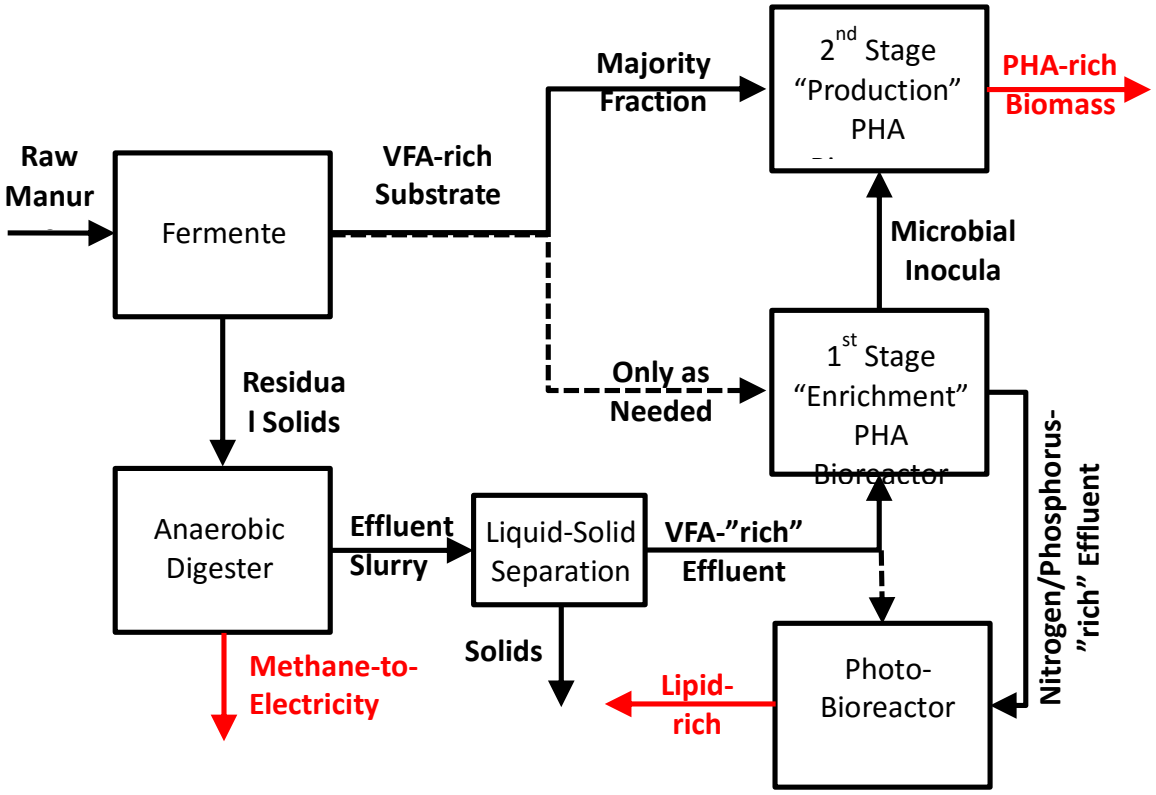


Figure 11: Proposed dairy manure to bio-products process diagram

The focus of this research is not to investigate every stage in the proposed resource recovery plan – only the stages that compose the PHA process. However, overall context is important and in this case showcases the fact that this research is not simply an academic exercise or thought experiment, but a means to provide real benefit to society.

1.5 Research Focus

The field of PHA investigation is quite broad, with implications in microbiology, genetics, chemistry, engineering, and business. In order to keep the scope of this research manageable and in a position to make a meaningful contribution to the existing body of literature, the following topics were addressed.

- Within the context of dairy manure treatment and optimization of intracellular PHA content, what is the maximum yield (Cmmol PHA per Cmmol VFA) that can be accomplished, and equally important, can these results be obtained with consistency?
- Every waste stream has a unique set of challenges that require a unique solution. The general setup for the ADF PHA process as described previously is well known, but within the context of dairy manure, system stability and control needs to be investigated. Can stability be sustained and how should the reactors be operated and monitored to achieve the desired results?
- The final end goal of this PHA research is to achieve commercialization at a full scale. In order to accomplish this, a mechanistic or fundamental explanation of process performance is needed as opposed to a phenomenological or “black box” approach. Therefore, can the results of this study be accounted for mechanistically that would allow application for a potential scale up?

To begin answering these research questions, an in depth literature review has been comprehensively conducted to find the best current practices and explore avenues for improvement.

CHAPTER 2: LITERATURE REVIEW

2.1 Synopsis of PHA Microbial Metabolism

Microbial metabolism is a vastly complex topic and a field of research in its own right. The fundamentals covered here are not exhaustive but do place the literature review in context of a theoretical framework. Most notably, the PHA mathematical models (reviewed in the next section) directly incorporate these biochemical reactions as thermodynamic constraints on the solution.

2.1.1 Biological Thermodynamics

Like any chemical reaction, the biochemical functions of microbes must fundamentally obey the laws of thermodynamics. The first law states that energy cannot be created or destroyed. The second law has many formulations, one of which is that the entropy of the universe must be always increasing. The relevance of the second law for biological systems is that the flow of energy, whether that energy is stored as chemical potential or the transport of electrons, must always be directed from a high to a low energy state or else the process cannot occur spontaneously. In this regard, the predominant type of biochemical reaction is oxidation-reduction (redox) wherein one reactant is oxidized by losing electrons from its chemical structure, with electron transfer to another reactant which is subsequently reduced. At a constant temperature and pressure the Gibbs free energy (a thermodynamic property) of each half reaction can be calculated separately, but the second law is fulfilled only if two half-reactions, one oxidation and one reduction, are coupled together and if the overall Gibbs value is negative. The requirement for a

nonnegative Gibbs value is necessary because it indicates the amount of energy released or energy that is “free” to the surroundings. Reactions with larger magnitudes have more energy potential that is able to be captured by the microbes (Koretsky, 2004).

In biochemistry, the oxidized reactant is referred to as the electron donor and the reduced reactant is the electron acceptor (Tchobanoglous et al., 2014). Because the half reactions are independent, many different combinations of electron donors and acceptors are possible, such as glucose or acetate (electron donors) being oxidized by oxygen (aerobic), nitrate (anoxic), or even glucose itself (anaerobic fermentation). The magnitude of the Gibbs free energy indicates which pairings are more energetically favorable within the microbial cell. The cell catalyzes the reaction and operates the same way an engine would, converting electrical energy to work such as growth and cell maintenance. Further, the metabolism of microbes is split into two distinct, yet coupled, processes: catabolism and anabolism (Kleerebezem and van Loosdrecht, 2010). Catabolism is the energy “generator” where the redox reaction from electron donor to acceptor is catalyzed by the cell and the energy is collected and chemically stored as adenosine tri-phosphate, or ATP. Anabolism is also a redox reaction wherein new cells are produced from a carbon and nitrogen source. Anabolism must consume ATP since the Gibbs energy is a large positive value and requires the energy release from ATP consumption (large negative Gibbs) to obey the second law. For heterotrophic bacteria, which are dominant in the ADF PHA process, the carbon substrate (commonly VFAs) is most often the electron donor for both the catabolism and anabolism and also the carbon source for biomass (Dias et al., 2006).

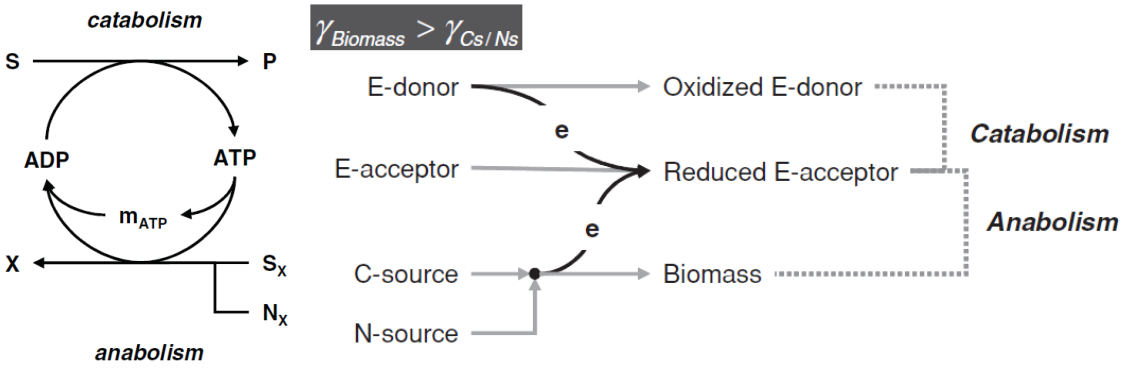


Figure 12: Coupled metabolism process (Kleerebezem and van Loosdrecht, 2010)

2.1.2 Substrate Uptake

In the ADF PHA process, VFAs are the most common and most important substrates utilized by the microbes, but to fully understand how they are consumed requires a brief review of glycolysis. Glycolysis is one of the most fundamental metabolic pathways in life. Glucose, the simplest of sugars, is converted via a multi-step process into pyruvate, a very common substrate-level intermediate (Madigan and Martinko, 2006). Glucose enters the cell passively but within the first five steps must consume two moles of ATP per mole of glucose, known as the investment phase. The remaining five steps, known as the payoff phase, produces four moles of ATP per mole of glucose, thus resulting in a net gain of two moles of ATP per mole of glucose. This method of ATP production is known as substrate level phosphorylation and is determined from stoichiometry. It should be distinguished from oxidative phosphorylation, covered more in depth later, which is more energy efficient but inexact in its ATP yield. Other substrates can enter this pathway at various stages by arriving from other metabolic processes; since these molecules enter glycolysis partway

through, the substrate level yield of ATP may be higher or lower than that of glucose (Madigan and Martinko, 2006).

Pyruvate has many functions in the cell, but the primary function is in a decarboxylation step, combined with the CoA enzyme, to produce acetyl-CoA. Here finally is where the fatty acids enter the cell's metabolism directly. While sugars and alcohols generally enter somewhere in glycolysis, VFAs enter after glycolysis but before respiration. Also, similar to how glucose requires ATP to become activated (before being recovered), VFAs require a net consumption of ATP per molecule to become activated. Longer chain fatty acids generally undergo decarboxylation (which consumes ATP) to generate acetyl- or propionyl-CoA and reducing equivalents. Reducing equivalents function as intracellular metabolites that catalyze the transfer of electrons from donors to acceptors (Madigan and Martinko, 2006). There are many different molecules of reducing equivalents, each having multiple notations. The two most relevant for the ADF PHA process are NAD^+/NADH and $NADP^+$/NADPH (in the form of oxidized/reduced). The difference between these molecules is minor, but important. NADH is the reducing equivalent responsible for transferring electrons from the donor (usually acetate) to the acceptor (usually oxygen) and producing energy as ATP via oxidative phosphorylation, whereas NADPH has a more specialized role in transferring electrons to acetate (more specifically, acetyl-CoA) to generate PHA (and other microbial macromolecules). Therefore, in order to store PHA, the cell must convert some NADH to NADPH via transhydrogenation or generate it through another pathway such as the pentose phosphate pathway (PPP).

Figure 13 illustrates the general pathway that VFAs take as they proceed towards PHA accumulation or respiration via the tricarboxylic acid cycle. Note that this pathway shows NADH rather than NADPH as the electron transport for PHA synthesis due to the similarity and often overlooked difference between the two. A closer look at Figure 13 reveals why VFAs are critical to process success, as they are already in the correct chemical form as the precursors to PHA. The steps that lead to PHA production are entirely optional for a fully functional prokaryotic cell, whereas substrate uptake and manipulation to acetyl-CoA are not. Elucidation of the pathway has shown that when PHA is produced, it does so by consuming acetyl- and propionyl-CoA and NADPH. When the cell is ready to use these reserves, the reaction is reversed: the CoA molecules and electron equivalents are restored but at the expense of some ATP consumption. Thus, storing PHA results in a net loss of energy compared to straightforward respiration, and a cell will only resort to it if strongly compelled (Dias et al., 2006; Madigan and Martinko, 2006).

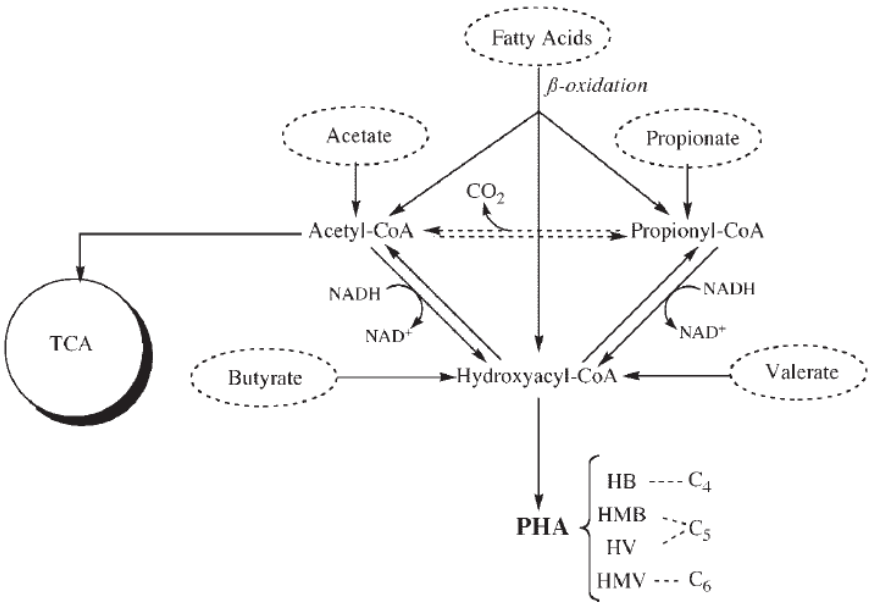


Figure 13: Fatty acid uptake, PHA storage and degradation (Dias et al., 2006)

2.1.3 Tricarboxylic Acid (TCA) Cycle

The TCA cycle is also known as the citric acid cycle (CAC) or the Krebs cycle. Acetyl-CoA is the main input, and the outputs are two moles of carbon dioxide and five moles of electron equivalents, some of which are NADH (Madigan and Martinko, 2006). The stoichiometry of the cycle is well defined, making it amenable for modeling purposes.

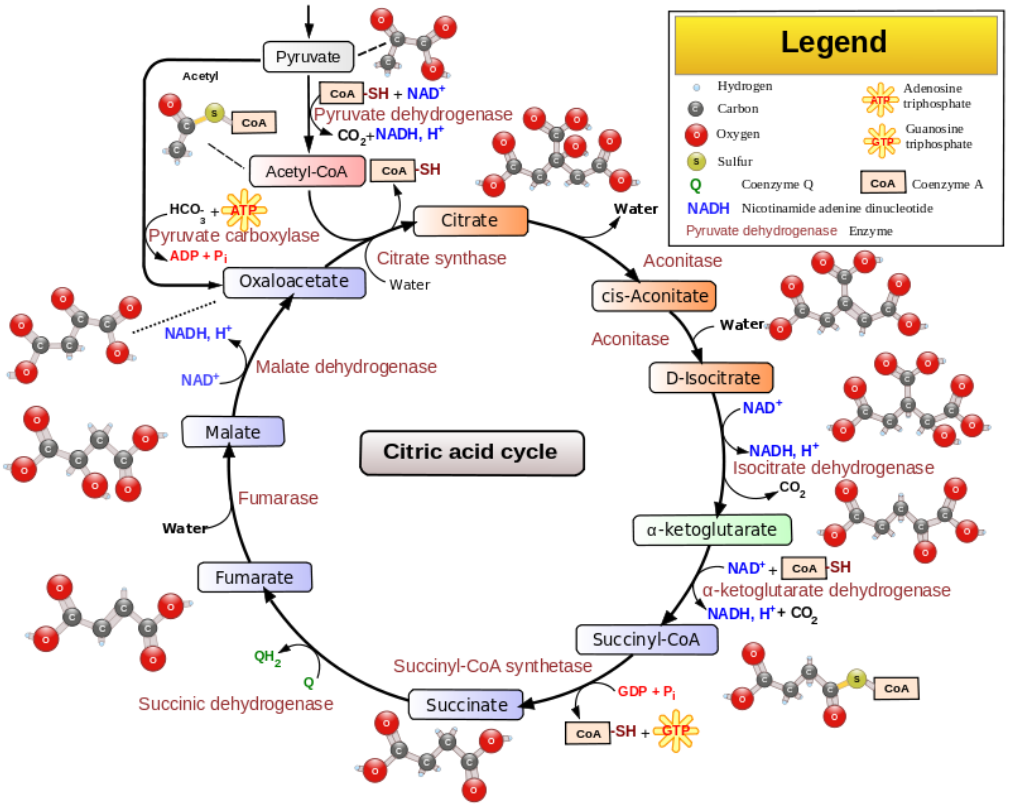


Figure 14: Tricarboxylic acid cycle (Narayanese et al., 2008)

Fundamentally, the chemical potential energy stored in the acetyl-CoA molecule (which often derives from a more energetic substrate like glucose) is harvested by stripping electrons from the chemical structure and oxidizing it to carbon dioxide. Like glycolysis, other molecules and intermediates may enter the TCA cycle at any point and increase or decrease the yield of reducing equivalents compared to acetyl-CoA. The cycle continues

indefinitely, unless inhibited, using acetyl-CoA as the primary input to primarily generate a continuous stream of carbon dioxide and NADH. At this stage the electron donor has completely shed all of its electrons and chemical potential to intracellular compounds. The electron equivalents will move to the next stage in respiration, oxidative phosphorylation, where that chemical energy is finally delivered to the electron acceptor and converted to ATP.

2.1.4 Oxidative Phosphorylation

In substrate-level phosphorylation, ATP is generated *in situ* from energy released in a chemical reaction when reactants are converted to products. Conversely, in oxidative phosphorylation ATP is still generated from a redox reaction, but the oxidation and reduction steps are separated and the driving force is a concentration gradient, otherwise known as the proton motive force (PMF). The generation of electrons from the donor have already been described in the previous two sections and all that remains is the final transfer of the electrons to the acceptor. Figure 15 provides a schematic of the PMF. In brief, NADH and other electron equivalents “unload” their electrons into enzymes embedded within the membrane of the cell which simultaneously pumps hydrogen ions outside the cell. The electrons reach their final destination with the electron acceptor which completes the redox reaction. The pumping of hydrogen ions outside the cells causes a concentration gradient across the membrane. According to chemical thermodynamics (Koretsky, 2004), hydrogen ions will spontaneously travel from a location of high concentration to low, thus minimizing Gibbs energy. This transport occurs passively via transport through another enzyme embedded in the membrane: ATP synthase. This enzyme works like an engine,

generating ATP by collecting the energy dissipated from an equalization of the hydrogen gradient (Madigan and Martinko, 2006).

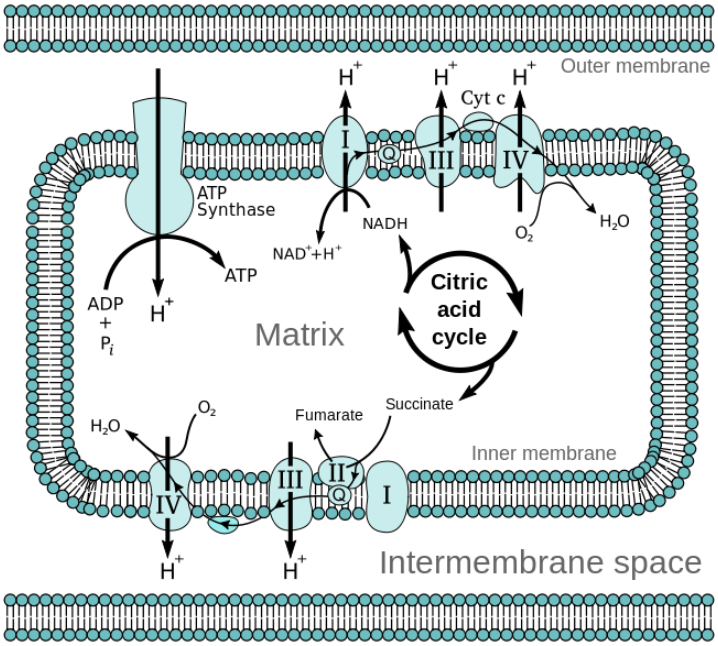


Figure 15: Oxidative phosphorylation with proton motive force (Fvasconcellos, 2007)

Since the PMF and ATP generation are the last step in a lengthy redox reaction, if an electron acceptor is not present, such as oxygen, all of oxidative phosphorylation cannot operate. The same is true of the TCA cycle and some parts of substrate uptake. In that case, the generation of ATP is usually restricted to substrate-level phosphorylation and must be attained via fermentation or other mode that is not respiration. The yield of ATP on NADH depends on how many hydrogen ions are pumped outside the cell and how efficiently ATP synthase collects energy from the PMF. This yield value is referred to as the P/O ratio and will vary substantially depending on external conditions. Theoretical estimates calculate the ratio to be about 3.0 mol ATP/mol NADH, but real cells are usually much less efficient,

attaining values ranging about 1.5-2.5 mol ATP/mol NADH (Dias et al., 2005; Madigan and Martinko, 2006).

2.1.5 Growth and Maintenance

The microbial cell generates ATP primarily for two general purposes: growth and maintenance. Growth is the generation of new biomass and commonly assumes a chemical formula of $C_5H_7NO_2$ (Tchobanoglous et al., 2014) but other formulae are possible (Tamis et al., 2014). Biomass synthesis requires a carbon, nitrogen, and electron source. In the PHA process, external substrate is used for both the carbon and electron source while ammonia is most often used for the nitrogen source. Anabolism is a well-characterized process and, for the purposes of this thesis, does not require a more detailed description with the exception of maintenance. Maintenance is used as a catch-all term for all the other non-growth cellular processes that consume ATP for various purposes. Since the other variables of interest (e.g., VFAs, oxygen, carbon dioxide, ammonia, etc.) are not involved in these reactions, it is only ATP that has an unbalanced loss term. In theory, an ATP balance can be calculated if the P/O ratio and the maintenance terms are known, which should sum to zero (Kleerebezem and van Loosdrecht, 2010). In practice, however, this balance is difficult to estimate.

2.2 Predictive PHA Model

Consistent with common scientific practice, a mathematical description of the ADF PHA process under fully aerobic conditions has been developed (building on previous literature) to better understand microbial behavior, confirm assumptions, and utilize for

scale-up and design. Fundamentally it is an initial value problem in which the state variables (such as substrate, biomass, and PHA concentration) are integrated with respect to time, subject to certain thermodynamic, stoichiometric, and kinetic constraints.

