Exploring Volatile Fatty Acids (VFAs) as a Novel Substrate for Microbial Oil Production
by
Sagar Chakraborty
M.S. Chemical Engineering Practice, Massachusetts Institute of Technology (2013)

Submitted to the Department of Chemical Engineering
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Chemical Engineering Practice
at the
MASSACHUSETTS INSTITUTE OF TECHNOLOGY
JUNE 2015

© 2015 Massachusetts Institute of Technology. All rights reserved.

Signature redacted

Signature redacted

Certified by.............................................
Gregory N. Stephanopoulos
Willard Henry Dow Professor of Chemical Engineering and Biotechnology
Thesis Supervisor

Accepted by .............................................
Richard D. Braatz
Edwin R. Gilliland Professor of Chemical Engineering
Chairman of the Committee for Graduate Students
Exploring Volatile Fatty Acids (VFAs) as a Novel Substrate for Microbial Oil Production

by

Sagar Chakraborty

Submitted to the Department of Chemical Engineering on May 27, 2015
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Chemical Engineering Practice

Abstract

Cost effective production of biofuels depends critically on feedstock cost and availability. As such, volatile fatty acids (VFAs) can play an important role in advancing sustainable biofuel production since they can be derived from low cost feedstock including gases and municipal solid waste. To this end, we studied fermentations of the oleaginous microbe *Yarrowia lipolytica* engineered for lipid overproduction. With acetate as sole carbon source, we conducted fed batch fermentations of *Y. lipolytica* in which acetic acid was maintained at low, non-inhibitory levels yielding high lipid titer of 50 g/L and productivity of 0.25 g/L/h, along with a lipid content of 60%. We also conducted fed batch fermentations with cell recycle to utilize dilute steams of acetic acid that essentially replicated the results of the fed batch process. Carbon balances were satisfied and no excess carbon dioxide production was detected beyond the amounts associated with biomass formation and product synthesis. Acetate is one member of the entire range of VFAs produced from municipal solid waste (MSW) via anaerobic digestion; thus, facilitating the use of MSW as a primary feedstock would be contingent on the ability of the above strain to grow on a mixture of VFAs. Given the insufficient literature examining microbial growth on VFAs, one of the goals of this project was to explore individual as well as mixed VFAs as a feedstock for *Y. lipolytica*. Dilute stream of mixed VFAs were successfully used as feed in bioreactor studies to obtain high cell density cultures. Similar results with respect to lipid production were obtained in comparison to the study on acetate. In addition, the microbe could tolerate perturbations in the feed composition and grow to similar cell densities. The success in establishing VFAs as a potential substrate for lipid accumulation in *Yarrowia lipolytica* raises the possibility of a two-stage commercial bioprocess enabling biodiesel production from MSW.

Thesis Supervisor: Gregory Stephanopoulos
Title: Willard Henry Dow Professor of Chemical Engineering and Biotechnology
Acknowledgements

I have cherished my time at MIT Chemical engineering and specially, the Stephanopoulos lab, where I was fortunate to interact with an extremely hard-working and brilliant group of people. First, I would like to thank Prof. Greg Stephanopoulos for his constant support and motivation throughout my time in the lab. He has always been a great inspiration for me and at the same time, a great critic of my work, which has helped me raise the bar for myself. Next, I would like to thank my committee members – Prof. Charles Cooney and Prof. Alan Hatton, for giving me great advice and fresh perspectives on my research directions.

I would next want to thank the wonderful GS group for the great experience it has been – both during the feisty debates in our group meetings as well as the fun times during TGs. Above all, I would like to thank Mitchell Tai, who created a great foundation for my thesis topic. He answered my innumerable questions related to the handling of the strain to the not-so-fun protocol development for the GC. Hussain, Adel and Vikram were some of the first to get me acquainted with a bioreactor. Several people have guided me along the way to perfect and better my measurement techniques and instrument handling – Andy, Haoran, Paulo, Hamid, Tom, Ryan, Peng, Aji, Joanne, Brian, Kang. I have also had the pleasure of working with and learning a lot from KJ. During the many late-night bioreactor samplings and other such fun moments, Mark was always around to chat about research among other interesting subjects such as American history and politics. The future of the lab lies in great hands – Steve, Ben, Jill and David, all of whom have helped make the lab a better place. I would like to finally thank Dr. Liu and Devin who have kindly agreed to continue my work and expand its scope.