2.2.1 Model Description

The first proposed mathematical model was published by van-Aalast-van-Leeuwen, et al. (1997) and contained seven fundamental reactions. All subsequent developments by other authors have only made slight modifications, leaving intact the general structure summarized in Table 3. In short, external substrate is taken up into the cell at the expense of ATP. Substrate is either stored as PHA at the expense of NADPH (usually modeled for simplicity as NADH) or consumed through the TCA cycle and oxidative phosphorylation to regenerate ATP. Alternatively, the source of electron donors for the TCA cycle may come from the degradation of PHA. Growth occurs by consuming more electron donors in conjunction with ATP. Finally, some ATP is lost to other cell processes, generally lumped into maintenance. More detailed explanations of these metabolisms can be found in the preceding section.

Table 3: Metabolic model overview for ADF (Tamis et al., 2014)

1	Substrate uptake	VFA + ATP $\rightarrow$ VFA-CoA + n_0CO_2 + n_1NADH
2	PHA production	VFA-CoA + n_2NADH $\rightarrow$ PHA
3	PHA consumption	PHA + ATP $\rightarrow$ VFA-CoA + n_3NADH
4	Catabolism	VFA-CoA $\rightarrow$ n_3NADH + n_4CO2
5	Oxid. phosphorylation	NADH + $0.5O_2$ $\rightarrow$ (P/O)ATP
6	Growth	n_5VFA-CoA + n_6NH_3 + $Y_{ATP,X}$ATP $\rightarrow$ X + n_7CO_2 + n_8NADH
7	Maintenance	ATP $\rightarrow$ 0

The original application of this model by van-Aalast-van-Leeuwen, et al. used acetate as the sole substrate in synthetic growth medium for a pure culture. These conditions were

appropriate at the time for validating a new model, but they are far from realistic conditions that would be found at a full scale ADF PHA facility using real waste substrate. Recognizing this fact, subsequent authors progressively extended the model to more relevant situations beginning with mixed cultures (Beun et al., 2002) and ADF/"feast-famine" operation (Dias et al., 2005; Johnson et al., 2009b).

The protocol for mathematically implementing the metabolic model is to write a mass balance for each of the compounds in the system, such as acetate, PHB (acetate forms only PHB), ammonia, and biomass. The metabolic reactions are incorporated into the reaction rates and place constraints on the system. The mass balance for component "*j*" can be written as follows.

$$\frac{dM_j}{dt} = Q_{in}C_{j,in} - Q_{out}C_j + r_jV \quad (general\ form) \tag{2.1}$$

$$\frac{dC_j}{dt} = r_j \quad (batch\ reactor) \tag{2.2}$$

For a batch reactor, of which a SBR is a special case, the flow in and flow out are zero which simplifies to equation (2.2). The reaction rate of each component "*j*" is a linear combination of the seven metabolic reactions presented in Table 3. In the publication of (Johnson et al., 2009b), five reactions are active in the feast phase (omitting maintenance and PHB consumption) but the degrees of freedom is reduced to two by applying conservation of the intracellular metabolites acetyl-CoA, ATP, and NADH.

$$\frac{d}{dt}\begin{bmatrix} C_S \\ X \\ C_P \\ C_N \end{bmatrix} = \begin{bmatrix} r_S \\ r_X \\ r_P \\ r_N \end{bmatrix} = \begin{bmatrix} -1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 \\ 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0.2 \end{bmatrix} \cdot \begin{bmatrix} r_1 \\ r_2 \\ r_3 \\ r_4 \\ r_5 \end{bmatrix} \tag{2.3}$$

$$\begin{bmatrix} r_{ATP} \\ r_{Ac\_CoA} \\ r_{NADH} \end{bmatrix} = \begin{bmatrix} -1 & 0 & 0 & \delta & -2.16 \\ 1 & -1 & -1 & 0 & -1.267 \\ 0 & -0.25 & 2 & -1 & 0.434 \end{bmatrix} \cdot \begin{bmatrix} r_1 \\ r_2 \\ r_3 \\ r_4 \\ r_5 \end{bmatrix} = \begin{bmatrix} 0 \\ 0 \\ 0 \end{bmatrix} \tag{2.4}$$

$$-r_S = q_S^{max}\left(\frac{C_S}{K_S + C_S}\right)X \tag{2.5}$$

$$r_X = \mu_{max}\left(\frac{C_S}{K_S + C_S}\right)\left(\frac{C_N}{K_N + C_N}\right)X \tag{2.6}$$

By defining any two additional rates the entire network becomes well defined and the ordinary differential equations can be solved simultaneously using numerical methods. Typically, acetate uptake and biomass growth are the easiest rate laws to implement using Monod kinetics (equations (2.5) and (2.6)); both reactions are zero order at non-limiting concentrations of substrate and ammonia. In some cases the rate of intracellular PHB synthesis is explicitly defined as the rate limiting step due to self-inhibition or the absence of external substrate (Jiang et al., 2011a; Johnson et al., 2009b). The parameters for the rate laws are determined either by assumption (e.g., half-saturation coefficients) or by calibrating to experimental data using a least-squares algorithm (e.g., maximum uptake and growth rates). Also, the stoichiometric coefficients are in many cases educated guesses, including the crucial "δ" term which is the P/O ratio for oxidative phosphorylation.

Real waste streams contain a myriad of compounds that necessarily complicate the ADF PHA model. The publication of Dias et al. (2008) conducted batch experiments using combinations of acetate and propionate – the first step towards an arbitrary substrate composition. However, this increased the number of equations to thirteen with only seven constraints, requiring six rate laws be determined in advance. Jiang et al. (2011a) simplified the Dias et al. model to eleven reactions with nine constraints, bringing the degrees of freedom back to two. A good fit was achieved in both cases, but the level of calibration to experimental data necessarily increased, making it difficult to compare results among researchers and limiting the model's generality and predictive power.

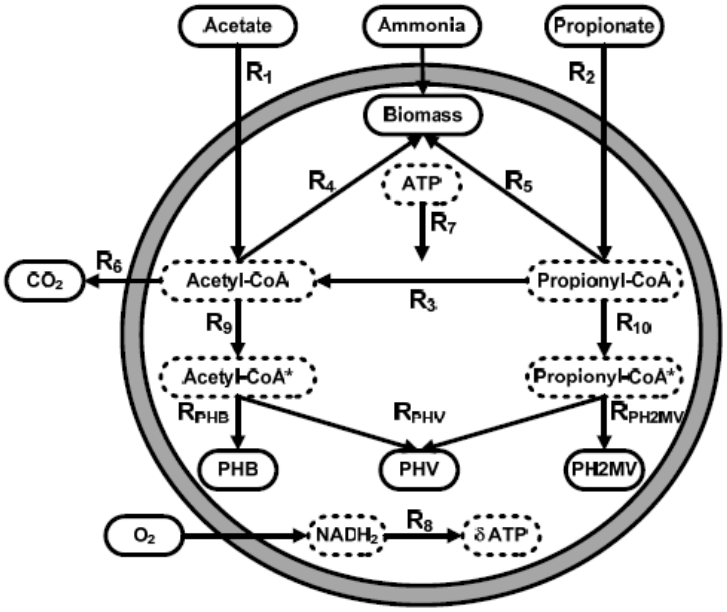


Figure 16: Schematic representation of metabolic model (Dias et al., 2008)

The first attempt to account for an arbitrary composition of substrate was by (Pardelha et al., 2012) which resulted in fourteen equations with six constraints. To overcome the increasing difficulty of producing a fully determined system with valid constraints, the authors employed a numerical technique called flux balance analysis (FBA) which seeks to

solve an undetermined system by minimizing a specified objective function. It was determined that flux through the TCA cycle should be minimized and the model was validated for dynamic conditions (Pardelha et al., 2014). However, this validation came at the cost of even more calibration with experimental data, negating any advancement as even parameters within the same paper operating under similar conditions did not match.

2.2.2 Limitations

The original model proposed by van-Aalast-van-Leeuwen, et al. (1997) is reasonably simple and general due to artificial conditions, but as others have made developments it has become more complicated and less useful the closer it approximates real systems (i.e., mixed microbial cultures and real waste streams). Complications are not intrinsically problematic, but an overly-parameterized model runs the risk of predicting only the exact data used for original inputs, which undercuts its validity altogether. The review paper by Tamis et al. (2014) addresses some of these concerns by identifying, among other things, the importance of finding a mechanistic (non-calibrated) explanation or description of substrate uptake, growth, and PHA consumption. Some success has been accomplished in relating the end-of-feast PHA content to the operational parameters cycle length (CL) and solids residence time (SRT) but neglects the effect of organic loading rate (Jiang et al., 2011b). Currently, PHA accumulation and consumption is modeled with a so-called "maximum" value, but this has been determined solely by model calibration and therefore has no predictive power. It has been suggested that a better approach is to model the flux of PHA as the net rate of gain due to substrate uptake minus first-order consumption rate,

which can also be related to the CL and SRT (Tamis et al., 2014), but this has yet to be implemented in the literature in any significant way.

As it regards the research questions of this thesis, a method to approximate PHA content would be highly useful for design and establishing a firm relationship between the enrichment and production phases. However, the reliance on data calibration hinders progress. A new approach may be needed that first seeks to establish a causal relationship between the stages which can replace calibrated parameters with calculated ones.

2.3 Culture Enrichment Strategies

Culture enrichment research for PHA synthesis is well studied, and the basic process mechanisms are understood, but a lack of consensus among researchers regarding best operation practices to maximize PHA production combined with non-standard presentation of data makes it difficult to compare and contrast results across publications. In this section, theoretical growth rates in a SBR form a fundamental basis of comparison and common interpretive framework.

2.3.1 Growth Rates in SBRs

In biological systems, the total mass of cells and their rate of growth dictate the rate of reaction of all the other variables, including substrate consumption and PHA synthesis. The growth rate is influenced by many factors including reactor operations and whether or not the biomass concentration changes with respect to time or space. For example, it can be shown that the net specific growth rate for a single CSTR at steady state is the inverse of the SRT (Tchobanoglous et al., 2014). However, for a SBR at steady state, although each

cycle is identical, the biomass concentration changes with time so that it is different at the beginning and end. This phenomenon was only recently analyzed and fully elucidated by (Guho, 2015-under review), a publication which is developing the theoretical equations for the net specific growth rate in a SBR generalized for all possible modes of operation. The derivation for SBR operation typically performed in the PHA process, adapted from Guho (2015-under review), is presented herein. The definition of terms and variables can be found in the "Nomenclature" section.

The growth of suspended biomass follows a first order reaction rate with the proportionality constant being the net average specific growth rate. The initial condition is simply that the biomass exhibits an initial concentration at time zero. It is assumed that there are no external limitations such as substrate or nutrient deficiency.

$$\frac{dX}{dt} = \mu_{net} \cdot X \,, \quad X(0) = X_0 \tag{2.7}$$

The analytical solution to the differential equation evaluated at the limits of integration yields the following expression for biomass as a function of time.

$$X_t = X_0 \cdot e^{\mu_{net} t} \tag{2.8}$$

At the end of a cycle, a certain fraction of the volume will be decanted, removing cells from the system, and replaced with feedstock that presumably has no cells in it, effectively diluting the remaining biomass. For a SBR at steady state, the biomass concentration at the beginning of every cycle must, by definition, be the same. The dilution factor can be

represented as the ratio of CL to SRT which is also the volume fraction of the reactor that is wasted every cycle. This can be mathematically related to the ratio of beginning and ending biomass concentrations according to equation (2.9). The main assumption with this equation is that the reactor is operated without a settling phase.

$$\frac{X_0}{X_f} = 1 - \frac{CL}{SRT} \tag{2.9}$$

Combining equations (2.8) and (2.9) yields the final expression for net specific growth rate per "division" in an SBR.

$$\mu_{net,div} = \frac{1}{\Delta t_{div}} \ln\left(\frac{1}{1 - \frac{CL}{SRT}}\right) \tag{2.10}$$

A division is the length of time during a cycle when growth may occur and is often represented by a substantial metabolic or operational shift. For example, in the PHA process the "feast" is a time period when all microbes may grow freely and the "famine" is growth restricted for all but PHA accumulators (provided internal PHA reserves are not depleted). In general, growth from endogenous decay during times of stress can be considered negligible.

The implications of this result are far-reaching as related to understanding how operations impact reactor performance on a fundamental level. It relates a crucially important kinetic parameter to easily controllable operational parameters without resorting to fitted experimental data or empirical correlations. Also, the equation can be applied to

different microbial phenotypes, such as PHA accumulators, non-PHA accumulators, and even autotrophs like ammonia oxidizing bacteria. With proper analysis, the design of experiments can be arranged to target a desired growth rate (or set of rates) that will most effectively cull out non-PHA producers and promote high carbon storage rates for PHA producers. It can also have application in modeling as illustrated by (Jiang et al., 2011b) and (Tamis et al., 2014). In those papers a special case of the growth rate equation was derived where the maximum feast PHA was predicted.

$$\frac{C_{PHA,end\_feast}}{X_0} = \frac{1}{Y_{X/STO}\left(\frac{SRT}{CL} - 1\right)} \tag{2.11}$$

While equation (2.11) requires an additional restriction that PHA reserves are fully depleted exactly at the end of the cycle, it nonetheless shows promise as a mechanistic explanation of PHA degradation in the famine that does not require fitting parameters to experimental data.

The review of operating conditions that follows are interpreted in light of this fundamental framework to find common ground and reconcile different conclusions.

2.3.2 Major Operational Parameters

For an enrichment culture operating under ADF conditions in an SBR, there are primarily three parameters the operator can easily manipulate and use as a basis for design: cycle length (CL), solids residence time (SRT), and organic loading rate (OLR). Finding the optimized combination of these parameters for ADF PHA operations has remained an elusive, long-standing research goal, with a general consensus only now beginning to form.

First, maximizing the unitless ratio of CL to SRT was identified by (Jiang et al., 2011b) as a potential selector for high intracellular PHA in the feast period. The publication by Johnson et al. (2009a) operated at a ratio of 0.5, about five times higher than previous studies, and achieved a mixed culture weight percent of 89%. This is the highest content achieved to date on mixed culture (albeit with synthetic feed) and the CL to SRT ratio is certainly useful in providing an explanation. Second, the feast to famine ratio (abbreviated F/F) was extensively analyzed by (Albuquerque et al., 2010b) who showed that this ratio should be minimized in order to exert the highest selective pressure for PHA accumulators. Empirically, a F/F value below 0.25 was recommended and below 0.20 was identified as "ideal." The F/F ratio is a mostly a function of OLR and CL (although SRT is non-negligible), and it is thus unclear how much control the operator has over this parameter, but it is preferentially reported over OLR because it is more conducive to dimensional analysis. The results from the literature shown in Table 4 present the major operating conditions along with best accumulation data. The selection summarized in Table 4 is by no means comprehensive but it is certainly representative and includes the most impactful and oft-cited publications. The order of entries is from least to most successful accumulation, with the exception of (Valentino et al., 2015) which is presented last for easy reference as it represents the closest comparison for the research presented herein.

Table 4: Compilation of results for literature reviewed ADF

Reference	Enrichment Operation[1]	Production PHA Results[2]
(Beun et al., 2002)	CL = 4 hr; SRT = 4 d; F/F = 0.081; synthetic	Single pulse, fed-batch: 40% VSS
(Dionisi et al., 2005; Dionisi et al., 2004; Dionisi et al., 2006)	CL = 2 hr; SRT = 24 hr; synthetic 1) F/F = 0.11, 8.5 gCOD/L 2) F/F = 0.25, 12.75 gCOD/L 3) F/F = 0.33, 20 gCOD/L 4) F/F ≈ 1.0, > 20 gCOD/L	Fed-batch, NH_3 limited: 1) 50% COD 2) 49-65% COD 3) 45% COD 4) ≈10% COD
(Villano et al., 2014)	CL = 6 hr; SRT = 24 hr; F/F = 0.29; synthetic	Fed-batch, NH_3 partially limited: 40-60% VSS (6 hr limit)
(Albuquerque et al., 2010b)	CL = 12 hr; HRT = 24 hr; SRT = 10 d; fermented sugar molasses 1) F/F = 0.21, 30 Cmmol/L 2) F/F = 0.22, 45 Cmmol/L	Fed-batch, NH_3 limited: 1) 30% VSS 2) 75% VSS
(Jiang et al., 2012)	CL = 12 hr; SRT = 24 hr; F/F = 0.091, full depletion; paper mill effluent	Fed-batch, NH_3 limited: 77% VSS
(Serafim et al., 2004)	CL = 12 hr; HRT = 24 hr; SRT = 10 d; F/F = 0.073; synthetic	Fed-batch, NH_3 partially limited: 78.5% VSS
(Jiang et al., 2011b)	SRT = 24 hr; synthetic 1) CL = 4 hr; F/F = 0.15 (20°C) 2) CL= 4 hr; F/F = 0.047 (30°C) 3) CL = 12 hr; F/F = 0.20 (20°C) 4) CL = 12 hr; F/F = 0.049 (30°C)	Fed-batch, NH_3 limited, VSS: 1) 18% (En.) 2) 22% (En.) 3) 51% (En.) 72% (Pr., 11 hr) 4) 53% (En.), 82% (Pr., 4 hr)
(Johnson et al., 2009a)	CL = 12 hr; SRT = 24 hr; F/F = 0.091; synthetic	Fed-batch, NH_3 limited: 89% TSS (low ash content)
(Valentino et al., 2015)	CL = 12 hr; HRT = 24 hr; SRT = 6 d; F/F = N/A; fermented whey permeate	Semi-continuous, substrate limited, controlled growth: 50-70% VSS

[1] Units: hr = hour, d = day, Cmmol/L = carbon-millimole per liter

[2] COD, VSS, and TSS indicate the basis of calculation and units used for determining percent. En. = enrichment phase, Pr. = production phase; time indicates reaction length when weight percent was determined.

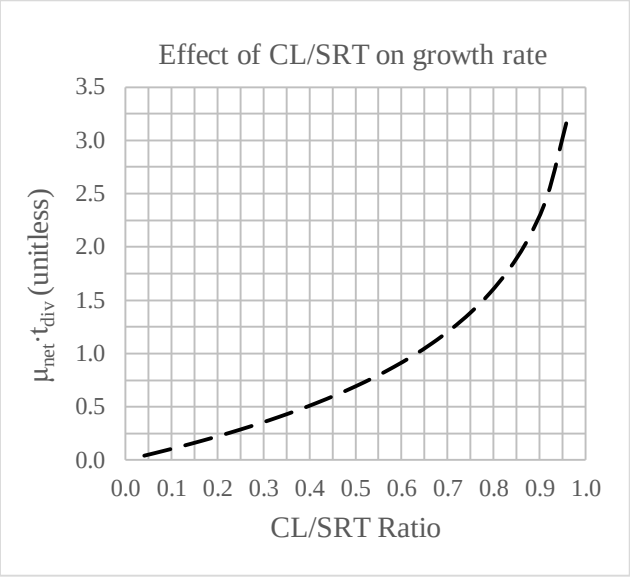


Figure 17: Graphical interpretation of equation (2.10)

Several important trends emerge from this collection of data that require discussion. First is the confirmation that the CL to SRT ratio for comparable F/F ratios has a significant impact on culture selection for PHA production. Most notable is the data from Jiang et al. (2011b) where an increase from 0.167 to 0.50 resulted in a significant increase in PHA content at the end of the feast cycle, from 18% to 51%. Indeed, an unmistakable correlation exists between research that used a CL to SRT of 0.50 (12 hour CL and 24 hour SRT) and high intracellular PHA content – a correlation that is independent of temperature and whether the HRT equals SRT. From an analysis of equation (2.10) and its graphical interpretation (Figure 17), it is no surprise why this should be the case. Increasing the CL from 4 to 12 hours (for 24 hour SRT) represents nearly a four-fold increase in the unitless growth rate. This exerts a tremendous selective pressure on the biomass as the mass balance requires that the microbes either adapt for faster growth or be culled out of the system. Thus, carbon utilization will occur very quickly, giving a natural advantage to PHA accumulators as this process is faster and less energy intensive than growth. Figure 17 is only valid for chemostats, but empirical evidence from Table 4 suggests that HRT does not need to equal SRT to exert similar pressure. In those cases, HRT seems to be the dominant parameter.

The second important trend is the confirmation that a minimized F/F ratio, for comparable CL to SRT ratio, exerts selective pressure as well. From equation (2.10) the F/F ratio is simply a measurement of the ratio of the net specific growth rates for the two main microbial populations: PHA accumulators and non-accumulators.

$$F/F = \frac{t_{feast}}{t_{famine}} = \frac{\mu_{net}^{accum.}}{\mu_{net}^{non\_accum.}} \quad (2.12)$$

For sufficiently high CL to SRT and low F/F ratios, the required growth rate of non-accumulators needed to sustain their presence in the system becomes so large that it effectively culls them out completely, leaving a highly enriched PHA-accumulator population. This is confirmed by Dionisi et al. (2006) when a significant drop in accumulation potential occurs after the F/F increases above 0.25, and also in Jiang et al. (2011b) when the maximum intracellular content increased from 72% to 82% when the F/F was reduced from 0.20 to 0.05, indicating that the former had a larger non-accumulator population. Importantly, it was shown by Albuquerque et al.(2010b) that increasing the OLR (for a constant CL to SRT ratio) barely increases the F/F ratio due to an increase in population resulting from a higher substrate concentration. Fundamentally, from equation (2.11), the growth of non-accumulators remained unchanged and the extra carbon introduced with a higher OLR went almost exclusively to PHA storage as was demonstrated when a 50% increase in OLR (with almost no change in the F/F ratio) resulted in a 150% increase in intracellular content. The extra biomass resulting from the increase in OLR can be interpreted to be almost exclusively PHA-accumulators, hence the reason why volumetric productivity was greatly enhanced. There is evidence however that suggests

there is a lower limit to the F/F ratio. An attempted duplication of results from Johnson et al. (2009a) by Jiang et al. (2011b) could only reach 82% compared to 90% with the only difference being a F/F ratio of 0.05 instead of 0.10, respectively. A possible explanation is that both F/F ratios virtually eliminate non-accumulators, but the former has capacity for a larger OLR due to lower pressure on the PHA-accumulators.

The third and final trend emergent from the summary in Table 4 is the effect of real waste streams used in lieu of synthetic medium. As discussed in Albuquerque et al. (2010b) and Jiang et al. (2012), fermented products derived from real waste are rarely composed entirely of readily biodegradable VFAs and in fact will contain many different carbon compounds including an array of slowly biodegradable carbon. The net effect, from a growth rates perspective, is that non-accumulators can grow on slowly biodegradable carbon after the readily biodegradable compounds are gone (i.e., after the feast period), thus lowering their required specific growth rate. Alternatively, the feast period may be elongated due to the inability of PHA-accumulators to consume certain substrates, thereby introducing competition for a specialized class of non-accumulators. As a case study, Jiang et al. (2012) duplicated the conditions of Johnson et al. (2009a) including the F/F ratio but could only reach 77% on paper mill effluent compared to 90% on synthetic medium. Similar results were achieved by Albuquerque et al. (2010b) and Valentino et al. (2015) for sugar molasses and whey permeate, respectively, with the latter employing controlled residual growth in the production reactor.

2.3.3 Minor Operational Parameters

Minor ADF PHA operational parameters include secondary, less controlled elements of design including temperature, pH, and chemical (e.g., nutrient) addition. Adding these additional layers of control increases the overall cost of production and perhaps unnecessarily complicates the system (Valentino et al., 2015). For example, Serafim et al. (2004) attempted to control the pH for a reactor operating with synthetic medium to be consistent with other reports but found that it actually decreased productivity and better results were obtained when the pH was allowed to "settle" around 7.5. The research by Dionisi et al. (2005) showed the optimal range for a synthetic reactor is in the range 7.5-8.5 with a large decrease in activity outside that range. Manipulating pH could be a method to control final polymer composition but it comes at a cost. Similarly, operating temperatures of 30°C have been shown to dramatically increase rates of reaction (Jiang et al., 2011b) but the same article also showed that the CL to SRT ratio was a better selector and was independent of temperature. Furthermore, increased kinetics primarily serves to decrease the F/F ratio which can be accomplished by altering the major operational parameters (CL, SRT, and OLR). Higher temperatures can arguably improve culture selection in other ways but the cost of energy to sustain those temperatures negatively impacts profitable potential (Fernandez-Dacosta et al., 2015). Because these parameters are of secondary importance and increase cost, their use should be avoided or minimized.

2.4 PHA Production Strategies

The PHA accumulation, or production, reactor utilizes inocula from the enrichment reactor and mixes it with external substrate to induce maximum PHA storage and

intracellular yield (see Figure 9). The purpose, of course, is not to induce a feast-famine response, but rather to induce an extended feast. As such, the production reactor is biologically similar, if not identical to, the enrichment reactor in terms of metabolism and mechanisms but largely different in terms of operation and control. The following subsections will focus on the latter, with the assumption that the enrichment was metabolically the same except where noted.

2.4.1 Substrate Dosing Method

The enrichment reactor most often is operated as a SBR, and therefore receives the entire dose of external substrate in one large pulse at the beginning of the operational cycle. When the carbon is depleted, the famine period begins. In principle, the production reactor may be operated similarly except with a larger mass of carbon necessary to achieve maximum yields. However, by far the most common technique for substrate dosing is fed-batch, first described in Majone et al. (1999) and partially implemented in Beun et al. (2002) and Dionisi et al. (2004), wherein the mass of carbon is dosed in discrete pulses, with each new pulse coming at the depletion of the previous. An illustrative example of this technique from the literature is shown in Figure 18.

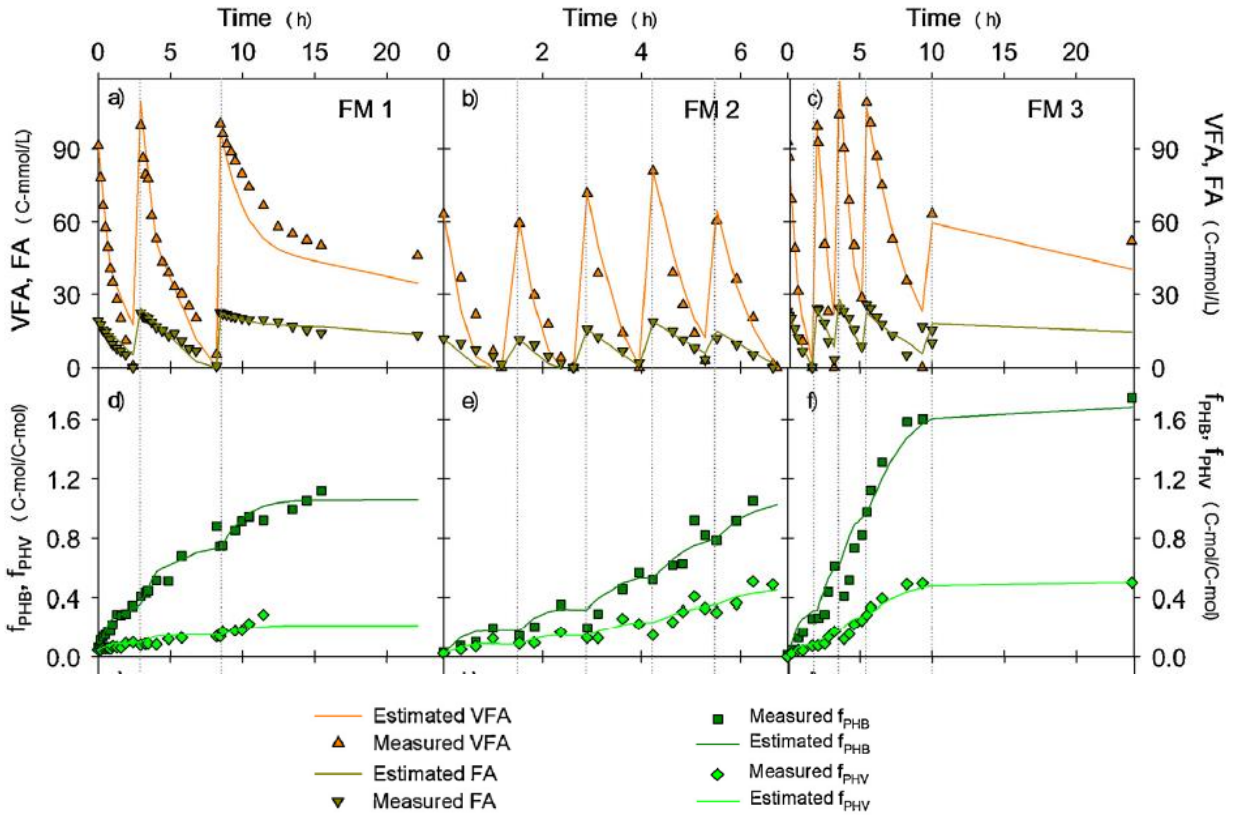


Figure 18: Example of fed-batch substrate dosing method (Pardelha et al., 2014)

There are two major benefits to splitting the carbon substrate dose into multiple, discrete pulses. The first is that it simulates the enrichment reactor more closely by providing substrate gradient conditions to which the microbes are most adapted, while simultaneously preventing immediate dilution of the biomass concentration, an effect that can dramatically slow the rate of reaction. The second is that it keeps the substrate concentration below inhibition levels. In the work of Serafim et al. (2004) a single pulse at 180 Cmmol/L yielded the highest intracellular PHA content but the rate of reaction was an order of magnitude lower than single pulses at lower initial concentrations. By switching to a fed-batch mode, splitting the 180 Cmmol/L into three pulses of 60 Cmmol/L, the authors

were able to retain the high PHA content while increasing the rate of reaction and overall productivity.

Although fed-batch is nearly universally used in PHA production, it is not without its own set of challenges. Foremost, it can be difficult to accurately determine when the next pulse should be dosed, a problem addressed in the subsequent section. Also, as mentioned previously, it is difficult to scale up a discrete-pulsed batch reactor to full size and it would be ideal to find a more convenient and simple mode of operation. One alternative is a fed-batch system with smaller initial pulse concentrations and more frequent feedings that would, in the limit, approach a continuous fed batch reactor. The only paper to consider this option seriously is Serafim et al. (2004) where it was concluded that the low residual VFA concentration was limiting the reaction and underperformed its fed-batch equivalent in terms of maximal PHA accumulation. However, in that case the production reactor was operated under growth limited conditions. The work of Grousseau et al. (2013) operated in continuous mode for a pure culture under growth-allowed conditions and was able to achieve better comparative results. Extending that idea to mixed culture was Valentino et al. (2015) who operated in quasi-continuous mode by having several small pulses and a VFA-limited reaction, also with growth allowed. Both of the previous papers were studying the effects of growth and not the viability of a continuous fed reactor as compared to a fed-batch. It remains to be tested whether a continuous fed system under VFA excess (non-limiting and non-inhibiting) is an acceptable alternative more amenable to process control and scale up than fed-batch.

2.4.2 Growth Restrictions

The objective of the PHA production reactor is to maximize intracellular PHA content and eliminate or reduce the carbon flux to any other process. Cellular growth is a large consumer of external substrate, requiring it as the carbon source for biomass and the electron donor for ATP generation. Limiting growth, then, seems like a reasonable strategy. In Dionisi et al. (2004) growth was not restricted and the authors noted that PHA saturation within the cell was likely not achieved as a result, either due to growth of a flanking population or adaptation of the PHA producers to more favorable external conditions. Growth limitation was specifically addressed in the follow up publication (Dionisi et al., 2005) where it was observed that restricting ammonia did have a slight, but not overwhelming, advantage over non-growth limited reactors; the authors admitted that the literature is inconclusive regarding mixed cultures and more research should be done. Since then, nearly every published accumulation experiment, with the intent to maximize intracellular PHA, has performed the reaction with ammonia restrictions to prohibit growth, the most notable result being a maximum yield of 89% on synthetic medium (Albuquerque et al., 2010a; Johnson et al., 2009a).

Recently, the conventional wisdom of limiting growth has been challenged. Specifically, the electron equivalent needed for PHA storage is NADPH, not NADH, which is more efficiently generated through the Entner-Doudoroff (ED) pathway and not the TCA cycle (Grousseau et al., 2013). However, when growth is limited, a buildup of ATP occurs which prevents activation of the ED pathway; the TCA cycle is still active and can generate NADPH but the theoretical yield decreased from 0.82 to 0.67 Cmmol/Cmmol. Experimental

results showed that, despite the loss of some carbon to biomass, controlled residual growth increases productivity without sacrificing intracellular yield. This idea was extended to mixed cultures by Valentino et al. (2015) who showed that high productivity can be achieved without restricting nutrients. A growth restriction of a different sort was utilized in that paper in that external carbon was maintained at low concentrations but not low enough to prevent growth. According to the authors, this selected for growth of PHA accumulators only while preventing growth of flanking populations. Perhaps most importantly, the entire nutrient starvation strategy has little practical value for most waste-based substrates, as ammonia and phosphorus are already present in excess. In these cases, non-limiting restrictions on substrate (Valentino et al., 2015) or oxygen (Third et al., 2003; Watson, 2015) may be more appropriate ways to control the flow of carbon to PHA, if it is necessary at all.

2.4.3 Process Control Techniques

Process control for the enrichment reactor mostly involves tuning of the easily alterable parameters such as cycle length, solids residence time, organic loading rate, and residual oxygen concentration. VFA and PHA depletion and accumulation are monitored rather than controlled. However, in the process of PHA production it is crucial to maintain the VFA concentration between the bounds of inhibition and limitation to ensure continuous “feast” conditions and prevent loss of intracellular PHA (through famine). Thus, for a fed-batch system (and to a lesser extent, a continuous fed), knowing the rate of VFA consumption and when to add the next pulse of substrate needs to be determined with utmost accuracy. Online VFA probes are not available at this present time, which has led

researchers to pursue primarily three different control strategies: pH, residual dissolved oxygen (D.O.), and oxygen uptake rate (OUR).

Control based on pH has only been attempted a few times, most notably by Johnson et al. (2009a). In that work, the feedstock was purely synthetic with low alkalinity and the subsequent pulses were presumably dosed when the pH approached the baseline of VFA depletion. Although some have found success with this method (e.g. (Jiang et al., 2012), (Marang et al., 2013)), irregular conditions, excess alkalinity (which will buffer pH changes), and the ambiguous nature of shifting pH in a biological system have encouraged researchers to try other methods. For example, Serafim et al. (2004) and Dias et al. (2005) opted instead to leverage a sudden D.O. spike following VFA depletion to indicate the need of the next substrate pulse. The main drawback of this process control technique is that it is entirely reactive, requiring the concentration to pass through the VFA-limitation stage all the way to depletion, potentially (likely) causing PHA reserves to temporarily decline. Such an outcome was partially avoided (Albuquerque et al., 2010b) by observing the pO_2 instead of O_2 concentration and by Valentino et al. (2015) who employed sophisticated D.O. monitoring equipment to dose the next pulse when the concentration reached 30% of its VFA-depleted baseline. Implementing the latter method requires highly sensitive and very finely tuned devices, as time delays in sensors and erratic D.O. patterns can easily confound this method. The third technique, control based on the OUR, alleviates some of the intricacy by being performed with standard residual D.O. sensors or even online OUR detectors (Dionisi et al., 2006; Johnson et al., 2009b). In short, the rate of change in oxygen concentration is the oxygen transfer rate (OTR) minus the OUR. If the oxygen flow is temporarily ceased, the

OTR becomes zero and the OUR can be measured from the slope of the oxygen concentration versus time plot. A sudden “flattening” in the slope indicates a loss of metabolic activity which implies the substrate concentration is nearly depleted. In theory, this technique can preempt full VFA depletion, but it is still reactive by nature, and the shift to “famine” conditions can occur so rapidly that the shift in slope may not be detected until it is too late.

Most researchers have had apparently little trouble obtaining sufficient process control with the techniques previously described, but they may not scale up well to larger systems or transfer easily to any type of waste-based substrate. For example, previous research conducted on dairy manure has observed highly buffered feedstock and unstable D.O. readings even at bench-scale, rendering all three methods unhelpful (Guho, 2010). Reactive techniques are intrinsically weak due to the necessity that VFAs must be nearly or completely depleted, an entirely unnecessary and potentially counterproductive event. In contrast, a proactive solution would know the rate of consumption in advance and make adjustments accordingly, but there are none in current practice. One simple proactive idea implemented by (Coats et al., 2016-under review; Watson, 2015) was to add pulses every 30 minutes which, based on the authors’ experience, would keep the concentration within the bounds of inhibition and limitation. This approach is not easily extendable or sufficiently generalized, but conceptually it may be workable with more development. In a review article (Dias et al., 2006) it was suggested that model predictive control could be a viable alternative. In other words, with reasonable starting estimates for process parameters and a well-tuned model (described in a previous section), the time to each subsequent pulse can

be determined conditionally rather than be an assumed constant value. Current model iterations are not yet this sophisticated, but reasonable process behavior can still be estimated. Combining a proactive control strategy with quantifiable, real-time data as a "check" might serve as a good compromise but has not been considered in the literature.

CHAPTER 3: HYPOTHESES

The research questions at the end of Chapter 1 were the driving force for the literature review in Chapter 2. With sufficient background material as a basis, three hypotheses were developed for experimental confirmation. The first examines the variations inherent to a biological system grown on a biological substrate and whether process stability can be achieved.

1. Given a constant operation for the enrichment reactor, despite fluctuations from the upstream dairy manure fermentation process, the effluent quality (i.e., VFA quantity, concentration, and speciation) will exhibit low variance. Likewise, given identical operation, the PHA yield and total accumulation in the production reactor will be consistent with low variance.

The PHA process using fermented dairy manure has been evaluated previously by the Coats Environmental Engineering research group (Guho, 2010; Watson, 2015), but the focus was on enrichment optimization, not enrichment stability. Conversely, synthetic feedstocks are still commonly used in the literature because they are more easily controlled. However, any real application will need comparable levels of consistency on waste streams which has not been confirmed for dairy manure. In addition, since upstream processes affect downstream processes, it is hypothesized that the production reactor will remain stable as long as the enrichment reactor is stable. To test this hypothesis, VFAs, PHA, D.O., total suspended solids (TSS), and volatile suspended solids (VSS) in the enrichment reactor were monitored for an extended operational period; multiple production experiments were conducted.

The second hypothesis examines an untested alternative to operating the production reactor. In fed-batch mode, a few high magnitude pulses of substrate are added to the reactor, but the same results could be theoretically obtained with many lower magnitude pulses. As the number of pulses increases (with decreasing pulse volume), it approaches a continuous feeding system.

2. Operating the production reactor with a continuous feed as opposed to the industry standard fed-batch mode will produce comparable results. The continuous mode will be more advantageous due to easier and more accessible control techniques.

Previous research (Guho, 2010; Watson, 2015) has shown that maintaining VFAs between inhibition and limitation in fed-batch operational mode is difficult due to high levels of noise in D.O. readings and highly buffered feedstock negating the usefulness of pH, D.O., and OUR control. A continuous fed mode, if successful, would be much easier to implement at full scale and would reduce the level of sophistication needed for control and operation.

The third hypothesis examines the assumption that the production reactor, since it receives inocula from the enrichment, is biologically identical and performs similarly.

3. With the enrichment reactor operating at pseudo-steady-state (i.e. each cycle profile is statistically identical), the production reactor can be modeled as an extension of the “feast” period during an enrichment cycle. As a result, an estimate of the maximum intracellular yield can be calculated *a priori*.

The rates of VFA uptake, PHA storage, and biomass growth are all the inputs needed for a simplified model and can be obtained from the steady-state enrichment cycle or enrichment operations. Combined with the production reactor mass balance, an estimate of PHA intracellular content can be determined.

CHAPTER 4: METHODS AND MATERIALS

4.1 Feedstock & Microorganisms Source

Dairy manure was collected from the University of Idaho dairy farm approximately every two weeks. The collection site was near the feeding stalls to avoid bedding material in the sample. Total solids (TS) and volatile solids (VS) measurements were taken with every new batch of manure collected with average values (sample size n=34) of 15.6% ± 1.7% and 83.6% ± 3.3% (of total solids), respectively.

The inocula for the enrichment reactor used in this research was obtained from an existing ADF reactor fed with fermented dairy manure since August 2011. The original inocula was grown in the laboratory using ADF to select for PHA-accumulators from bacteria that naturally occur in dairy manure.

4.2 Reactor Operations

A three-stage PHA process, as described previously (Figure 9), was employed for the duration of this research. All the reactors were maintained in a temperature-controlled room between 21-24°C.

The fermenter was operated with a working volume of 16 liters and a SRT/HRT of 4 days. The organic loading rate was maintained at 11.0 g-VSS (liter-day)$^{-1}$, with the wet weight of manure calculated from the total solids (TS) and volatile solids (VS) data. The fermenter was operated in SBR mode, with 4.0 liters decanted every day and refilled with 4.0 liters (diluted, wet manure) at the same time every day to maintain the target SRT. The

mass of wet manure was calculated according to equation (4.1). VFAs were measured regularly with average values (n=34) of 1956 ± 422 mg/L (acetate), 747 ± 155 mg/L (propionate), 316 ± 72 mg/L (n- and iso-butyrate), 248 ± 98 mg/L (n- and iso-valerate), and 70 ± 43 mg/L (caproate). Total VFAs were on average 4880 ± 850 as mg-COD/L and 136 ± 24 as Cmmol/L.

$$M_{manure} = \frac{OLR * V_{fermenter}}{1000 * (TS) * (VS)} \quad (4.1)$$

The enrichment reactor was operated as a SBR with a working volume of 10 liters. The SRT and HRT were maintained at 4 days and the total cycle length was 1 day. The reactor was decanted and fed (2.5 liters) every day at the same time (digital timers used to maintain constancy) with two Watson Marlow (400 rpm max), model 323 peristaltic pumps. The feed for the enrichment reactor was from the fermenter effluent centrifuged at 8000 rpm for 5 minutes. The target OLR was set at 10% of the reactor volume (1.0 liter of fermenter effluent and 1.5 liter of tap water for the feed container). Fluctuations in the VFA concentration from the fermenter caused the actual OLR to vary. This effect was a point of investigation and is discussed at more length in later sections. In July 2015, the OLR was increased to 15% of the reactor volume (1.5 liters of fermenter effluent and 1.0 liter of tap water for the feed container). The justification for this change is discussed in the results section. Rarely, but occasionally, the fermenter feed volume was adjusted to maintain a constant OLR when sudden changes in the VFA concentration from the fermenter occurred. The reactor was aerated with a 9 inch diameter Sanitaire Silver Series II membrane fine

bubble disc diffuser. A three-bladed, 6 inch diameter impeller was used for mixing using an Oriental Motor Co., LTD. (1500 rpm max at 60 Hz), model 5GN3.6SA. The reactor was covered to prevent excess foaming, but vented to the atmosphere.

The production reactor was operated as a batch reactor in a 3 liter beaker (actual volume increased over time, but always less than 3 liters). Four experiments were conducted using a fed-batch operation mode and six were conducted using a continuous fed operation mode. Dissolved oxygen, pH, VFAs, PHA, TSS, NH_3, and NO_3 were monitored by periodic sampling. Aeration was controlled by an Aera PI-98 Series pressure insensitive mass flow controller (1000 SCCM max) through a stainless steel diffuser set at 60% of the maximum flow rate (excess oxygen maintained). The reactor was mixed with a Teflon-coated stir bar and a magnetic stir plate. A water jacket was placed around the beaker to ensure consistent temperature. The feed to the reactor was undiluted fermenter effluent centrifuged for 3 hours at 9000 rpm. For fed-batch production reactions, the feedstock was supplied manually, and for continuous flow reactions, the feedstock was supplied via a Fisher Scientific variable flow mini peristaltic pump.

Figure 19: Enrichment reactor with feed and decant pumps

4.3 Analytical Techniques

Samples were collected to monitor pH, D.O., NO_3, NH_3, VFAs, TS, TSS, VS, VSS, and PHA. For soluble constituents, samples were first centrifuged to remove biomass and then filtered through a 0.22 µm syringe filter (Millipore Corp., Billerica, MA, USA) prior to testing. Soluble NO_3 was determined in accordance with Hach method 10020, and soluble NH_3 testing followed Hach method 10031. A Spectronic® 20 Genesys™ spectrophotometer (Thermo-Fisher Scientific Corp, Waltham, MA, USA) was utilized to measure the absorbance of the reacted sample at a wavelength of 410 nm for NO_3 and 655 nm for NH_3. NO_3 and NH_3 concentrations were determined utilizing a standard curve (R^2>0.99).

TS, VS, TSS and VSS were measured in accordance with Standard Methods 2540 D and 2540 E (Clesceri LS, 1998), respectively. Measurement of pH was accomplished with a Thermo-Fisher Scientific Accumet AP85 Waterproof pH/Conductivity Meter. D.O. measurements were collected using a Hach HQ30d Meter with a LDO101 D.O. Probe.