My friends in ChemE and other departments at MIT have lent me great support in good and bad times. I would most remember the rejuvenating table tennis sessions in the evenings with my buddies – Nachiket, Siva, Vishnu, Sayalee, Atulya, Harshad, Mehul, Chiraag and others. My roommates Nachiket and more recently, Arun are the best chefs at MIT, and have cooked some of the best Indian dishes I have had away from home. Home – my parents, and my brother; these are the people I have missed the most during my time here at MIT. They have been the single biggest sources of strength for me. From giving them updates on my research to cribbing about the cold – they have listened to it all and in return, always given me courage by expressing their relentless faith in my ability. Ma-Baba, I really could not do it without you! Lastly, I am thankful to Dolon who has been another pillar of strength for me. She is a source of energy – always full of life and joy and I have been lucky to feed off that vivacity during my intense graduate school experience. Speaking of energy, I need to whole-heartedly thank ARPA-E (Advanced Research Projects Agency – Energy) for funding this fantastic project which I have genuinely loved working on. Thank you everybody!
Contents

1. INTRODUCTION................................................................................................................................... 9
  1.1. INTRODUCTION TO BIOFUELS – NEED AND MOTIVATION..................................................... 9
  1.2. THESIS OBJECTIVES.................................................................................................................. 12

2. BACKGROUND ................................................................................................................................... 15
  2.1. OVERVIEW OF PAST BIOFUEL TECHNOLOGIES ................................................................... 15
    2.1.1. BIOETHANOL ......................................................................................................................... 15
    2.1.2. BIODIESEL ............................................................................................................................ 16
  2.2. ELECTROFUELS............................................................................................................................. 17
  2.3. MUNICIPAL SOLID WASTE AS FEEDSTOCK...................................................................... 21
  2.4. MICROBE OF CHOICE – YARROWIA LIPOLYTICA ...................................................................... 23
    2.4.1. MICROBES ASSOCIATED WITH BIOFUEL PRODUCTION ........................................ 23
    2.4.2. Y. LIPOLYTICA – TARGET MICROBE............................................................................... 25

3. THEORETICAL ANALYSIS OF LIPID ACCUMULATION ON VFAS.................................. 28
  3.1. BIOCHEMISTRY OF LIPID SYNTHESIS ......................................................................................... 28
  3.2. PATHWAY ANALYSIS .................................................................................................................. 33
    3.2.1. GLUCOSE ................................................................................................................................ 34
    3.2.2. ACETATE ................................................................................................................................ 38
    3.2.3. PROPIONATE .......................................................................................................................... 41
    3.2.4. BUTYRATE ............................................................................................................................ 42
    3.2.5. PENTANOATE ........................................................................................................................ 43
    3.2.6. HEXANOATE ........................................................................................................................ 44
  3.3. YIELD CALCULATIONS .............................................................................................................. 44
  3.4. ENGINEERED ACC-DGA STRAIN ............................................................................................. 53
    3.4.1. ACETYL-COA CARBOXYLASE (ACC)............................................................................. 53
    3.4.2. DIGLYCEROL ACYLTRANSFERASE (DGA)........................................................................ 54

4. EXPLORING HIGH CELL DENSITY GROWTH ON ACETATE............................................... 56
  4.1. INTRODUCTION............................................................................................................................ 56
  4.2. MATERIALS AND METHODS ...................................................................................................... 57
    4.2.1. STRAINS AND CULTURE CONDITIONS ............................................................................ 57
    4.2.2. BIOREACTOR RUN – MEDIA AND PROCESS PARAMETERS .............................................. 58
    4.2.3. PRINCIPLE OF ACTION OF HOLLOW FIBER MEMBRANE ............................................. 59
    4.2.4. MEASUREMENTS AND CARBON BALANCE .................................................................... 60
    4.2.5. LIPID EXTRACTION AND QUANTIFICATION ............................................................... 61
  4.3. RESULTS ...................................................................................................................................... 62
    4.3.1. BATCH FERMENTATION USING 50 G/L SODIUM ACETATE – BATCH RUN .................. 62
    4.3.2. FED-BATCH FERMENTATION USING CONCENTRATED ACETATE FEED (30% V/V ACID FEED) – CONCENTRATED FED BATCH (CFB) RUN 64
Chapter 1

Introduction
1. INTRODUCTION

1.1. Introduction to Biofuels – Need and Motivation

In spite of the remarkable efforts made in the area of renewable energy, petroleum still continues to serve as the primary source to meet our liquid fuel demand (United States Energy Information Administration 2011). Not surprisingly, consumption of liquid fuels is projected to increase by as much as 30% between 2008 and 2035 and the transportation sector would account for 82% of this increase (U.S. Energy Information Administration, International Energy Outlook, 2011). With limited oil reserves and rising greenhouse gas emissions, it is imperative that we investigate clean and renewable sources of liquid fuels to sustain the demands of the transportation sector in the near future (Bp 2012; Chu & Majumdar, 2012).

The situation can be better assessed using the figures published in reports related to petroleum use and its future availability. The BP Statistical Report of 2012 reports that the proven oil reserves would last for approximately 50 more years given the projected oil demand. The Scripps CO₂ program has been recording the CO₂ concentration in the atmosphere and the data suggests that the concentration has increased by as much as 25% over the last 50 years from 320 ppm to 400 ppm. To better illustrate the concern over energy security, in 2011, US imported nearly 45% of its petroleum needs ((United States Energy Information Administration 2011).