VFAs (acetic, propionic, butyric, isobutyric, valeric, isovaleric, and caproic acids) and methanol were quantified using a Hewlett-Packard 6890 series gas chromatograph (GC) (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a flame-ionization detector (FID) and a Hewlett-Packard 7679 series injector. The system was interfaced with the Hewlett-Packard GC ChemStation software version A.06.01. VFA separation was achieved using a capillary column (Heliflex® AT™-AquaWax-DA , 30 m x 0.25 mm ID, W. R. Grace & Co., Deerfield, IL, USA) which was ramped from an initial 50°C to 200°C in three steps (following 2 min at 50°C, ramp to 95°C at 30°C min^{-1} then to 150°C at 10°C min^{-1} and hold for 3 min; finally, ramp to 200°C at 25°C min^{-1} and hold for 12 min) with helium as the

carrier gas (1.2 mL min^{-1}). The split/splitless injector and detector were operated isothermally at 210 and 300°C, respectively. Prior to analysis, samples were acidified to a pH of 2 using nitric acid. 0.5 µL of each sample was injected in 20:1 split mode. VFA concentrations were determined through retention time matching with known standards (Sigma-Aldrich Co., St. Louis, MO, USA; Thermo Fisher Scientific Inc., Waltham, MA, USA) and linear standard curves (R^2>0.99).

Biomass PHA content was determined by gas chromatography/mass spectrometry (GC-MS) as described in Braunegg, et al.(1978). Dried biomass samples were digested at 100°C in 2 mL of acidified methanol (3% v/v sulfuric acid) and chloroform. Benzoic acid was added as an internal standard to the chloroform at 0.25 mg/mL. After digestion, 2 mL of deionized water was added and vortexed to separate into chloroform and water phases. The chloroform phase was extracted and filtered through sodium sulfate anhydrous to remove excess moisture and particulates. GC-MS was performed on a Thermofinnigan PolarixQ iontrap GC-MS instrument (Thermo Electron Corporation). The sample was introduced using split injection. Separation was achieved on a ZB1 (15 m, 0.25 mm ID) capillary column (Phenomenex, Torrance, California, USA) with helium as the carrier gas (1.2 mL min^{-1}) and an initial temperature of 40°C (2 min) ramped to 200°C at 5°C min^{-1}. The compounds were confirmed by retention time and mass spectral matching with known PHA standards (PHB and PHB-co-HV: Sigma Aldrich; NaHB: Alfa Aeser) as methyl ester derivatives, and quantified based on the internal standard. The Xcalibur software program (Thermo Electron Corporation) was used to facilitate PHA quantification, and the optimal molecular weight for PHA quantification was determined to be 103 g mol^{-1}. PHB eluted at

approximately 5.4-5.6 min, and PHV eluted at approximately 7.9-8.4 min. The benzoic acid standard eluted at 11.9-12.1 min. Total intracellular PHA content was determined on a percent dry weight basis (mass PHA per mass TSS, w/w) and a percent cell weight basis (mass PHA per mass VSS, w/w).

4.4 Calculations

PHA yield on substrate and specific substrate uptake rate are important metrics discussed in the results section that have a non-elementary calculation. PHA yield was calculated as the cumulative mass of PHA stored divided by the cumulative mass of VFAs consumed. "C_P" and "C_S" are the concentrations of PHA and substrate (as Cmmol/L), respectively; "Q_{in}" is the flow rate into the reactor and is a function of time; "V_r" and "V_{sample}" are the reactor volume and sample volume, respectively.

$$PHA\ Yield = \frac{C_P V_r + \sum_i C_{P,i}\, V_{sample,i}}{\int_0^t C_{S,in} Q_{in}(t)\, dt - C_S V_r - \sum_i C_{S,i}\, V_{sample,i}} \tag{4.2}$$

For the enrichment reactor (with a large volume and few samples), this reduces to the concentration of PHA at the end of the feast divided by the concentration of VFAs at the start of the cycle. For the production reactor, however, frequent sampling reduces the amount of VFAs available for consumption and removes PHA from the system. Flow into the system (for continuous fed mode) must also be accounted for.

The specific substrate uptake rate was calculated by rearranging the mass balance for substrate. It is defined as the mass rate of change of substrate divided by mass of biomass; "X" is the concentration of biomass (in mg-VSS/L).

$$Specific\ Substrate\ Uptake = \frac{\frac{d}{dt}(C_S V_r) - C_{S,in} Q_{in}(t)}{X V_r} \quad (4.3)$$

The enrichment reactor and fed-batch production mode did not have flow into the system (feed addition was modeled as an instantaneous pulse), so equation (4.3) reduces to the slope of the substrate concentration line (rate of change with respect to time) divided by the biomass concentration. It was observed that the uptake proceeded linearly for no flow conditions resulting in a constant measurement for the rate of change term. For continuous fed, the slope of the line varied substantially depending on flow rate changes and changes in metabolic behavior. The slope of the line for these cases was determined by polynomial fitting of degree 2 to 6.

The calculations for the enrichment reactor and the fed-batch production reactor were performed exclusively with Microsoft Excel® software. The PHA yield and substrate uptake rate calculations for the continuous fed production assessments were performed using MATLAB® and the remaining were calculated with Excel. All statistical calculations were also performed with Excel.

CHAPTER 5: RESULTS AND DISCUSSION

The primary focus of this research was to analyze the enrichment reactor in combination with two modes of operation for the production reactor (fed-batch and continuous fed). In order to sufficiently cover the results, each reactor and production mode is first discussed independently, followed by a holistic assessment of the enrichment and production reactors in series. In the last section the dependent relationships are established and provide a basis for engineering design.

5.1 Enrichment Reactor

5.1.1 Stability Assessment

The enrichment reactor provides the biomass for PHA production and must be able to achieve consistent and reliable effluent for any full scale process to be successful. Real waste substrates such as dairy manure (along with full scale operation) will inevitably result in natural fluctuations in the feed composition. The enrichment phase must be capable of "absorbing" this variable input while still producing stable output. Stability can be interpreted in different ways and is difficult to measure quantitatively. For the purpose of this discussion, stability is assessed by analyzing some of the SBR cycle profile characteristics over time – specifically, initial biomass concentration, organic loading rate, feast length, and PHA yield on VFAs. The enrichment reactor was operated continuously with constant set points (except where noted) from February 2015 to April 2016 with profile sampling occurring every one to two weeks (see Chapter 4 for details).

A typical profile for the enrichment reactor provides a significant amount of information regarding the "health" of the mixed culture. The primary constituents (NH_3, VFAs, PHA, and D.O.) during a single enrichment cycle is illustrated in Figure 20. TSS was measured but omitted from Figure 20 for graphical clarity. VFAs and PHA are reported on a carbon millimole per liter (Cmmol/L) basis so the constituents (e.g., acetate, propionate, etc. for VFAs, and PHB, PHV, etc. for PHA) can be summed together. The PHA yield, or flux of carbon to storage, is the mass of PHA at VFA depletion (the end of the feast) divided by the mass of VFAs consumed. The full 24 hour profile and a zoomed in profile on the "feast" period are presented for full resolution. The organic loading rate (expressed as Cmmol/(L*day)) is the initial VFA concentration times by the numbers of cycles per day.

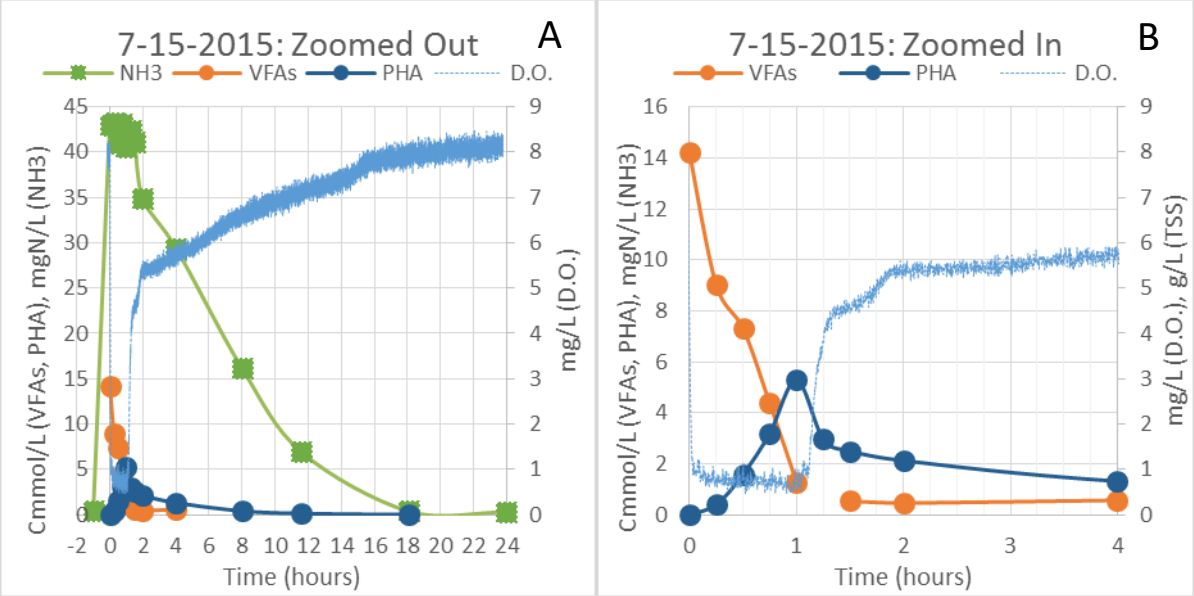


Figure 20: Enrichment profile from 7-15-2015

The sudden spike in residual oxygen concentration (at approximately one hour into operation; Figure 20B) indicates the depletion of readily biodegradable carbon (VFAs). Since the oxygen transfer rate does not change during the course of the cycle, it can be presumed

that the oxygen uptake rate drastically declined as the result of less substrate being available for consumption. Regarding the ammonia concentration, it was observed that no nitrification occurred during the feast period; significant nitrification did occur throughout the famine. The D.O. increased sharply again at about 15 hours (Figure 20A) likely corresponding to complete nitrification. The ammonia concentration continued to decrease even after internal PHA depletion (at about 8 hours) indicating that autotrophs were the primary driver for ammonia loss.

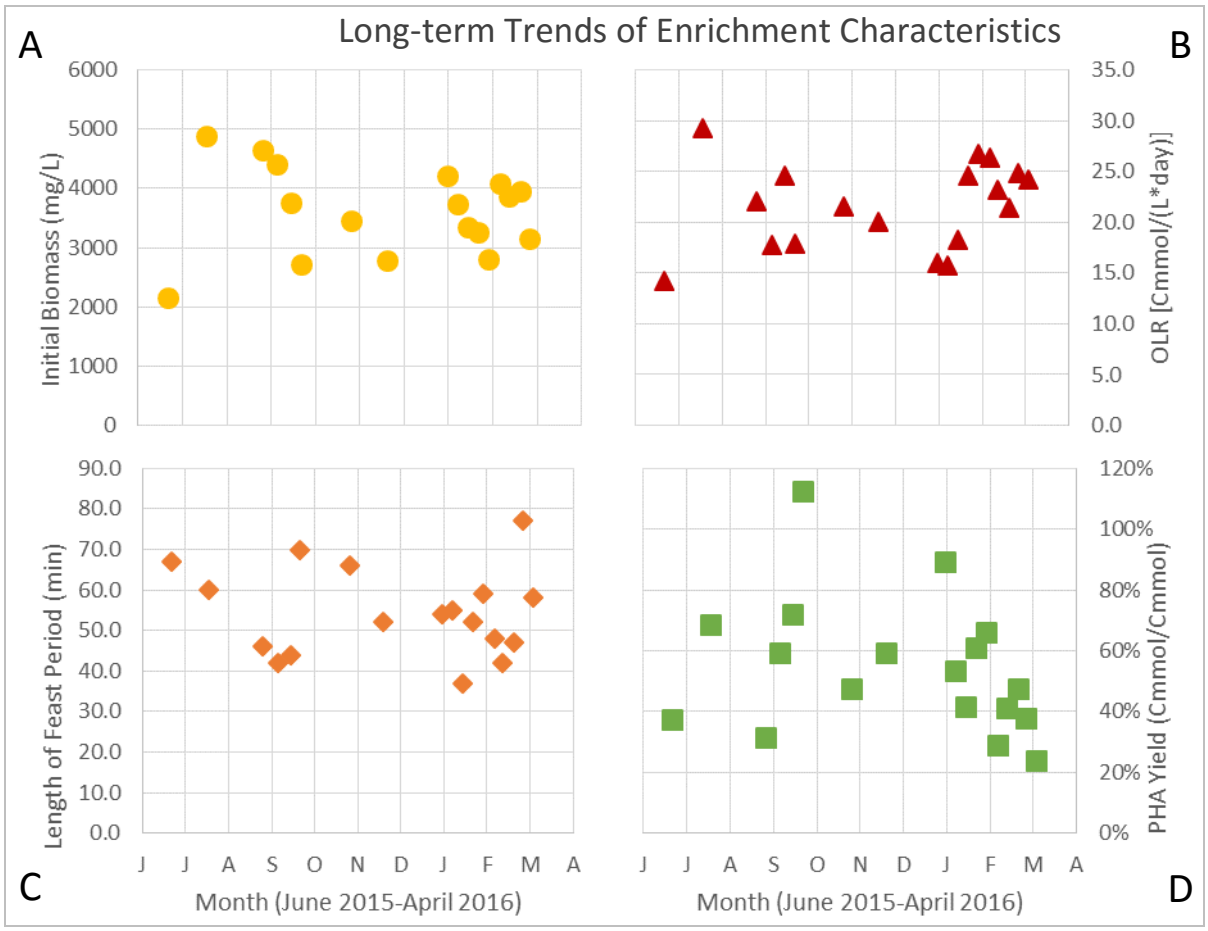


Figure 21: Stability data for enrichment reactor

The operational stability criteria (initial biomass, OLR, feast length, and PHA yield) were ascertained from profile data collected over the span of enrichment reactor operation. The results are summarized in Figure 21 and Table 5.

Table 5: Summary statistics for enrichment reactor

(n=19, all variables)	**Initial Biomass** *mg/L*	**OLR** *Cmmol/(L*day)*	**Feast Length** *minutes*	**PHA Yield** *Cmmol/Cmmol*
Mean ± Std. Dev.	3540 ± 770	21.1 ± 4.6	54 ± 11	54% ± 22%
Minimum	2150	13.0	37	24%
1st Quartile	2800	17.8	46	38%
Median	3600	21.6	53	48%
3rd Quartile	4120	24.6	62	66%
Maximum	4880	29.2	77	113%

As can be seen, there was significant variation in all variables without any discernable trend. In particular, PHA yield, an important metric in terms of effective downstream production performance, ranged from 24% to 113%. The natural fluctuations from dairy manure (the substrate) affected fermenter operation and explains the variance in the OLR. VFAs were monitored regularly to exert some control over the OLR (by increasing or decreasing feed volume as needed) but it was insufficient to prevent deviations from the mean. One possible solution to counter this is to increase the OLR so that the standard deviation is a smaller percentage of the mean, resulting in a more consistent feeding pattern. There are other advantages to increasing the OLR that are discussed later. The CL to SRT ratio was fixed at 0.25 (1 day CL, 4 day SRT) for the duration of the enrichment investigations which could have contributed to inconsistent performance. From the literature review (Chapter 2.3 and Table 4) there was a clear

advantage (in terms of highest measured intracellular PHA) for experiments conducted with cultures enriched at a CL to SRT ratio of 0.5 over those that operated at 0.25 or less. As the ratio increases, the microbes in the mixed culture must substantially increase their growth rate to prevent being washed out. As a result, PHA producers gain a better advantage since they can remove the VFAs from bulk solution (stored as PHA) more quickly than non-producers. By operating at 0.25, it is likely that the non-PHA producers, or flanking population, were not forcibly or consistently outcompeted and removed from the system. A constantly shifting population can explain some of the variances observed in the enrichment data, particularly PHA yield since even a small flanking population would have a significant detrimental impact.

Another criterion identified in Chapter 2 used to assess culture selection for PHA accumulators is the feast to famine (F/F) ratio. As the ratio decreases, similar to the impact of the CL to SRT ratio, PHA producers are better adapted to survive compared to non-PHA producers. The latter group can grow only during the feast period while the former can grow all throughout the famine. In other words, PHA producers have more time to grow relative to non-PHA producers as the F/F ratio decreases. The F/F ratio is not controlled directly but is a function of the CL, SRT, and organic loading rate (OLR). A review of the literature showed that higher OLR resulted in better performance as long as the F/F ratio remained at or below the recommended 0.2 value. Considering that the enrichment reactor always maintained a F/F ratio well below 0.2, it would seem logical that the fluctuations in the OLR data would correlate strongly with the fluctuations in the output variables (biomass, feast length, and PHA yield), but no such correlations are observed. One possible

cause is the effect of operational changes to the enrichment reactor. Despite the best intentions to maintain a constant operation there were two parameters that were adjusted over the course of the investigation period. The first was an increased air flow rate in June 2015, thus imparting a higher dissolved oxygen concentration during the feast period that was not oxygen limited. The second was an increase of the OLR in July 2015 from 10% reactor volume to 15% reactor volume. An unintentional third change occurred in February 2016 in which the air flow rate was accidentally decreased resulting once more in an enrichment feast period that was oxygen limited. The original two changes were performed with the purpose of reducing the number of experimental variables by removing oxygen limitation and providing excess substrate carbon. The PHA yield data is presented again in Figure 22 with the vertical bands showing the date range in which both the higher flow rate and higher OLR were in effect. The horizontal lines show the trend of PHA yield data inside and outside that range. Interestingly, the average PHA yield with “ideal” conditions (higher air flow and OLR) is larger than the time periods experiencing low D.O. in the feast or 10% reactor volume OLR – about 65% and 35%, respectively.

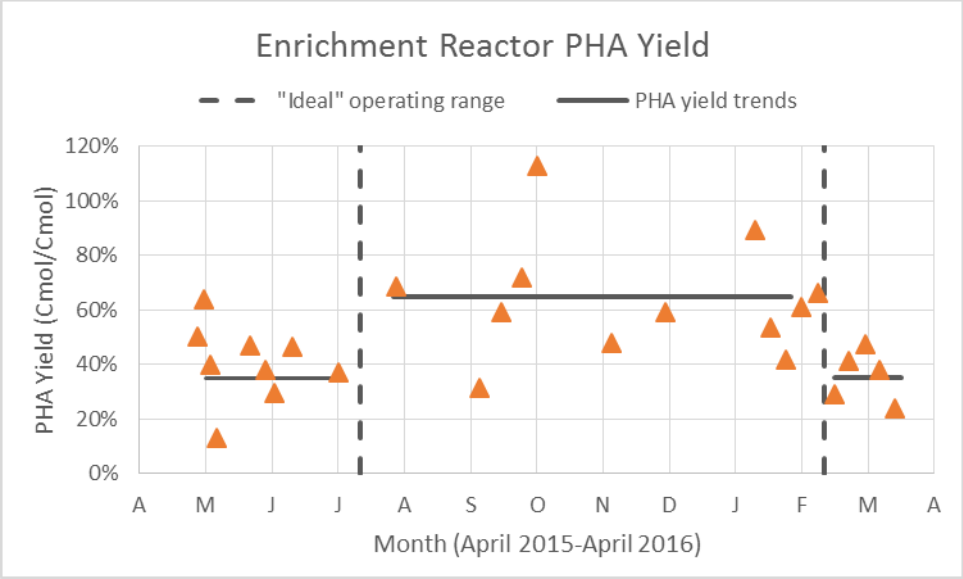


Figure 22: Enrichment PHA yields with operational changes

5.1.2 Effect of Organic Loading Rate

A low CL to SRT ratio and changes in operational conditions have been identified as two of the causes for inconsistent enrichment reactor output, but much of the results remains unexplained. For example, while low, a CL to SRT of 0.25 has in the literature successfully enriched for PHA producers. Also, the PHA yield in the "ideal" operating range is still quite variable. Another consideration is that, despite the increase of OLR from 10% to 15% of reactor volume, it was still too low to obtain stable reactor performance. Since PHA reserves are depleted anywhere from 8-12 hours into the cycle (leaving 12-16 hours of endogenous decay – see Figure 23A-B for examples), contrary to the literature where PHA is either kept in reserve or depleted only at the end of cycle, it may simply be that the PHA accumulators cannot keep the biological advantage over their competitors. Figure 23 illustrates how an increase in OLR could be beneficial in enhancing that advantage. The profile from July 15 was a "low" OLR cycle at 15 Cmmol/(L*day) compared to August 10

which was a “high” OLR at 30 Cmmol/(L*day); these two dates were on either side of the operational change in OLR from 10% to 15% reactor volume. The total mass of PHA was higher in the second case due to the higher mass of VFAs used to feed the reactor. This had the effect of increasing the famine length by 50%, from 8 hours with the low OLR to just over 12 hours with the high OLR. The feast length remained essentially unchanged, thus decreasing the F/F ratio from 0.157 to 0.099. Most important however is the change in PHA yield which doubled from 33% to 67%, suggesting that PHA accumulators were a higher fraction of the biomass, more efficient at PHA storage, or a combination. In any case, the higher OLR resulted in a microbial culture more capable of PHA synthesis.

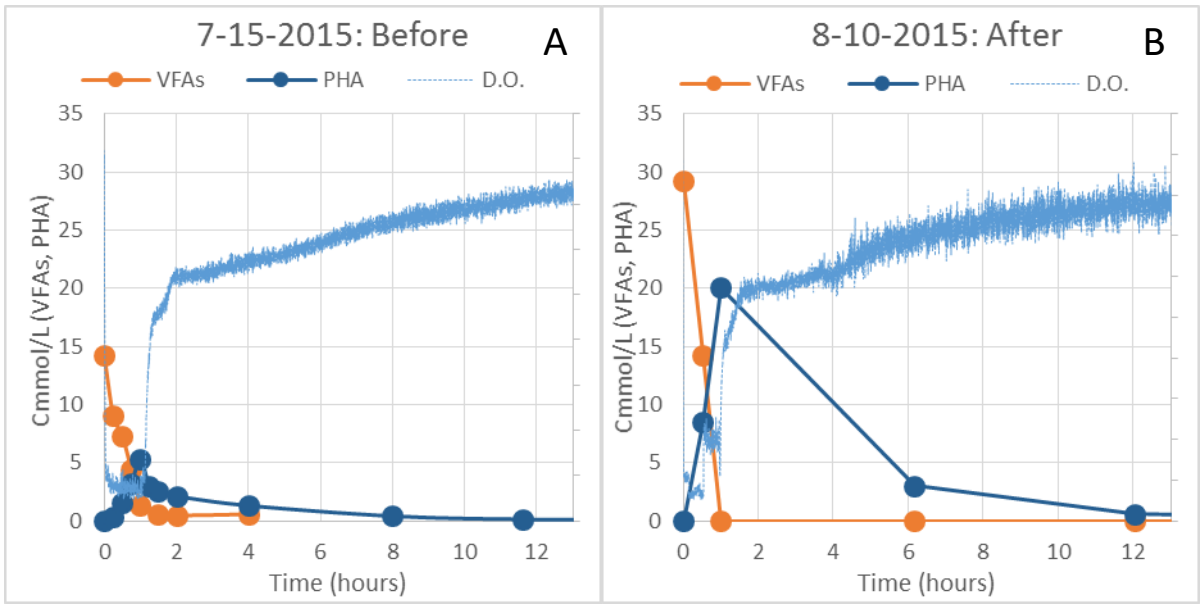


Figure 23: Profile comparison before and after OLR increase

Although the higher OLR was beneficial, there is good reason to suppose increasing it more would further enhance those benefits. For example, the endogenous period with the high OLR is still half of the total cycle length (12 hours), a time which the PHA accumulators do not have a selective advantage. There could be reasons for allowing the PHA reserves to be

depleted before the end of the cycle (Hanson et al., 2016-accepted-a) but preliminary evidence (Figure 23) suggests it should be less than 50% of the cycle. In addition, the F/F ratio is well below the empirical threshold of 0.2 needed to maintain a competitive advantage; considering there is no benefit to operating below this point (see Table 4 and also Albuquerque et. al. (2010b)), an increase in OLR could provide additional selective pressure by increasing the growth period of PHA producers relative to non-producers at the cost of a larger (but still less than 0.2) F/F ratio. In fact, evidence from Jiang et al. (2011b) suggests that operating with a F/F below 0.2 could be detrimental because it indicates that the biomass has a higher OLR capacity and a higher PHA storage potential. However, the main reason for suggesting a higher target OLR is that the enrichment reactor would be less impacted by variability in the waste substrate. The PHA yield on VFAs increased substantially (by 100%) when the OLR was increased from 15 to 30 Cmmol/(L*day) and Figure 21 (B, D) gives evidence that the reverse is also true – a sudden decrease in OLR significantly drops the PHA yield. The standard deviation was very large compared to the mean (21.8%) resulting in a wide, unstable set of PHA yield data. If the OLR range were increased, for example, between 50-60 Cmmol/(L*day), the standard deviation as a percentage of the mean would only be about 7%. Presumably, the PHA yield would fluctuate much less and correlate more strongly with the OLR.

A proposed increase in the OLR is consistent with findings in the literature that suggest that the organic loading should be maximized, provided the F/F is maintained near 0.2 and the PHA is fully depleted at the end of cycle for chemostats ((Albuquerque et al., 2010a; Dionisi et al., 2006). A recommended ceiling of 60 Cmmol/L (Serafim et al., 2004) is

theoretically non-inhibiting and could be applied at the beginning of each cycle thereby tripling the average OLR obtained from the reactor. One potential disadvantage of this operational change is that the extra volume of fermenter effluent diverted to the enrichment decreases the amount available for production which ultimately impacts the economics of the overall process. This can be partially compensated for by reducing the enrichment reactor volume and decreasing the SRT. The F/F ratio would likely increase slightly, but would not cause a problem provided it remained below 0.2, a likely occurrence since it hovered consistently around 0.1 for the conditions used in this study. Additional selective pressure should be added by also increasing the CL to SRT ratio as recommended by Jiang, et. al. (2011b). The best results in the literature were obtained at a value of 0.5 but as a starting point the SRT could be decreased from four days to three days, increasing the ratio from 0.25 to 0.33. These two changes would bring the enrichment operation more in line with successful attempts described in the literature.

5.2 Fed-batch PHA Production

5.2.1 Results Overview

A total of four fed-batch production investigations were conducted which started in July 2015 and concluded December 2015. Briefly, biomass was taken from the end of the enrichment cycle and placed in a 1.0 Liter beaker with a stir bar and stainless steel aerator. The enrichment reactor was operated continuously with the same CL and SRT for the entire fed-batch experiment timeframe. Centrifuged effluent from the fermenter was used as the feedstock (the same used for enrichment feed) and fed to the reactor in pulses. Sampling

occurred at least three times per pulse to maintain a high resolution of VFAs, PHA, and biomass concentrations. The experiment was arranged so that the pulse volume and total sample volume per pulse were approximately equal, thus maintaining a constant working volume – this was performed for convenience only to prevent high volume accumulation and would not be performed at full scale (and was not performed for the continuous fed experiments described later). More information is found in Chapter 4.

The profiles of each assessment (VFAs, PHA, D.O., and TSS) are presented in Figure 24 and the summary of data is in Table 6. The vertical lines indicate new substrate pulses, resulting in an influx of VFAs and an instantaneous dilution of PHA and TSS concentrations. The D.O. fluctuated significantly but can generally be correlated with the VFA concentration (oxygen saturation when VFAs were depleted). The D.O. curves trended towards saturation as the reaction progressed, implying that the loss of biomass due to sampling resulted in decreased oxygen uptake rate. The experiment on October 13 was the most successful, reaching a PHA concentration close to 120 Cmmol/L with no periods of extended VFA depletion or inhibition. The other experiments had varied success, with results PHA concentrations ranging from 25-75 Cmmol/L PHA. The run on July 21 had several periods of VFA depletion while November 17 and December 10 experienced VFA accumulation over time, with the latter reaching limiting concentrations after pulse five.

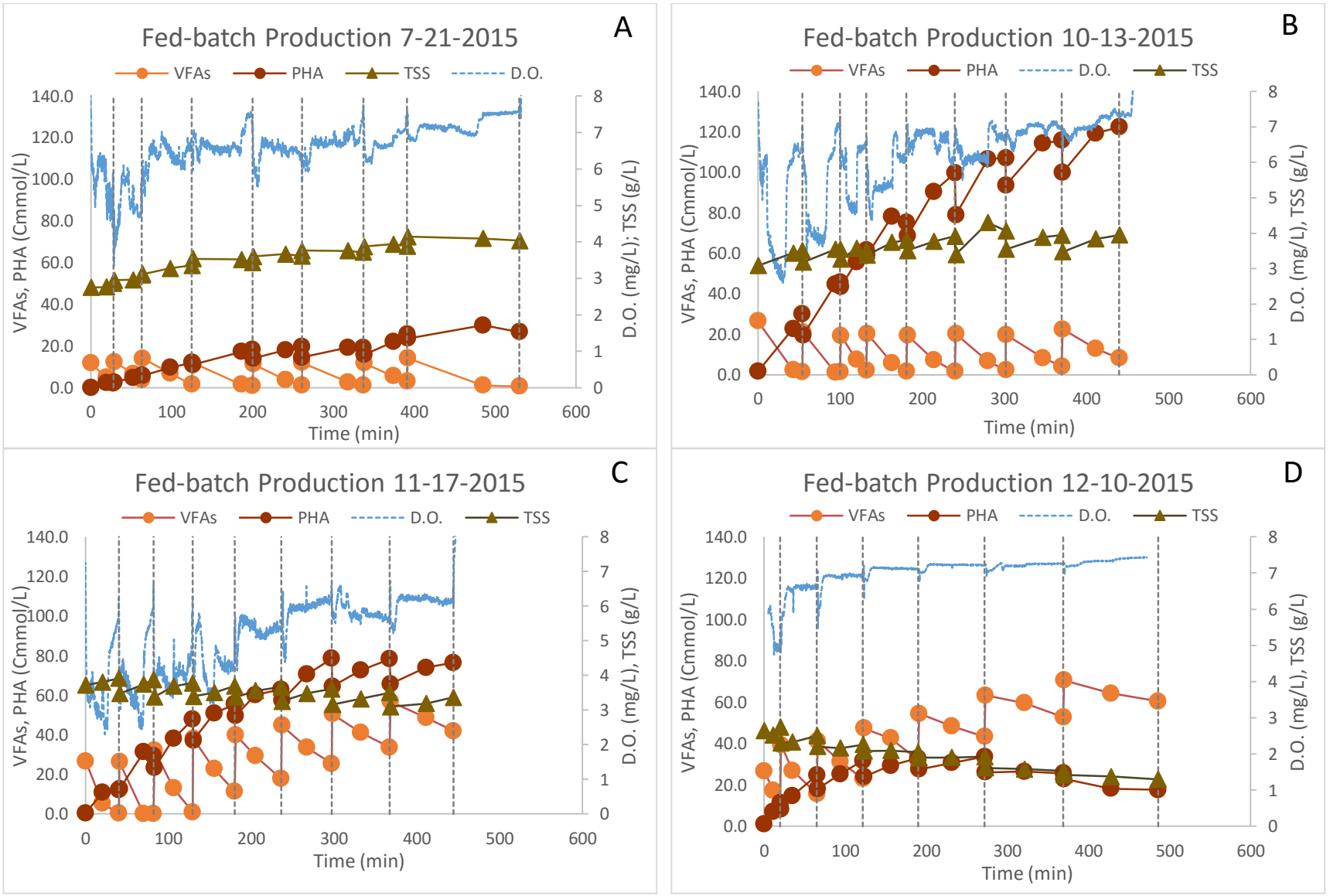


Figure 24: Summary of fed-batch production experiments

Table 6: Summary of data for fed-batch production experiments

		7/21/2015	10/13/2015	11/17/2015	12/10/2015
Volume, mL	*Initial*	800	650	500	400
	Final	485	540	510	310
Feed VFAs & Pulse Volume	*Cmmol/L*	115	252	190	163
	Avg., mL	60	80	100	80
Maximum PHA	*Cmmol/L*	29.9	122.5	78.5	33.6
	Wt%, TSS	15.5%	64.7%	48.2%	36.3%
	Wt%, VSS	18.5%	74.8%	58.0%	40.8%
PHA Yield %	*Cumulative*	41.1%	114%	75.1%	57.8%
	Average	38.4%	114%	66.3%	63.0%
Uptake Rate, Cmmol/ (gVSS*hr)	*Initial*	8.90	14.2	20.5	22.1
	Average	4.84	14.6	15.7	16.8
	Stable	3.41	14.1	11.7	13.1

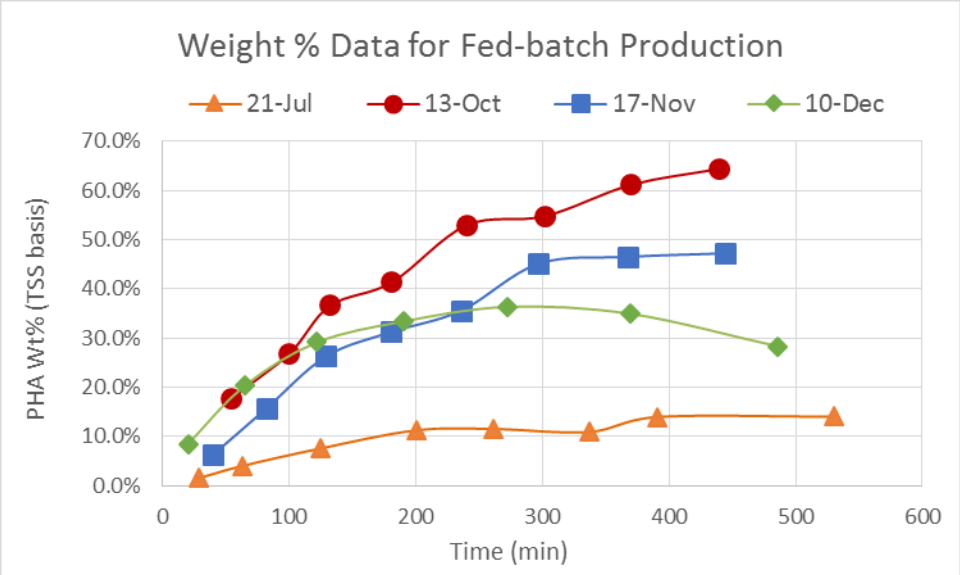


Figure 25: Weight percent, TSS basis, fed-batch production experiments

The cumulative PHA weight percent for each experiment is shown in Figure 25 (TSS basis), and maximum values presented in Table 6 (TSS and VSS basis). The weight percent reported on a TSS basis is more valuable to engineers because it represents the final product that would need to go to an extraction phase and thus has the largest economic

impact (i.e., low intracellular %PHA would require more chemicals for polymer recovery, on a mass of polymer recovered basis). However, for scientists it is usually reported as VSS for easier comparison across publications and to obtain a more realistic assessment of reactor performance (VSS is more representative of the microbial fraction of the TSS, which would be responsible for PHA synthesis). The highest obtained value of 74.8% is comparable to reported values of 75% (Albuquerque et al., 2010b), 77% (Jiang et al., 2012), and 78.5% (Serafim et al., 2004).Of note, all these other experiments were conducted under growth limitation in the production and the latter was performed with synthetic feed, whereas the research presented herein was conducted on real waste and without any growth limitation (ammonia, phosphorus, oxygen, and substrate all in excess). The research by Valentino et al. (2015) is the closest comparison to this work (growth allowed but substrate limited, fermented whey substrate) and reported VSS weight percent ranging from 50-70%. The results from this research are comparable, possibly even outperforming, except with a lower rate of consistency. The inconsistency may be explained by the variant nature of the upstream enrichment reactor (described in the previous section) and such an analysis is conducted later in this section after the continuous fed production results.

5.2.2 PHA Yield and Substrate Uptake

In analyzing PHA production data, the two most important measurements besides maximum PHA content are the PHA yield on substrate and the specific rate of substrate utilization. Yield is a measure of the thermodynamics, the conversion of the substrate carbon into PHA, biomass, or carbon dioxide. Substrate utilization measures the kinetics, the rates at which the metabolic reactions occur. An analysis of the thermodynamics and

kinetics are necessary to fully understand the microbial behavior and are essential for developing design criteria or a mathematical model. In the enrichment reactor both are calculated during the relatively short feast period; it was observed (see for example Figure 23B) that the VFAs were consumed linearly and PHA was stored linearly, thus resulting in no change in PHA yield or substrate uptake rate with respect to time for a single cycle length. By contrast, the "feast" period for the production reactor (ideally, the entire reaction time) is sufficiently long that these metrics may differ significantly from the corresponding enrichment reactor cycle or change as the reaction time increases. The comparison to enrichment discussion is deferred to the final section of this chapter. Figure 26 and Figure 27 show how the PHA yield and specific substrate utilization changed throughout the reaction, respectively.

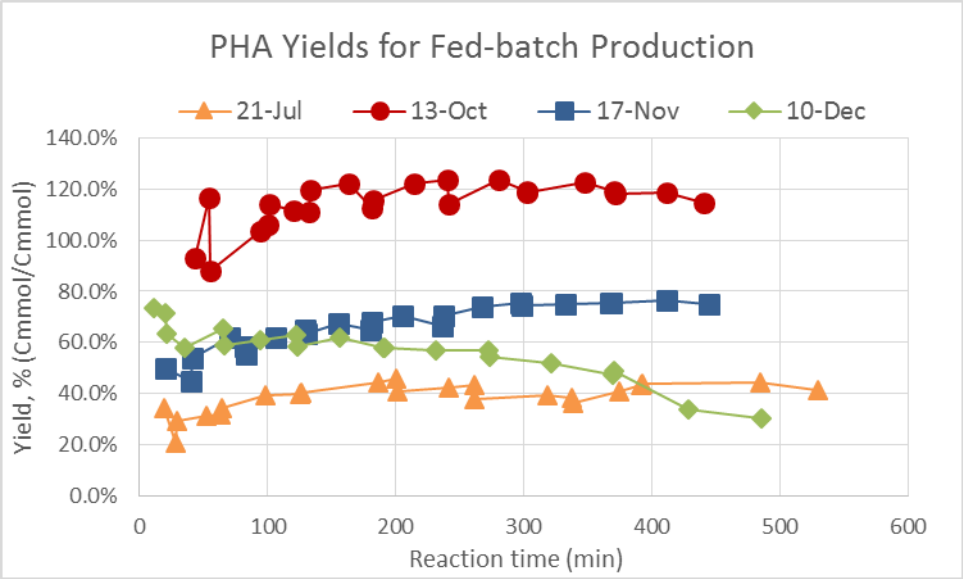


Figure 26: Fed-batch production cumulative PHA yield data

Regarding PHA yield, as Figure 26 shows, the cumulative PHA yield (determined as mass of PHA in the cells divided by total mass VFAs consumed since time zero, corrected for

sampling volume) remained essentially constant for all fed-batch experiments. The one exception was from December 10 which reached inhibitory VFA concentrations at about 200 minutes. The inhibition point correlates with a sudden decrease in yield – an expected result considering inhibition has a similar effect to limitation in that the microbial activity slows significantly. This constancy in yield throughout the entire production reaction is important for modeling and design purposes. Intracellular PHA content, the standard measurement for determining economic feasibility, is defined as the ratio of PHA mass (as VSS) to total VSS mass (PHA plus biomass) – all easily quantifiable values. The thermodynamics show that from the yield data, the mass of VFAs required to achieve a desired intracellular content can be calculated with reasonable certainty.

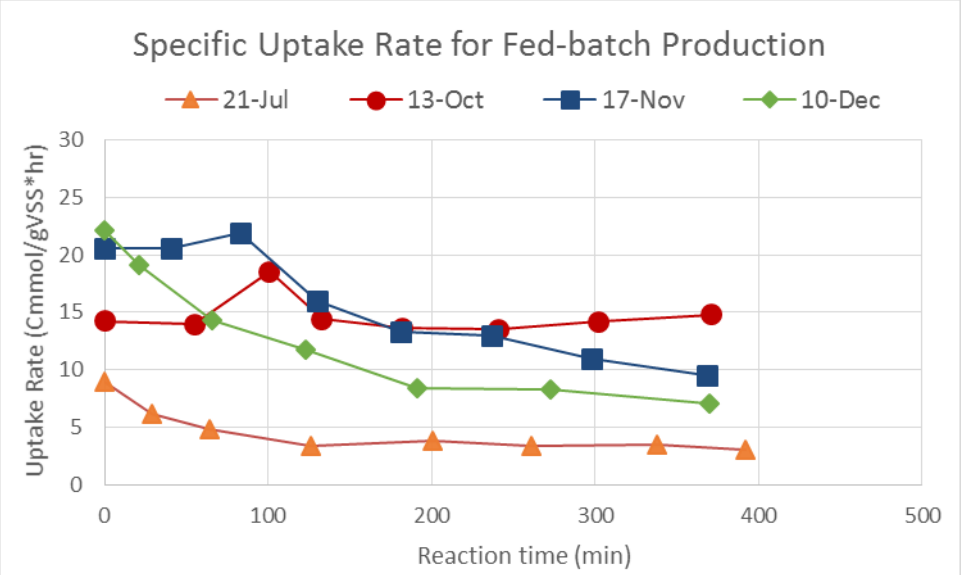


Figure 27: Fed-batch production specific substrate uptake rate data

Substrate utilization can be normalized to the reactor volume (volumetric uptake) or to the amount of biomass (specific uptake). The latter provides more clarity in describing the kinetics because it measures the activity of the microbes directly and is not affected by

growth or removal of biomass due to sampling. Contrary to the results of PHA yield data, as is shown in Figure 27, the specific substrate uptake rate did not remain constant over time during a production assessment (with the possible exception of October 13) and actually declined before eventually stabilizing. This suggests that the biomass somehow adapted to the extended "feast" conditions of the production reactor without decreasing the PHA yield on substrate (as Figure 26 confirms). As discussed in Chapter 2, microbes will preferentially grow instead of storing PHA due to a higher energy efficiency. If the microbes became less stressed, it is presumed that the growth rate would increase and PHA storage would decrease. However, the constant PHA yield shows this is not the case. Perhaps an alternative explanation for the drop in substrate utilization is the overall higher D.O. concentration in the production reactor compared to a typical enrichment cycle. The microbes may be adapted to consuming nitrate or nitrite in addition to oxygen (due to high competition) during the enrichment process, but then have unlimited access to the more energetically favorable oxygen for the production. This would have the effect, as was observed, of an initially high uptake rate followed by a decline. Although the specific substrate uptake rate did not remain constant, it appeared to have little effect on the final results (except for maintaining process control – more discussion to follow).

Like all chemical reactions, the microbial biochemistry of the ADF PHA process is fundamentally linked to and controlled by the thermodynamics and kinetics. Proper system design can prevent thermodynamic control (e.g., running out of feedstock too early in the reaction) making the ADF PHA process fundamentally limited by the kinetics. Even in theory, an indefinite reaction time would not result in sequentially higher PHA content because of

biomass growth and adaptation. As was observed (most clearly in Figure 24C-D), a PHA maximum was reached and then declined. A full-scale system would need knowledge about when to finish the reaction to obtain maximum which requires knowledge of the kinetics. However, as was discussed, this would be difficult given the unpredictable behavior of the specific substrate uptake rate. If the rate had been constant, it could be combined with the PHA yield and initial biomass data to provide an estimate of reaction time needed to reach completion. Until a better explanation of reaction rates can be elucidated, reaction time design must continue to be determined from experience.