There is therefore, an urgent need for an alternative technology to fossil fuels to meet our growing liquid fuel demand. Such an alternative fuel technology needs to be cost-competitive with petroleum in order to gain acceptance as a substitute for petroleum, apart from being eco-friendly and sustainable.

Biofuels are a promising alternative in the quest to find an eco-friendly, sustainable and affordable source of energy. Two of the major biofuel products currently available are bioethanol
and biodiesel (Fortman et al. 2008). Bioethanol is currently produced from corn and sugarcane. There are certain drawbacks associated with the use of ethanol as a fuel - it has a lower caloric value than gasoline, it is hygroscopic and, distillation is energy-intensive. (Atsumi & Liao 2008; Hill et al. 2006). Biodiesel contains mono alkyl esters of long chain fatty acids and represents a much more attractive option due to its similarity to petroleum in properties such as fatty acid profile and calorific value (Ma & Hanna 1999). Moreover, it scores over petroleum diesel in features such as reduction in emissions, inherent lubricity, and higher flash point; and it is also compatible with the existing fuel distribution infrastructure (Knothe 2009). Biodiesel has been traditionally obtained using crops which store high quantities of oils such as soy, jatropha and palm.

However, this path has certain disadvantages such as requirement of arable land and clean water resources. The alternative route is to depend on microbes for converting carbohydrates and other organic substrates to lipids, which can then be transesterified to biodiesel. The advantages in transitioning from vegetable oil to microbial oil production for the oil feedstock include: adaptability to diverse feedstocks, reduced land requirements, efficient process cycle turnover, and ease of scale-up (Beopoulos et al. 2011)

Oleaginous microbes are the class of microbes which store more than 20% of their cell mass as lipids (Meng et al. 2009). The oil stored by such microbes, referred to as single cell oil is similar in quality to plant oils (triglycerides) used currently for biodiesel production (Oil et al. 1993). Yarrowia lipolytica belongs to a family of oleaginous microbes, which can assimilate a variety of substrates such as glucose, glycerol, short chain organic acids, alkanes, fats (Coelho et al. 2010; Barth & Gaillardin 1997) and store triacylglycerides (TAGs) within their cell body inside lipid vacuoles. Y. lipolytica has been studied quite extensively in literature for use in a
variety of applications (De Felice 1997; Papanikolaou et al. 2001; Papanikolaou & Aggelis 2003a; Fickers et al. 2009; Papanikolaou & Aggelis 2009). Most importantly, its genome has been sequenced and genetic engineering toolkits have been developed (Xuan et al. 1988; Chen et al. 1997; Beopoulos et al. 2008).

Our laboratory has developed an engineered strain of *Y. lipolytica* (ACCDGA) which can store up to 60% or higher of its dry cell weight as lipids (Tai & Stephanopoulos 2012) as opposed to 12% stored by the wild type strain. The above mentioned study, as most other studies, has investigated oil accumulation on glucose. The economic analysis by Fei et al., 2011, demonstrates that feedstock costs account for 80% of the cost of the final biodiesel. The dependence on precious land resources for feedstock (such as glucose) leads to high costs of production, which prohibits commercial production of biodiesel from glucose. Thus, enabling large-scale biodiesel production requires the exploration and investigation of cheaper feedstock options such as acetate.

Municipal Solid Waste (MSW) is a promising feedstock candidate for the production of acetate and its family of short chain fatty acids called volatile fatty acids (VFAs), which apart from acetic acid includes propionic acid, butyric acid, pentanoic acid and hexanoic acid. The safe disposal of MSW would be posing a serious issue in the near future given the current and projected rates of MSW generation (World Bank Report, 2012). In addition, MSW decomposes in landfills to generate harmful greenhouse gases such as methane (Johari et al. 2012). In US, landfills are the third largest source of methane emissions (US EPA, 2011). Thus, there is sufficient incentive to discard MSW through safe means. It can therefore be anticipated that MSW would be available at low prices. Hence, exploiting MSW as a source of carbon feedstock would be very effective in facilitating low-cost biofuel production. Literature, however, suggests
that MSW degradation through anaerobic digestion produces a mixture of VFAs (Bassetti et al. 1995). Thus, enabling use of MSW as feedstock would be contingent on the ability of \textit{Y. lipolytica} to grow on a mixture of VFAs and convert them to intracellular lipids.