5.2.3 Process Control

Three out of the four production assessments experienced operational difficulty in maintaining VFAs in the target range between limitation and inhibition (July 21 and December 10 being most strongly affected). This outcome can be attributed to practical difficulties in process control and predicting the time to full VFA depletion. More specifically, as discussed in the literature review, observation of D.O. shifts upon VFA depletion is unreliable in the best circumstances but particularly difficult with dairy manure due to significant noise and readout delays (and also excess ammonia, which requires oxygen for its biological oxidation). A preemptive method was adopted for all fed-batch assessments in which the time to depletion for the first pulse was estimated using enrichment reactor uptake rate data as an initial guess. Then, the guess was modified as needed with the online D.O. measurement, ideally allowing for substrate addition before complete depletion. In this revised mode, all subsequent substrate pulses were assumed to operate with the same uptake rate, properly adjusted for the dilution factor resulting from

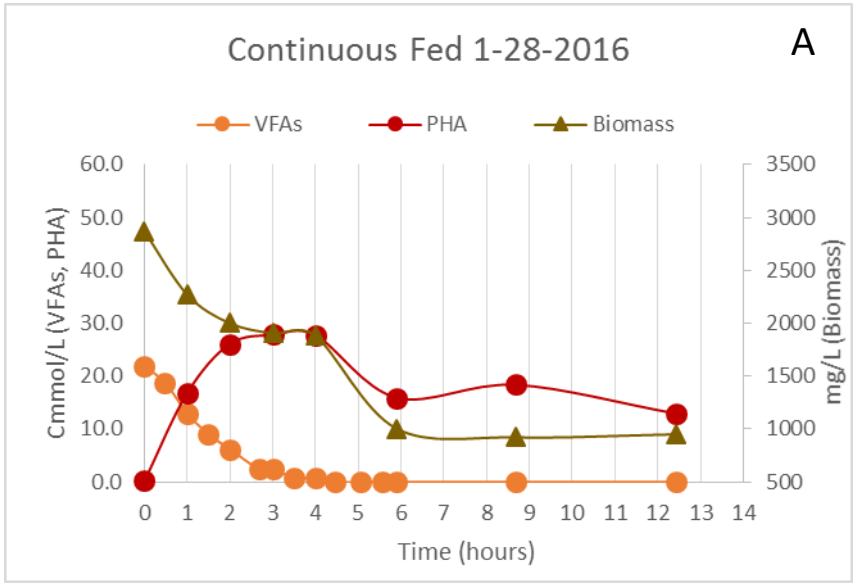
increasing reactor volume and removal of biomass due to sampling. While partially successful, this technique was still difficult to execute and resulted in significant problems for some of the experiments, particularly December 10 when a miscalculation for the first pulse compounded into VFA inhibition later in the reaction. Also, as discussed previously, in most cases the constant uptake rate assumption is invalid which resulted in VFA accumulation in two of four experiments. Consequently, the experiment on October 13 experienced the best process control due to relatively constant uptake rate. The dynamic nature of fed-batch mode has intrinsic difficulty with process control, a problem largely overcome in switching to continuous fed mode as discussed in the next section.

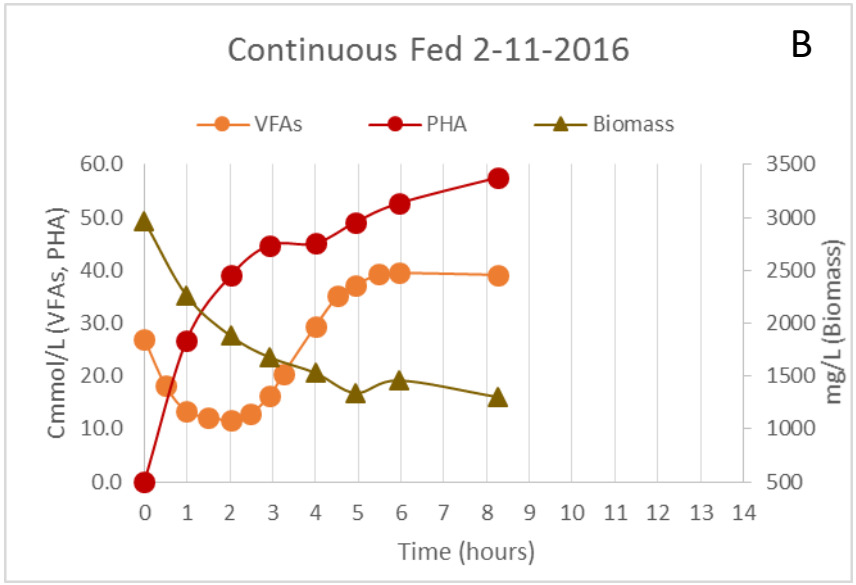
5.3 Continuous fed PHA Production

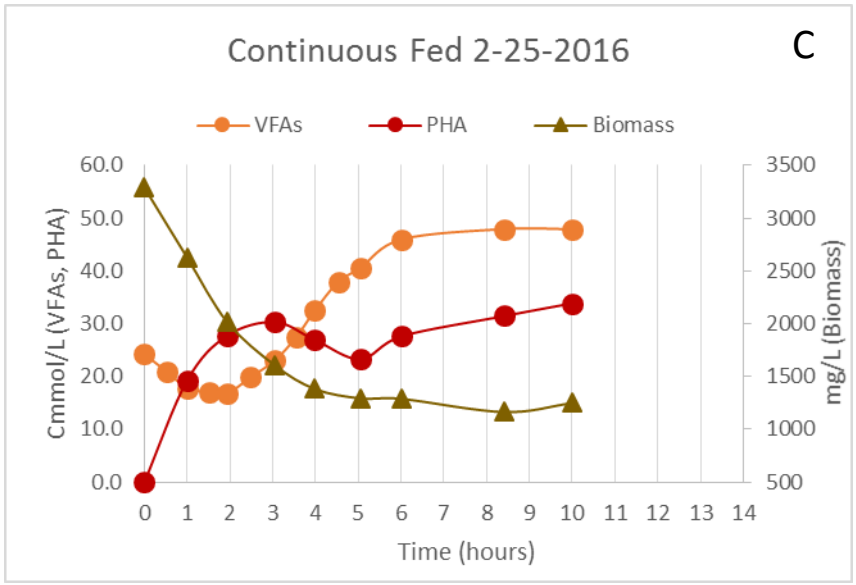
5.3.1 Results Overview

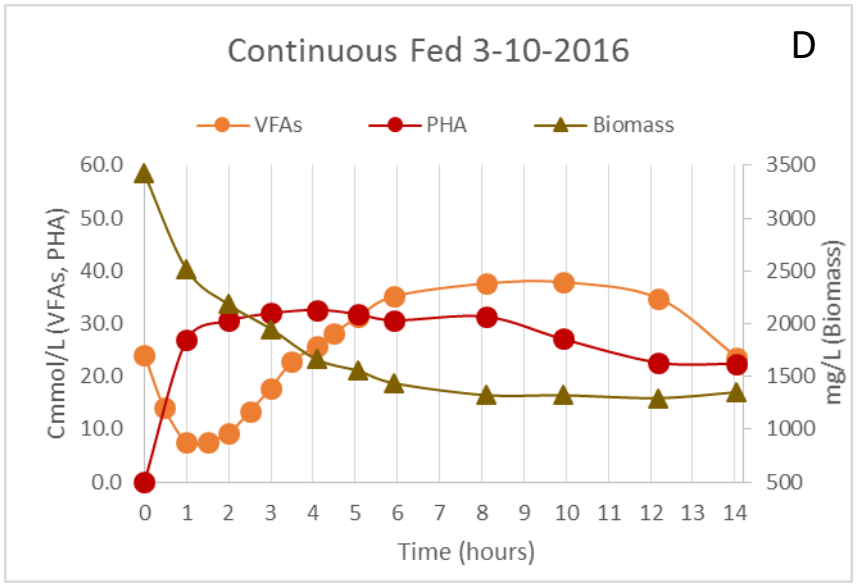
The motivation for conducting continuous fed production assessments was a desire to streamline the production reactor operation, improve process control, and appeal to full scale implementation. The premise is to operate the reactor exactly as done with fed-batch except to add more frequent pulses with much smaller volumes – in the limit, the influent flow becomes continuous. As with fed-batch, the target VFA concentration was to remain in excess without becoming inhibitory; it was not necessary to let it decrease close to zero. To examine the potential to enhance and maximize PHA production by applying a continuous substrate addition, six PHA production experiments were conducted from January 2016 to March 2016. The experimental set up was unchanged from the fed-batch investigations, with the exception that volume was accumulated due to a less frequent sampling regimen.

The results for each of these (VFAs, PHA, and biomass) are shown in Figure 28. Biomass concentration was calculated by subtracting PHA from the VSS measurements. As shown, four of the six assessments (B-D, F) maintained VFA concentrations between limiting and inhibiting values (0-60 Cmmol/L), while the other two (January 28 and March 17) had significant VFA depletion for part of the experiment. The experiment from January 28 was particularly affected due to the VFA concentration never recovering. However, it should be understood that even though VFAs appear to have been limiting in these cases, the continuous feeding still added substrate to the reactor, maintaining to some degree the metabolic activity required for PHA accumulation. This is in direct contrast to fed-batch where a total loss of VFAs causes PHA synthesis to cease immediately.


Continuous Fed 1-28-2016
A
VFAs
PHA
Biomass
Cmmol/L (VFAs, PHA)
mg/L (Biomass)
Time (hours)


Continuous Fed 2-11-2016
B
VFAs
PHA
Biomass
Cmmol/L (VFAs, PHA)
mg/L (Biomass)
Time (hours)


Continuous Fed 2-25-2016
C
VFAs
PHA
Biomass
Cmmol/L (VFAs, PHA)
mg/L (Biomass)
Time (hours)


Continuous Fed 3-10-2016
D
VFAs
PHA
Biomass
Cmmol/L (VFAs, PHA)
mg/L (Biomass)
Time (hours)

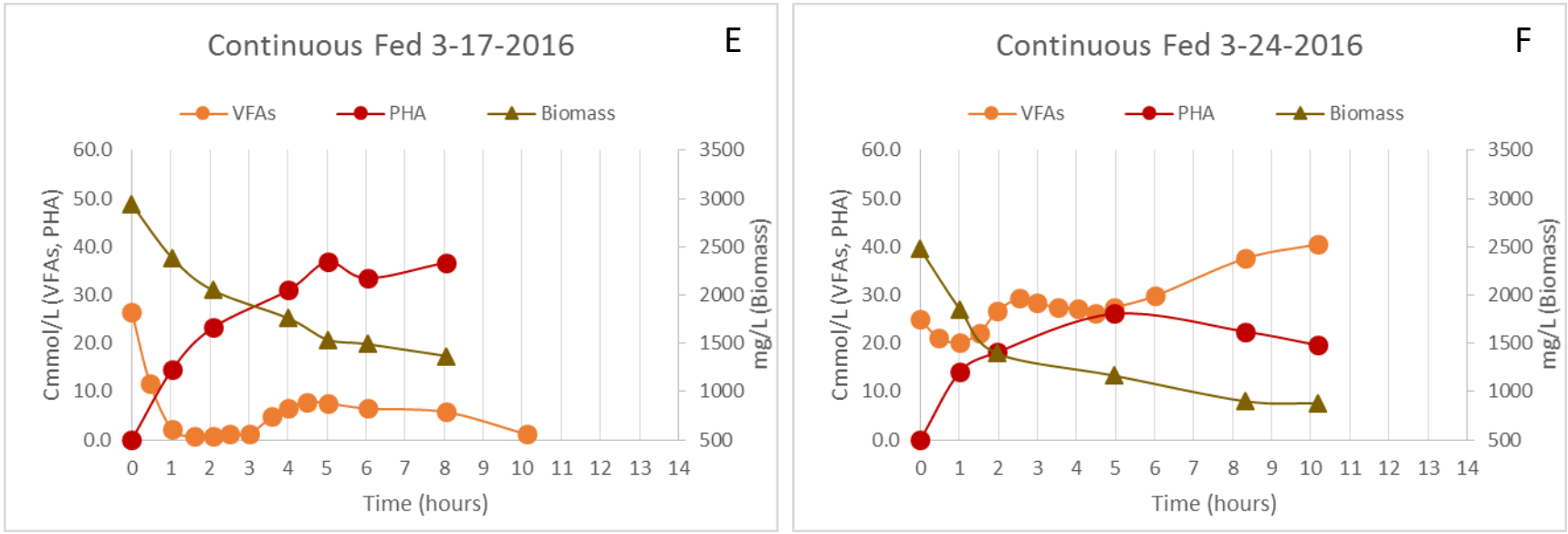


Figure 28: Summary of continuous fed production experiments

As shown, PHA was successfully accumulated in all six reactor assessments; the weight percent data is presented in Figure 29 (TSS basis) and Table 7 (TSS and VSS basis). In considering these continuous fed PHA production results, there are two important differences in comparison to the fed-batch experiments. First is that the results are much more consistent, reaching maximum PHA content between 31.6%-49.3% VSS compared to 18.5%-74.8% in the fed-batch investigations. The second difference is the continuous fed maximum intracellular PHA accumulation results are much lower than observed in the fed-batch investigations, with only one of six nearing 50% PHA content on a VSS basis, compared to two out of four reaching 60% or higher in the fed-batch studies. This apparent underperformance is discussed further in the next section.

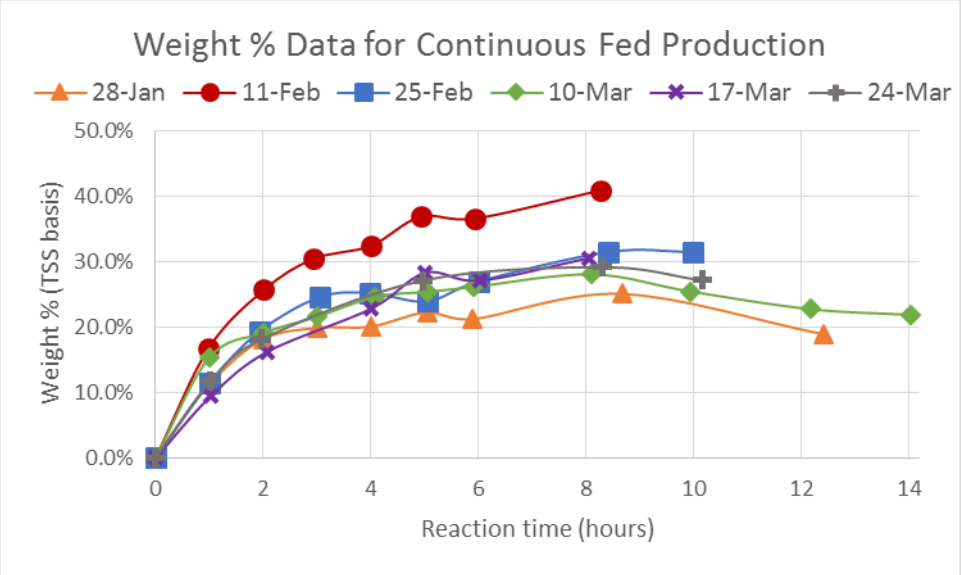


Figure 29: Weight percent, TSS basis, continuous fed experiments

Table 7: Summary of data for continuous fed production experiments

		1/28/2016	2/10/2016	2/25/2016
Volume, mL	*Initial*	500	750	500
	Final	1590	2260	1980
Feed VFAs & Flow Rate	*Cmmol/L*	114	158	132
	mL/min	1.49	3.10	2.50
Max PHA	*Cmmol/L*	28.5	57.6	33.9
	Wt%, TSS	25.1%	40.8%	31.5%
	Wt%, VSS	31.6%	49.3%	41.4%
PHA Yield %	*Cumulative*	48.7%	80.2%	46.7%
	Average	49.8%	76.2%	45.9%
Uptake Rate, Cmmol/ (gVSS*hr)	*Initial*	28.0	15.9	11.8
	Average	12.4	11.7	8.5
	Stable	9.0	9.5	7.3
		3/10/2016	**3/17/2016**	**3/24/2016**
Volume, mL	*Initial*	500	750	500
	Final	2230	2690	2740
Feed VFAs & Flow Rate	*Cmmol/L*	140	140	135
	mL/min	2.07	3.22	3.66
Max PHA	*Cmmol/L*	32.6	36.8	26.1
	Wt%, TSS	28.0%	30.5%	29.1%
	Wt%, VSS	35.4%	37.1%	36.4%
PHA Yield %	*Cumulative*	20.5%	33.1%	22.6%
	Average	37.5%	34.1%	29.5%
Uptake Rate, Cmmol/ (gVSS*hr)	*Initial*	8.9	26.2	23.7
	Average	8.8	14.1	16.4
	Stable	6.6	11.3	13.2

5.3.2 PHA Yield and Substrate Uptake

The PHA yields and specific substrate uptake rates as functions of reaction time are shown in Figure 30 and Figure 31, respectively. It was observed for the fed-batch experiments that the PHA yield remained constant for the entire reaction period; this was

also true for continuous fed mode, with two notable exceptions – January 28 and March 10. The former is easily explained by the fact that VFAs became limiting (Fig. 27A) and the microbes resorted to consuming internal PHA reserves to maintain maximum metabolic activity. However, the latter has no good explanation and appears to be an exceptional case. It is possible that for an unknown reason the first hour experienced unusually high conversion of VFA to PHA, followed thereafter by "typical" values. Another potential explanation is that growth metabolisms became more prevalent late in the operational cycle, thus re-directing VFAs away from PHA production. Alternately, it is possible that around 6-8 hours the flanking population became significant, consumed a higher proportion of VFAs and dropping the yield further. One of the experiments, February 11, resulted in significantly higher PHA yield compared to the others which incidentally corresponds to the experiment with the highest maximum PHA content. The next section discusses whether the intracellular content is actually a function of PHA yield or simply a one-time coincidence.

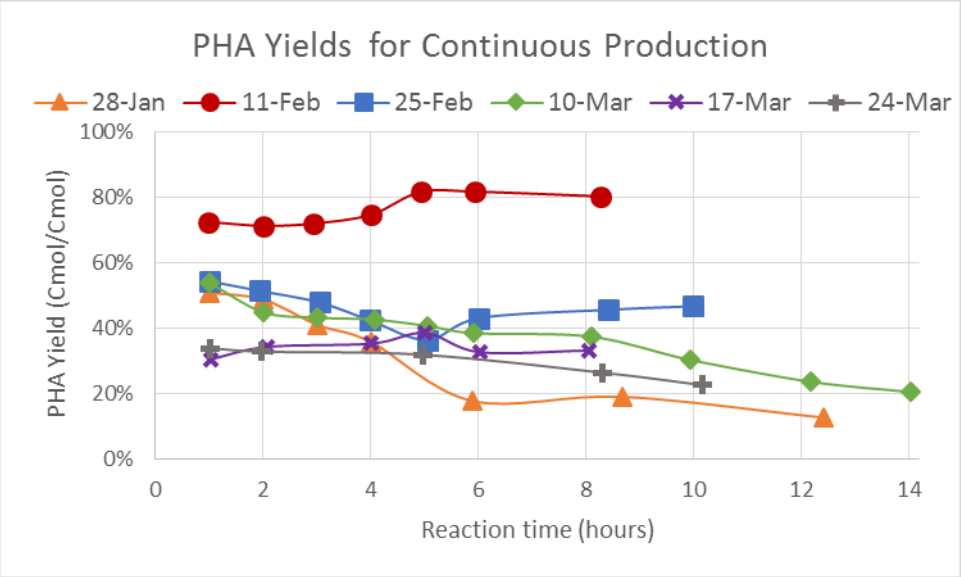


Figure 30: Continuous fed production cumulative PHA yield data

The trends in the specific substrate uptake rate are similar to that observed and reported for the fed-batch experiments. Specific uptake rates start high but quickly fall to a stable rate (Figure 31). The fed-batch data has a slightly higher variance in the "stable" region but corresponds closely with the continuous fed in the range of 5-15 Cmmol/(g-VSS*hr).

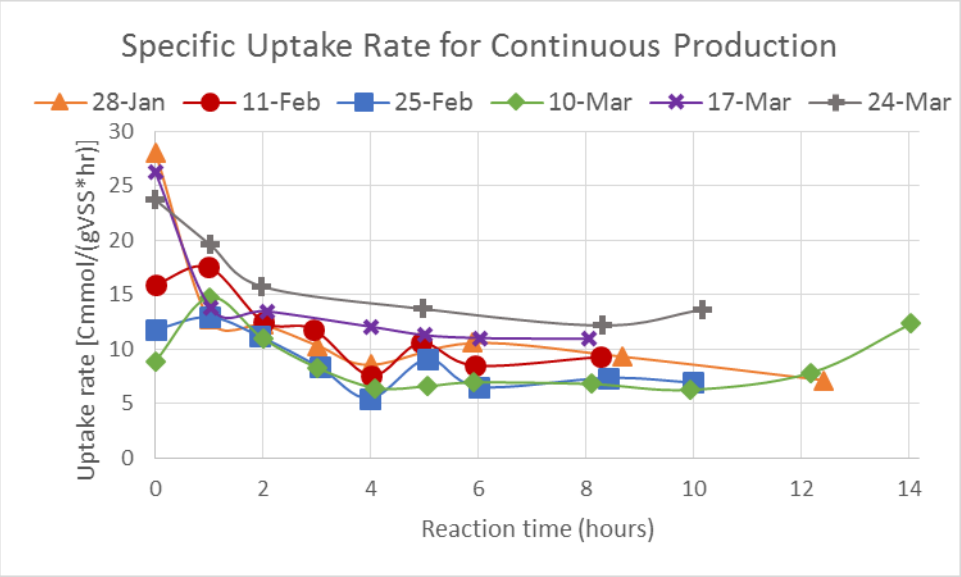


Figure 31: Continuous fed production specific substrate uptake rate data

A final observation from the continuous fed data that needs explaining is the trend of the biomass concentration. Considering that the reactor volume is initially increasing at a faster rate than new growth (as evidenced by the initial drops in biomass concentration) it is expected that the concentration will continue to decline for the entire length of the reaction. However, that is not what was observed. Instead, the biomass reached a minimum and in some cases may have begun to increase (Figure 28C-D). There are three possibilities to explain this phenomenon. First, the microbes gradually increased their metabolism resulting in a higher growth rate over time. Second, the microbes switched from PHA

storage to growth also resulting in a higher growth rate over time. Third, a fast growing flanking population was initially a small fraction of the total biomass but gradually overtook the slow growing PHA accumulators resulting in a higher observed (but not actual) growth rate. The first option cannot be true because Figure 31 clearly shows that the specific substrate uptake rate (and consequently the overall metabolic activity) did not increase with time but actually substantially decreased. Therefore, some combination of options two and three is most likely. Evidence from the literature and the weight percent data (Figure 29) suggests that growth of a flanking population is a significant, if not dominant, factor. The work of Jiang et. al. (2012) on fermented paper mill effluent demonstrated that non-accumulating microbes cannot be completely eliminated from a waste-fed enrichment reactor. Therefore, if growth is not restricted in the production reactor it can be assumed that non-accumulators will divert carbon away from storage at a rate proportional to their fraction of overall biomass. According to the growth rate equation for SBRs (see Chapter 2), since the feast period is shorter than the famine, the net specific growth rate of non-PHA accumulators (that grow only during the feast) must be higher than the PHA accumulators (that grow through the famine). This means that the fraction of non-accumulators will increase over time at an exponential rate. As was confirmed experimentally by Valentino et. al. (2015) on fermented whey permeate, the non-accumulator fraction will eventually grow large enough to cause the intracellular weight percent to reach a maximum and decline. The production assessments from this research with a lower maximum PHA content (March 10, March 24) also showed a trend of maximum and decline – a pattern that may have extended to all assessments if the reaction time were longer. In these cases, a larger initial

non-accumulator fraction simultaneously explains why the maximum was lower (more diversion of carbon to non-accumulator growth) and why the peak was reached sooner (the rate of growth is proportional to the concentration). This data could also be explained in part by the PHA producers switching metabolically from storage to growth but it is difficult to reconcile with Figure 30 which shows the yield of PHA on substrate staying consistent for each assessment period.

Regardless of whether the observed increase in biomass growth is a result of changing metabolisms, the overtaking by a flanking population, or some combination, the final analysis confirms the discussion from the fed-batch investigations that the ADF PHA process is fundamentally controlled by kinetics. In other words, there is an optimum reaction time that will maximize the PHA content for a given set of conditions – the results cannot be improved by extending the reaction time or by consuming extra substrate.

5.3.3 Process Control

The continuous mode of operation for PHA production offered several operational advantages over fed-batch and streamlined process control to maintain VFAs within the target range to avoid both limiting and inhibiting VFA concentrations. The first assessment, January 28, was operated with an initial assumed substrate uptake rate equal to that observed in the enrichment reactor. Ultimately it proved to be highly underestimated, but because feedstock was constantly being fed it was over three hours before VFAs became limiting, and even then substrate was still being introduced, thus maintaining a partial "feast" metabolism (Figure 28A). In contrast, for fed-batch mode such an underestimated

uptake rate would cause a major process upset within minutes and switch completely to a "famine" period. To avoid a repeat of VFA loss, all subsequent continuous fed experiments were operated with periodic checks of VFA concentrations with the GC-FID to ensure the correct range was sustained. Indeed, the substrate flow rate was adjusted at least three times for each reaction according to the rate of change in VFA concentration. In each case, the initial substrate uptake rate was underestimated (as evidenced by the initial negative slopes in Figure 28) but were successfully corrected. The one exception was the experiment March 17 in which the initial specific uptake rate was so large that VFA depletion occurred before flow adjustments could correct it; nevertheless, the VFA concentration did eventually recover and remain in excess.

The common practice for process control to maximize PHA production is to use a surrogate for VFA measurements such as D.O. shifts, pH, or oxygen uptake rate measurements (see Chapter 2 for a review). However, by operating in continuous substrate addition mode (i.e., non-traditional for PHA production), it was not necessary to use a surrogate and instead the actual VFA measurements were used as feedback to maintain process control. Another advantage of operating with continuous feed is operational simplicity. After setting the feed pump, the operator can periodically sample at any convenience and as often as desired – usually with more frequent testing in the beginning to ensure a proper flow rate. In fed-batch, each successive pulse must be carefully timed with only a small margin of error allowed regarding feeding and sampling. Since there is no outflow (except relatively small sampling volume) the continuous method maintains the same biomass residence time as fed-batch. That is, all discrete elements enter and exit the

reaction at the same time (i.e. temporal plug flow). One difference between the two modes is that in continuous fed operation the speciation of VFAs is more likely to shift due to some species potentially being preferentially taken up over others. In fed-batch, assuming no accumulation of VFAs, each pulse “resets” the concentration and the speciation to match the feedstock. It is unclear how strong of an impact this difference makes, if any exists at all, but the operational advantages of continuous feed are unambiguous and represent a step forward towards process viability.

5.4 Comparative Assessment

The previous three sections were devoted to independently presenting and discussing the results from the enrichment reactor, fed-batch production reactor, and continuous fed production reactor. In this section, the relationships among the three stages are explored in more detail. In particular, it is desired to know whether knowledge about the enrichment operation (PHA yield, substrate uptake rate, stability or lack thereof) is able to be correlated with performance in the production reactor. If so, the design of larger systems becomes simplified and business ventures can have increased confidence that the process will ultimately succeed. Also, the continuous fed mode, while operationally superior, underperformed the fed-batch experiments in terms of maximum weight percent PHA. It is important to learn if this was the result of unstable enrichment operation upstream or an intrinsic handicap of continuous operation.

5.4.1 Enrichment & Production Correlations

For all production experiments, same day or previous day PHA yield, substrate uptake, and biomass concentration were measured from the enrichment reactor. The results are summarized in Table 8.

Table 8: Summary of same-day enrichment and production reactor metrics

	Enrichment Data			Production Data		
Date	**Initial biomass** *mg/L*	**Uptake rate** *Cmmol/ (gVSS*hr)*	**PHA yield** *%*	**Uptake rate, stable** *Cmmol/ (gVSS*hr)*	**PHA yield** *%*	**Maximum PHA** *Wt%, TSS*
7/21/2015	2160	5.9	37.2%	3.4	38.4%	15.5%
10/13/2015	2720	5.6	113%	14.1	114%	64.7%
11/17/2015	3450	5.7	47.8%	11.7	66.3%	48.2%
12/10/2015	2780	8.3	59.3%	13.1	63.0%	33.6%
1/28/2016	3730	4.6	53.6%	9.0	49.8%	25.1%
2/11/2016	3260	8.7	60.9%	9.5	76.2%	40.8%
2/25/2016	4080	8.0	55.3%	7.3	45.9%	31.5%
3/10/2016	3950	6.9	47.4%	6.6	37.5%	28.0%
3/17/2016	3160	6.1	38.1%	11.3	34.1%	30.5%
3/24/2016	3100	8.1	23.9%	13.2	29.5%	29.1%

The most important metric that indicates an enrichment reactor is highly enriched in PHA producers is the PHA yield – as more substrate carbon flows to PHA, the less it flows to growth (both of the PHA producers and the flanking population). PHA yields approaching 100% indicate a high accumulating biomass fraction and a high level of efficiency in storage metabolism. The yield data for enrichment and production from Table 8 is plotted in Figure 32 and shows a remarkably high level of correlation. Note that the identity line in the plot is not a least-squares linear regression but indicates where the enrichment and production

yields are equivalent. There are several important implications from this data. First, the "health" of the enrichment reactor is an excellent predictor of production behavior – in other words, whatever yield is observed in the former is what can be expected in the latter, within a certain margin of error. The robustness of this assertion is confirmed by the wide range of data points - as low as 24% to a maximum of 114%. Second, there is random scatter around the identity line for the fed-batch experiments, continuous fed experiments, and the combination of both. In essence, there is no underlying variable controlling this pattern (independence of variance) and the two operating modes appear to be equally effective. Figure 32 (and the upcoming Figure 34) confirms that continuous operation is as effective as fed-batch, and the apparent underperformance is a result of poor enrichment stability.

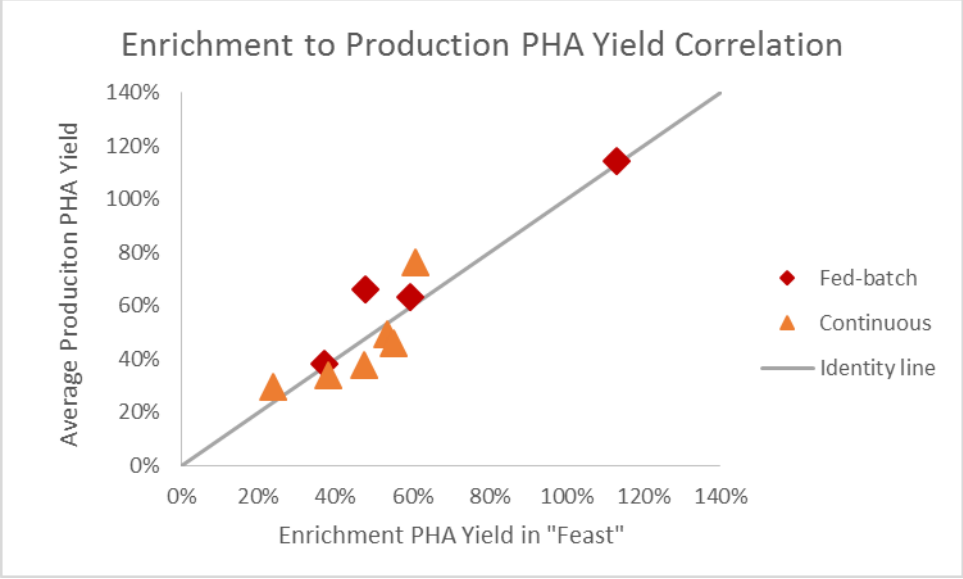


Figure 32: Correlation of enrichment and production PHA yields

The second most important metric to try to correlate between enrichment and production is the specific substrate uptake rate, which is an indication of the metabolic

activity of the mixed culture (specifically to VFAs). If a correlation exists for specific substrate uptake rate as well as PHA yield, a simple model can be composed to predict the intracellular PHA content as a function of time. Such a model would be useful for design and lead to greater understanding of the PHA process as a whole. Unfortunately, as Figure 33 shows, no such correlation exists from the data collected in this research.

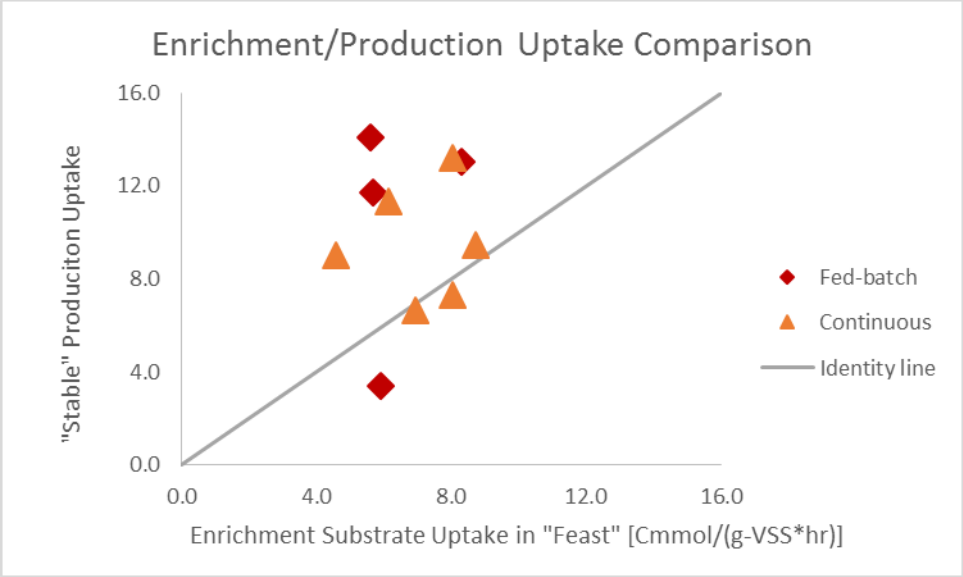


Figure 33: Correlation of enrichment and production specific substrate uptake rate

In most cases the substrate uptake rate in the production reactor (using the stabilized value as a basis) was many times larger than that observed in the enrichment, and there appears to be no clear relationship of any kind between these two variables. There is at this point no good explanation why a stronger relationship does not exist. Indeed, it seems natural that if both reactors are operated similarly (well mixed, excess oxygen and VFAs) then the rate of substrate consumption should be similar. One possible explanation hinted at in previous discussion is the potential reliance of the microbes in the enrichment phase on alternative electron acceptors (nitrate and nitrite) over oxygen to consume substrate. Due to reactor

configuration, it is possible that limitations were placed on the oxygen transfer rate even though the D.O. remained above 1.0 mg/L in most cases. For some production assessments (particularly March 10 through March 24) the corresponding enrichment cycle was truly oxygen poor, with D.O. well below 1.0 mg/L (an operational misstep discussed in the first section of this chapter). If true, it would signify a definite metabolic difference between the two phases as an unlimited oxygen supply in the production would result in different microbial behavior. A better reactor design may be all that is required to correlate the uptake rate more effectively.

5.4.2 Implications for Design

The final correlation, and perhaps the most important, is that shown in Figure 34 wherein the maximum PHA weight percent in the production reactor is strongly linearly dependent on the average PHA yield in the production reactor. It confirms that the PHA yield is the best indicator of overall reactor performance – higher yields leads to higher weight percent.

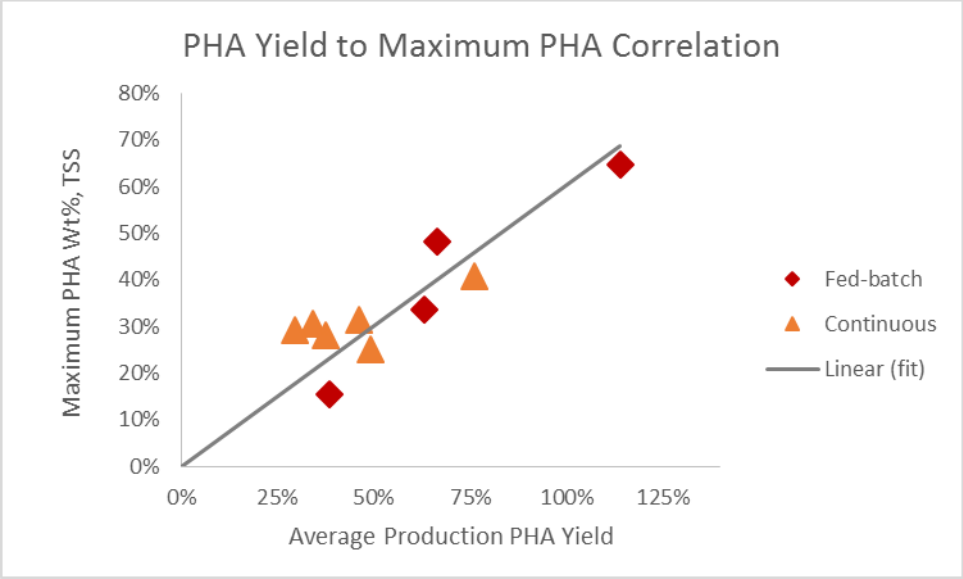


Figure 34: Correlation of production yield to maximum weight percent

This empirical data set has real practical value for design. The starting point is to pick a desired weight percent that will result in financial stability and economic success. From Figure 34, the average PHA yield in the production reactor needed to reach that value can be determined and, from Figure 32, is the same needed in the enrichment reactor. Optimal enrichment performance was not obtained in this research, but it has been discussed previously how this might be accomplished. Briefly, the CL to SRT ratio should be increased to the highest practical value, followed by the highest non-inhibitory organic loading rate that sustains the feast-to-famine ratio below the empirical threshold of 0.2. The PHA correlations can also be used in reverse as a troubleshooting tool. If low PHA weight percent is being produced and the yield values in the enrichment and production are far apart, then the production reactor is not operating at optimum. Conversely, if there is low weight percent but the yields are close (such was the case for the latter half of the continuous fed assessments) it can be concluded that the problem lies with the enrichment. Thus, a protocol is established to separately optimize each stage and identify problems.

As a final note, the figures shown above are empirical in nature and are quantitatively only valid for fermented dairy manure until proven otherwise. Qualitatively, however, the results have universal implications. Namely, higher enrichment yields lead to higher production yields which in turn lead to higher weight percent values. In situations where growth is fully or partially restricted (applied in almost every case – see Table 4) it may be possible to obtain very high weight percent data from subpar enrichment performance. However, growth restriction is often only possible when synthetic medium is used for growth because real waste streams have all the nutrients required for growth (hence the reason they are good choices to begin with). Trying to remove an essential growth element from a real waste stream is completely impractical in most cases. As a reminder, the data presented herein was collected from a true waste resource, was not pH adjusted, was not temperature adjusted, and was not limited in any growth element including ammonia, phosphorus, VFAs, and oxygen. Despite these suggested disadvantages and despite the fact that the reaction is kinetically limited by the growth of biomass, it was still possible to obtain weight percent numbers comparable to the literature, possibly even exceeding it for the most similar cases. Moreover, such results were generated without intricate monitoring equipment or complicated process control. In essence, the data were obtained with the most favorable economic factors and at conditions, including operation, which would be found at full-scale.

CHAPTER 6: CONCLUSION

6.1 Summary

Resource recovery, the practice of converting traditional waste streams into valuable commodities, is an ever growing field of interest in science and engineering. In the research presented and discussed herein, dairy manure was used as a model waste substrate capable of being converted into a bioplastic called polyhydroxyalkanoate, or PHA. With physical and mechanical properties similar to conventional plastics, PHA has shown great promise as an alternative that is renewable, biodegradable, and sustainable. Previous research in this field has been dominated by synthetic substrates and conditions detrimental to economic viability, such as elevated temperature and artificial growth restrictions. Indeed, several life cycle assessments (Fernandez-Dacosta et al., 2015; Gurieff and Lant, 2007) have identified operating cost as the limiting factor preventing expansion of this field into the full scale industry. The overarching objective of this research was to explore whether dairy manure, with the fewest possible interventions, is competitive with other substrates (including synthetic) in the PHA research literature and to identify practical guidelines for scale-up design that is applicable for any waste substrate. To this end, a three-stage PHA process was constructed with elements including a fermenter, an enrichment reactor, and a production reactor. Three hypotheses were developed and evaluated. The first hypothesis tested the stability of the enrichment reactor (and subsequent production reactor) over a long time period, given that dairy manure is intrinsically variable. The primary findings can be summarized as follows:

- The loading to the enrichment reactor was volumetrically based, therefore the organic loading rate (OLR) varied as expected at 21.1 ± 4.6 Cmmol-VFA/(L*day).
- The biomass concentration, feast length, and PHA yield on VFAs varied substantially, with PHA yield having the widest range. These variables were not well correlated to the shifts in OLR.
- Considering the wide range of PHA yields, the best metric for stability, it was concluded that the enrichment reactor was not sufficiently stable over the time period assessed.
- Some of the instability can be accounted for by oxygen limitation at the beginning and end of the assessment period. The remaining can be best explained by having too low of an OLR. It is expected that stability could be achieved by increasing the OLR to a maximum that is non-inhibitory and below a feast-to-famine ratio of 0.2. The cycle length to SRT ratio should be increased as well.
- The production reactor, especially when operated as fed-batch, experienced a wide range of reported PHA values, as is expected considering it is downstream of the enrichment reactor.

The second hypothesis compared two modes of operation for the production reactor against each other and against literature reported values. The first mode was the traditional, industry standard fed-batch operation and the second was a continuous fed-batch reaction. In summary:

- The maximum PHA content, on a VSS basis, was obtained under fed-batch mode and reached 74.8%. The maximum reached with continuous mode was 49.3% VSS. Both results are comparable to Valentino et. al. (2015), who operated on a waste substrate with growth allowed and achieved a range between 50-70% VSS.
- The continuous mode was operationally superior to fed-batch mode due to lower required operator attention and easier sampling. Most importantly, continuous mode was able to maintain excellent process control by using real VFA measurements as feedback, something not possible in fed-batch mode.
- The apparent underperformance of continuous mode compared to fed-batch (for some reactions, not all) can be attributed to instability in the upstream enrichment reactor. Confirmation of this fact is provided by the independent variance of the two methods in the PHA yield correlations.
- Considering operational superiority and equal production performance, continuous fed-batch is the preferred mode of operation.

The third and final hypothesis tested the assumption that the production reactor is merely an extension of the enrichment reactor and will have comparable biological activity. If true, the intracellular PHA content can be calculated *a priori* from enrichment data without needing to be empirically fit to production data. The following points summarize the results:

- The PHA yields in the production reactor remained nearly constant for the entire reaction length. The specific uptake rate was not constant, beginning at a high value and gradually declining to a stable point later in the reaction.

- The PHA yield in the enrichment reactor is highly correlated with the average PHA yield. The scatter is around the identity line, meaning that the yield obtained in the enrichment was the average yield obtained in the production.
- It was not possible to correlate specific substrate uptake rate from the enrichment to the production reactor. Thus, it is not possible to calculate weight percent *a priori* without fitted parameters.
- Although a theoretical framework for determining weight percent was unsuccessful, an empirical relationship was developed that is useful for design. The weight percent is linearly dependent on the production PHA yield, which in turn is linearly dependent on enrichment PHA yield (as stated earlier). Therefore, this metric is the most important factor for success in the overall process.
- With the PHA yield relationships in mind, it is possible to identify which reactor – enrichment or production – is underperforming when final weight percent is low.

Despite some setbacks, particularly unstable enrichment performance, it was shown that dairy manure is a competitive, viable substrate for the PHA process. The results presented are qualitatively and possibly quantitatively applicable for any waste substrate operating under growth allowed conditions. Finally, the conclusions can be utilized by scientists and engineers seeking to develop a full-scale design.

6.2 Design Flowchart

One of the objectives of this research was to aid in design and optimization of the PHA process. The design flowchart in Figure 35 provides step-by-step guidance on how to

select reactor volumes and operating conditions, drawing from the results of this paper and the body of literature as a whole. It is constructed in a way that is applicable for any waste fed, three-stage PHA process in which the enrichment reactor is operated in SBR mode as a chemostat (i.e., no settling phase for the biomass).

From the fermenter, a known volume of VFA-rich liquor is produced at a certain rate and has a known concentration of VFAs. Fermenter optimization is not covered in this paper, but any full-scale PHA design should nevertheless have these input values readily available. In the enrichment, the CL is chosen prior to SRT and matched so that the ratio is at least 0.25 or greater. This fraction represents the volume percent that is wasted and fed every cycle and also the dilution factor of the influent feed. Then, the OLR per cycle is chosen, but it cannot exceed the maximum achievable concentration determined by the fermenter effluent and the dilution factor. Finally, the enrichment reactor volume is chosen and several calculations are performed to ensure an optimal choice. It is assumed that a new production experiment will start at the conclusion of every enrichment cycle using the wasted volume as inocula. Using enrichment data as feedback, the ratio of fermenter volume to inocula volume, along with total production volume, can be calculated. The rate of fermenter liquor required by the enrichment and production reactors cannot exceed the total volume available from the fermenter. Finally, several quality control checks are employed to ensure optimal choices for operational parameters. The F/F ratio must be less than 0.2 and the OLR must be maximized, otherwise the OLR needs to be adjusted.

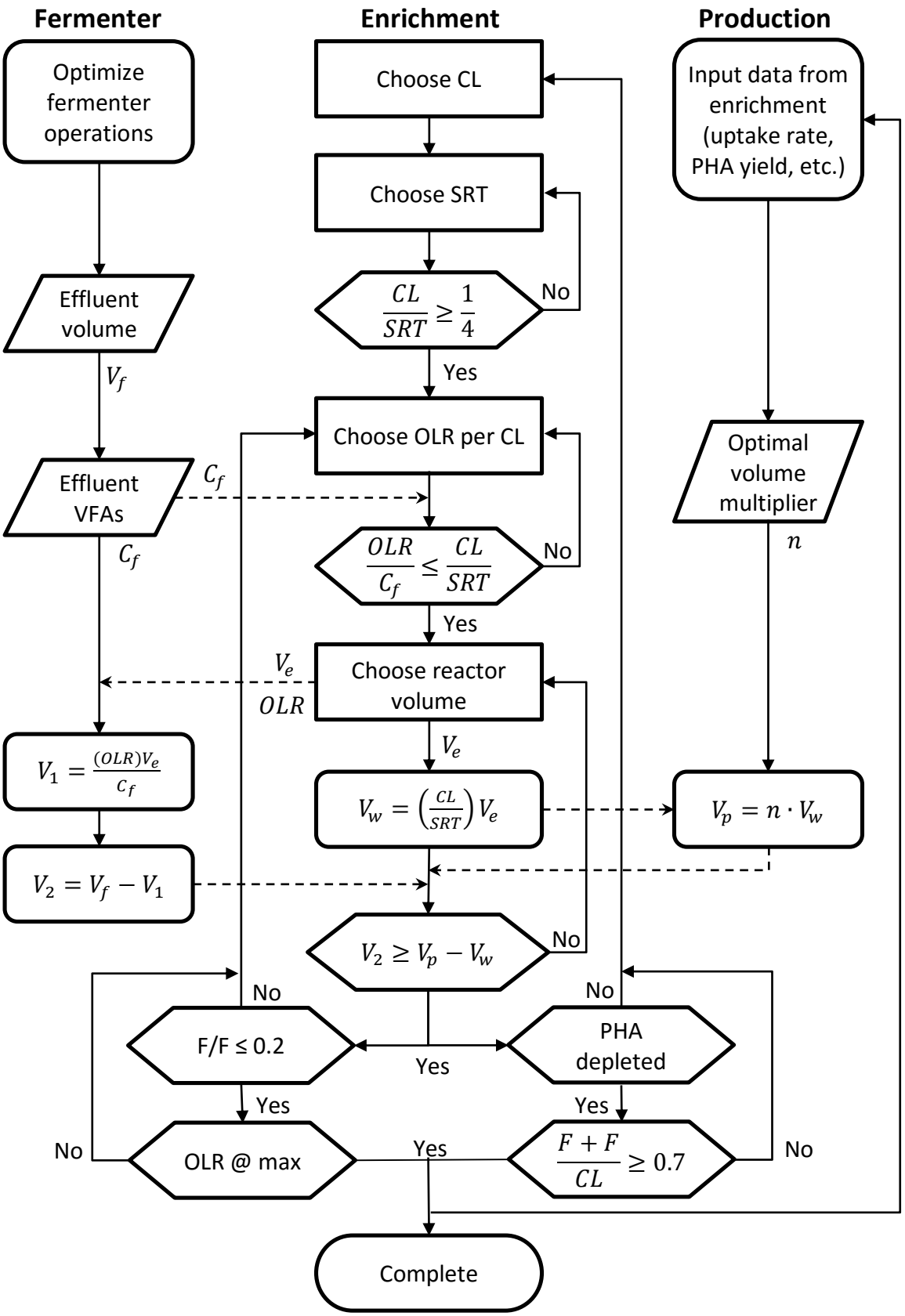


Figure 35: Design flowchart for optimization of 3-stage PHA process

The PHA content at the end of the cycle should be fully depleted but also not depleted too early, otherwise the cycle length needs to be adjusted. This information is used as feedback for the production reactor and the design is at completion.

The information in Table 9 shows how the flowchart can be applied using the laboratory fermenter used in this research as an example. From experience it is known that four liters of fermenter effluent with a concentration of 150 Cmmol/L can be obtained per day. With a CL and SRT of 1 and 3 days, respectively, the maximum attainable OLR is 50 Cmmol/(L*day). Choosing an enrichment volume of three liters allows one liter per day of fermenter liquor to be used as enrichment food with the other three being used for production. From experience it requires about four times the initial volume in a production reaction to reach maximum weight percent, so the effective production volume is four liters.

Table 9: Example scenario for design flowchart application

Parameter	Symbol	Value	Unit
Fermenter effluent	V_f	4.0	L/day
Fermenter concentration	C_f	150	Cmmol/L
Cycle length	CL	1	day
Solids residence time	SRT	3	day
Organic loading rate	OLR	50	Cmmol/(L*day)
Enrichment volume	V_e	3.0	L
Waste volume	V_w	1.0	L/day
Enrichment feed volume	V_1	1.0	L/day
Production volume multiplier	n	4	--
Production volume	V_p	4.0	L
Production feed volume	V_2	3.0	L/day

Thus, 75% of the original fermenter volume is used for PHA production. Assuming a PHA yield of 110% (a reasonable estimate if 60-70% PHA as TSS is desired) and an average molecular PHA weight of 90 g/mol, this scenario could produce as much as 45 g-PHA per day.

Taking it one step further, the average weight of wet manure fed to the fermenter is approximately 1.40 kilograms per day (this value changes with seasonality). The PHA production is then 32 g-PHA per kilogram of wet manure. If a cow produces 30 kilograms of waste per day, then a small dairy farm with 100 head of cow could ideally produce 96 kilograms of PHA every day. At U.S. $1.40 per kilogram (the price of its competitor, PET), this hypothetical farm could see revenue close to U.S. $50,000 per year. Operation costs and return on investment must still be accounted for in order to calculate profit, but the potential gain is appealing. This simple example is not intended to be a replacement for experimental results and pilot testing but rather to illustrate the usefulness for design and usefulness for making business evaluations.

6.3 Future Work

The results and conclusions from this research affirm the use of dairy manure as a model substrate and contribute to the body of knowledge for PHA research as a whole. There are, of course, many ideas and research topics remaining, ranging from practical to theoretical, that can and should be explored in the future. Chief among them is to make the necessary changes to the enrichment reactor as outlined previously to obtain stability and leverage the greatest possible selective pressure on the mixed microbial culture. Once

achieved, more productions experiments should be conducted to check for maximum possible weight percent and associated variance. At a broader level, dairy manure is high in ammonia and previous research (Coats et al., 2016-under review; Watson, 2015) has discovered that nitrate and nitrite can be used as electron acceptors in place of oxygen while still maintaining a culture enriched for PHA accumulators. Studying the role of nitrogen cycling in more depth could lead to simultaneous denitrification and high yield PHA accumulation, increasing the appeal of the process with regards to resource recovery and dairy manure treatment. Ammonia oxidation requires oxygen which may be partially responsible for the gap between the enrichment and production reactors for specific substrate uptake rate. More investigation on this point may provide a way to predict uptake rate in the production and revive the possibility of developing a predictive model capable of calculating weight percent *a priori*. Another research avenue is to investigate the phylogenetic properties of the mixed microbial consortium. Determining the quantity and type of bacteria present may help confirm the results of this research or open up new questions altogether. Alternatively, experimental determination of growth rates combined with a thermodynamic balance of carbon and nitrogen could be compared with theoretical values from the SBR growth rate equation and offer insight about how to maximize the PHA yield on VFAs.

All these suggestions are merely a sample of the future research possibilities, all of which would likely reveal more research questions of their own. The vast field of PHA research has room for more growth, with topics including molecular microbiology, biochemistry, thermodynamics, engineering design, business economics, and others. It is

the combined contribution of all these areas that will ultimately determine the relevant questions and influence the direction of PHA research and resource recovery as a whole. It is with honor that this thesis can contribute to this global discussion, however small, and encourage others to do the same.

CHAPTER 7: REFERENCES

Albuquerque, M. G. E., S. Concas, S. Bengtsson, and M. A. M. Reis, 2010a, Mixed culture polyhydroxyalkanoates production from sugar molasses: The use of a 2-stage CSTR system for culture selection: Bioresource Technology, v. 101, p. 7112-7122.

Albuquerque, M. G. E., C. A. V. Torres, and M. A. M. Reis, 2010b, Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses: Effect of the influent substrate concentration on culture selection: Water Research, v. 44, p. 3419-3433.

Appel, F. J., 2015, Advancing a Post-Anoxic Biological Nutrient Removal Process Selecting for Nitritation, University of Idaho, Moscow, ID, 75 p.

Beccari, M., L. Bertin, D. Dionisi, F. Fava, S. Lampis, M. Majone, F. Valentino, G. Vallini, and M. Villano, 2009, Exploiting olive oil mill effluents as a renewable resource for production of biodegradable polymers through a combined anaerobic-aerobic process: Journal of Chemical Technology and Biotechnology, v. 84, p. 901-908.

Bengtsson, S., A. R. Pisco, M. A. M. Reis, and P. C. Lemos, 2010, Production of polyhydroxyalkanoates from fermented sugar cane molasses by a mixed culture enriched in glycogen accumulating organisms: Journal of Biotechnology, v. 145, p. 253-263.

Beserker79, 2005, Chemical structure of polyhydroxyalkanoates PH3B, PHV and PHBV, Wikipedia Commons, Wikipedia.

Beun, J. J., K. Dircks, M. C. M. Van Loosdrecht, and J. J. Heijnen, 2002, Poly-beta-hydroxybutyrate metabolism in dynamically fed mixed microbial cultures: Water Research, v. 36, p. 1167-1180.

Beun, J. J., F. Paletta, M. C. M. Van Loosdrecht, and J. J. Heijnen, 2000, Stoichiometry and kinetics of poly-beta-hydroxybutyrate metabolism in aerobic, slow growing, activated sludge cultures: Biotechnology and Bioengineering, v. 67, p. 379-389.

Braunegg, G., B. Sonnleitner, and R. M. Lafferty, 1978, A rapid gas chromatographic method for the determination of poly-(beta)-hydroxybutyric acid in bacterial biomass: Applied Microbiology and Biotechnology, v. 6, p. 29-37.

Chen, G. Q., and M. K. Patel, 2012, Plastics Derived from Biological Sources: Present and Future: A Technical and Environmental Review: Chemical Reviews, v. 112, p. 2082-2099.

Choi, J. I., and S. Y. Lee, 1997, Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation: Bioprocess Engineering, v. 17, p. 335-342.

Clesceri LS, G. A., Eaton AD, 1998, Standard Methods for Examination of Water and Wastewater: Washington D.C., American Public Health Association.

Coats, E. R., F. J. Loge, W. A. Smith, D. N. Thompson, and M. P. Wolcott, 2007, Functional stability of a mixed microbial consortium producing PHA from waste carbon sources: Applied Biochemistry and Biotechnology, v. 137, p. 909-925.

Coats, E. R., A. Mockos, and F. J. Loge, 2011a, Post-anoxic denitrification driven by PHA and glycogen within enhanced biological phosphorus removal: Bioresour. Technol., v. 102, p. 1019-1027.

Coats, E. R., K. E. VandeVoort, J. L. Darby, and F. J. Loge, 2011b, Toward polyhydroxyalkonate production concurrent with municipal wastewater treatment in a sequencing batch reactor system: ASCE J. Environ. Engr., v. 137, p. 46-54.

Coats, E. R., B. Watson, and C. K. Brinkman, 2016-under review, Effect of Oxygen Mass Transfer on Polyhydroxyalkanoate Synthesis by Mixed Microbial Consortia cultured on Fermented Dairy Manure: Bioresour. Technol.

Dias, J. M. L., P. C. Lemos, L. S. Serafim, C. Oliveira, M. Eiroa, M. G. E. Albuquerque, A. M. Ramos, R. Oliveira, and M. A. M. Reis, 2006, Recent advances in polyhydroxyalkanoate production by mixed aerobic cultures: From the substrate to the final product: Macromolecular Bioscience, v. 6, p. 885-906.

Dias, J. M. L., A. Oehmen, L. S. Serafim, P. C. Lemos, M. A. M. Reis, and R. Oliveira, 2008, Metabolic modelling of polyhydroxyalkanoate copolymers production by mixed microbial cultures: Bmc Systems Biology, v. 2, p. 21.

Dias, J. M. L., L. S. Serafim, P. C. Lemos, M. A. M. Reis, and R. Oliveira, 2005, Mathematical modelling of a mixed culture cultivation process for the production of polyhydroxybutyrate: Biotechnology and Bioengineering, v. 92, p. 209-222.

Dionisi, D., M. Beccari, S. D. Gregorio, M. Majone, M. P. Papini, and G. Vallini, 2005, Storage of biodegradable polymers by an enriched microbial community in a sequencing batch reactor operated at high organic load rate: Journal of Chemical Technology and Biotechnology, p. 1306-1318.

Dionisi, D., M. Majone, V. Papa, and M. Beccari, 2004, Biodegradable polymers from organic acids by using activated sludge enriched by aerobic periodic feeding: Biotechnology and Bioengineering, v. 85, p. 569-579.

Dionisi, D., M. Majone, G. Vallini, S. Di Gregorio, and M. Beccari, 2006, Effect of the applied organic load rate on biodegradable polymer production by mixed microbial cultures in a sequencing batch reactor: Biotechnology and Bioengineering, v. 93, p. 76-88.

Fernandez-Dacosta, C., J. A. Posada, R. Kleerebezem, M. C. Cuellar, and A. Ramirez, 2015, Microbial community-based polyhydroxyalkanoates (PHAs) production from wastewater: Techno-economic analysis and ex-ante environmental assessment: Bioresource Technology, v. 185, p. 368-377.

Fvasconcellos, 2007, Schematic diagram of the mitochondrial electron transport chain, Wikipedia.

Grady, J., C.P. Leslie, G. T. Daigger, N. G. Love, and C. D. M. Filipe, 2011, Biological Wastewater Treatment: Boca Raton, FL, CRC Press.

Grousseau, E., E. Blanchet, S. Deleris, M. G. E. Albuquerque, E. Paul, and J. L. Uribelarrea, 2013, Impact of sustaining a controlled residual growth on polyhydroxybutyrate yield and production kinetics in Cupriavidus necator: Bioresource Technology, v. 148, p. 30-38.

Guho, N., 2010, Polyhydroxyalkanoate production coupled with waste treatment using a three-stage sequencing batch reactor system fed dairy manure: M.Sc. thesis, University of Idaho, Moscow, ID, 100 p.

Guho, N. M., 2015-under review, Theoretical assessment of the average net specific growth rate in inter-cycle steady-state sequencing batch reactors with implications for design and operation: Water Research, p. 35.

Gurieff, N., and P. Lant, 2007, Comparative life cycle assessment and financial analysis of mixed culture polyhydroxyalkanoate production: Bioresource Technology, v. 98, p. 3393-3403.

Hanson, A. J., N. M. Guho, A. J. Paszczynski, and E. R. Coats, 2016-accepted-a, Community Proteomics Provides Functional Insight into Polyhydroxyalkanoate Production by a Mixed Microbial Culture Cultivated on Fermented Dairy Manure: Appl. Microbiol. Biotechnol.

Hanson, A. J., A. J. Paszczynski, and E. R. Coats, 2016-accepted-b, Microbial Community Dynamics in a Sequencing Batch Reactor Operated under Aerobic Dynamic Feeding Conditions for the Synthesis of Polyhydroxyalkanoates from Fermented Dairy Manure: Microbiology.

Hanson, A. J., A. J. Paszczynski, and E. R. Coats, 2016-accepted-c, Proteomic profiling of an undefined microbial consortium cultured in fermented dairy manure: methods development: Electrophoresis.

Holmes, P. A., 1985, APPLICATIONS OF PHB - A MICROBIALLY PRODUCED BIODEGRADABLE THERMOPLASTIC: Physics in Technology, v. 16, p. 32-36.

Jiang, Y., M. Hebly, R. Kleerebezem, G. Muyzer, and M. C. M. van Loosdrecht, 2011a, Metabolic modeling of mixed substrate uptake for polyhydroxyalkanoate (PHA) production: Water Research, v. 45, p. 1309-1321.

Jiang, Y., L. Marang, R. Kleerebezem, G. Muyzer, and M. C. M. van Loosdrecht, 2011b, Effect of temperature and cycle length on microbial competition in PHB-producing sequencing batch reactor: Isme Journal, v. 5, p. 896-907.

Jiang, Y., L. Marang, J. Tamis, M. C. M. van Loosdrecht, H. Dijkman, and R. Kleerebezem, 2012, Waste to resource: Converting paper mill wastewater to bioplastic: Water Research, v. 46, p. 5517-5530.

Johnson, K., Y. Jiang, R. Kleerebezem, G. Muyzer, and M. C. M. van Loosdrecht, 2009a, Enrichment of a Mixed Bacterial Culture with a High Polyhydroxyalkanoate Storage Capacity: Biomacromolecules, p. 670-676.

Johnson, K., R. Kleerebezem, and M. C. M. van Loosdrecht, 2009b, Model-Based Data Evaluation of Polyhydroxybutyrate Producing Mixed Microbial Cultures in Aerobic Sequencing Batch and Fed-Batch Reactors: Biotechnology and Bioengineering, v. 104, p. 50-67.

Kleerebezem, R., and M. C. M. van Loosdrecht, 2010, A Generalized Method for Thermodynamic State Analysis of Environmental Systems: Critical Reviews in Environmental Science and Technology, p. 1-54.

Koretsky, M. D., 2004, Engineering and Chemical Thermodynamics: Hoboken, NJ, Hermitage Publishing Services.

Laycock, B., P. Halley, S. Pratt, A. Werker, and P. Lant, 2013, The chemomechanical properties of microbial polyhydroxyalkanoates: Progress in Polymer Science, v. 38, p. 536-583.

Liu, H.-Y., P. V. Hall, J. L. Darby, E. R. Coats, P. G. Green, D. E. Thompson, and F. J. Loge, 2008, Production of poly(hydroxyalkanoate) during treatment of tomato cannery wastewater: Water Environ. Res., v. 80, p. 367-372.

Madigan, M. T., and J. M. Martinko, 2006, Biology of Microorganisms: Brock: Upper Saddle River, NJ, Pearson Prentice Hall.

Madison, L. L., and G. W. Huisman, 1999, Metabolic engineering of poly(3-hydroxyalkanoates): From DNA to plastic: Microbiology and Molecular Biology Reviews, v. 63, p. 21-+.

Majone, M., K. Dircks, and J. J. Beun, 1999, Aerobic storage under dynamic conditions in activated sludge processes. The state of the art: Water Science and Technology, v. 39, p. 61-73.

Majone, M., P. Massanisso, A. Carucci, K. Lindrea, and V. Tandoi, 1996, Influence of storage on kinetic selection to control aerobic filamentous bulking: Water Science and Technology, v. 34, p. 223-232.

Marang, L., Y. Jiang, M. C. M. van Loosdrecht, and R. Kleerebezem, 2013, Butyrate as preferred substrate for polyhydroxybutyrate production: Bioresource Technology, v. 142, p. 232-239.

Marang, L., M. C. M. van Loosdrecht, and R. Kleerebezem, 2015, Modeling the competition between PHA-producing and non-PHA-producing bacteria in feast-famine SBR and staged CSTR systems: Biotechnology and Bioengineering, v. 112, p. 2475-2484.

Mozumder, M. S. I., L. Goormachtigh, L. Garcia-Gonzalez, H. De Wever, and E. I. P. Volcke, 2014, Modeling pure culture heterotrophic production of polyhydroxybutyrate (PHB): Bioresource Technology, v. 155, p. 272-280.

Narayanese, WikiUserPedia, YassineMrabet, and TotoBaggins, 2008, Tricarboxylic acid cycle, English, Wikipedia.

Oehmen, A., P. C. Lemos, G. Carvalho, Z. G. Yuan, J. Keller, L. L. Blackall, and M. A. M. Reis, 2007, Advances in enhanced biological phosphorus removal: From micro to macro scale: Water Research, v. 41, p. 2271-2300.

Pardelha, F., M. G. E. Albuquerque, M. A. M. Reis, J. M. L. Dias, and R. Oliveira, 2012, Flux balance analysis of mixed microbial cultures: Application to the production of polyhydroxyalkanoates from complex mixtures of volatile fatty acids: Journal of Biotechnology, v. 162, p. 336-345.

Pardelha, F., M. G. E. Albuquerque, M. A. M. Reis, R. Oliveira, and J. M. L. Dias, 2014, Dynamic metabolic modelling of volatile fatty acids conversion to polyhydroxyalkanoates by a mixed microbial culture: New Biotechnology, v. 31, p. 335-344.

Serafim, L. S., P. C. Lemos, M. G. E. Albuquerque, and M. A. M. Reis, 2008, Strategies for PHA production by mixed cultures and renewable waste materials: Applied Microbiology and Biotechnology, v. 81, p. 615-628.

Serafim, L. S., P. C. Lemos, R. Oliveira, and M. A. M. Reis, 2004, Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions: Biotechnology and Bioengineering, v. 87, p. 145-160.

Sudesh, K., H. Abe, and Y. Doi, 2000, Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters: Progress in Polymer Science, v. 25, p. 1503-1555.

Tamis, J., L. Marang, Y. Jiang, M. C. M. van Loosdrecht, and R. Kleerebezem, 2014, Modeling PHA-producing microbial enrichment cultures-towards a generalized model with predictive power: New Biotechnology, v. 31, p. 324-334.

Tchobanoglous, G., D. H. Stensel, R. Tsuchihashi, and F. Burton, 2014, Wastewater Engineering, Treatment and Resource Recovery: Metcalf & Eddy, AECOM: New York, NY, McGraw Hill Education.

Third, K. A., M. Newland, and R. Cord-Ruwisch, 2003, The effect of dissolved oxygen on PHB accumulation in activated sludge cultures: Biotechnology and Bioengineering, v. 82, p. 238-250.

Valentino, F., L. Karabegouic, M. Majone, F. Morgan-Sagastume, and A. Werker, 2015, Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels: Water Research, v. 77, p. 49-63.

vanAalastvanLeeuwen, M. A., M. A. Pot, M. C. M. vanLoosdrecht, and J. J. Heijnen, 1997, Kinetic modeling of poly(beta-hydroxybutyrate) production and consumption by Paracoccus pantotrophus under dynamic substrate supply: Biotechnology and Bioengineering, v. 55, p. 773-782.

Villano, M., F. Valentino, A. Barbetta, L. Martino, M. Scandola, and M. Majone, 2014, Polyhydroxyalkanoates production with mixed microbial cultures: from culture selection to polymer recovery in a high-rate continuous process: New Biotechnology, v. 31, p. 289-296.

Watson, B., 2015, Optimization of a Dairy Manure Based Polyhydroxyalkanoates Production through Reduced Aeration on Enrichment Cultures, University of Idaho, Moscow, ID, 112 p.

Wei, L., N. M. Guho, E. R. Coats, and A. G. McDonald, 2014, Characterization of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) biosynthesized by mixed microbial consortia fed fermented dairy manure: J. Appl. Polym. Sci., v. 131, p. 5516-5528.

Winkler, M., E. R. Coats, and C. K. Brinkman, 2011, Advancing post-anoxic denitrification for biological nutrient removal: Water Res., v. 45, p. 6119-6130.

Yates, M. R., and C. Y. Barlow, 2013, Life cycle assessments of biodegradable, commercial biopolymers-A critical review: Resources Conservation and Recycling, v. 78, p. 54-66.