1.2. Thesis Objectives

The main objective of this thesis is to explore and assess the feasibility of short chain organic acids, collectively referred to as to volatile fatty acids (VFAs) as an alternate feedstock for lipid production in an engineered lipid over-producing strain of \textit{Yarrowia lipolytica}. Previous work (Tai & Stephanopoulos 2012) on the growth of the engineered strain of \textit{Y. lipolytica} on glucose serves as the basis for this study. Chapter 2 provides background on the topic of biofuels, specifically describing the benefits of electrofuels (Reduction of CO\textsubscript{2} to fuels using electrons derived from renewable sources) over the more conventional route of biofuel production. It also gives an overview of the various microbial sources of fats and the justification for choosing \textit{Y. lipolytica} as our target oleaginous microbe. Chapter 3 provides the framework for studying lipid accumulation in \textit{Y. lipolytica} on glucose and VFAs. The mechanism of lipid accumulation as explained in literature serves as a basis for the media composition and the bioreactor operation described in the later chapters. In addition, the respective metabolic pathways and yields associated with the production of lipid on glucose and other VFAs are presented.

Chapter 4 explores the topic of lipid accumulation on acetate. Starting with a batch reactor study on acetate as baseline, fed-batch reactor studies were designed and conducted with the goal of improving the lipid titer and productivity of the system. High cell density cultures of \textit{Y. lipolytica} were obtained with improved titer and productivity relatively to the batch study. The chapter concludes with an analysis the fed-batch system - carbon balance, acetate losses during the run and the shortcomings in the design of the bioprocess. Chapter 5 uses the platform
developed in the previous chapter to study lipid accumulation for the entire group of volatile fatty acids. Shake flask studies involving growth on individual and mixed VFAs were performed followed by fed-batch studies on mixed VFAs simulating the composition of streams that can be obtained from anaerobic digestion of municipal solid waste. Next, a model was developed to control the VFA titer in the reactor. Moreover, a final bioreactor study was performed demonstrating the robustness of the system in the presence of perturbations in the VFA feed concentration. Chapter 6 concludes with the major conclusions of the study and recommendations for future work. One of the goals of the research described in this thesis is to enable low-cost biofuel production from organic municipal solid waste. Thus, as part of the Capstone paper requirement for the PhDCEP program, Chapter 7 provides an evaluation and comparison of current and novel strategies for organic waste management and valorization through the lens of commercial viability.
Chapter 2

Background
2. BACKGROUND

2.1. Overview of Past Biofuel Technologies

The objective of biofuels research has been to develop a cheap and eco-friendly liquid fuel alternative to petroleum to share the burden of needs of the transportation sector. Hill et al., 2006 lists the following criteria in order for a liquid fuel to be a viable alternative to petroleum fuel: have environmental benefits, be economically competitive, and be produced in large quantities without competing with food supplies. In addition, the biofuel should be as close as possible to petroleum in its chemical structure so as to minimize the modifications to the current infrastructure (e.g. design of internal combustion engines). In the following sub sections, some current biofuel technologies are discussed which have already shown promise but however, have certain drawbacks.

2.1.1. Bioethanol

Ethanol has been one of the oldest and most widely used biofuels. It has been produced traditionally from the fermentation of sugars obtained from corn and sugarcane followed by distillation. Ethanol has been referred to as first generation of biofuels. However, there have been numerous concerns with the use of ethanol as a biofuel. First, the fuel itself has inferior fuel properties compared to gasoline. Ethanol has a lower caloric value, its distillation is energy-intensive and its hygroscopic nature leads to the corrosion of the walls of engines and pipes (Atsumi & Liao 2008; Stephanopoulos 2007; Zhang et al. 2012).

Second, a lot of studies have focused on the harmful effects of ethanol production (Hill et al. 2006). The dependence on arable land and precious resources like clean water is one of the greatest concerns with bioethanol. Moreover, corn production requires large amounts of nitrogen,
phosphorus and pesticide inputs. Such compounds ultimately drain into water bodies and lead to algal bloom, which causes the destruction of aquatic life. Total life-cycle analysis has shown that total life-cycle emissions of five major air pollutants [CO, VOC, PM10, oxides of sulfur (SOx), and oxides of nitrogen (NOx)] are higher with the “E85” corn grain ethanol–gasoline blend than with gasoline per unit of energy released upon combustion (Brinkman et al. 2005). Second-generation biofuels such as cellulosic ethanol have been advertised as being more eco-friendly. Switchgrass, cornstover, miscanthus and wood chips are popular cellulosic crops for ethanol production. They can be grown on agriculturally marginal lands with use of minimal fertilizers and pesticide inputs. Reduced competition with food crops for arable land would drive down the cost of the fuel and make it more competitive to petroleum. However, the major obstacle in improving the cost-competitiveness of this technology has been the fact that cellulosic materials require significant processing through the pretreatment and hydrolysis steps in order to generate the sugar monomers for fermentation into ethanol.

2.1.2. Biodiesel

Biodiesel are the methyl esters of long chain fatty acids. They are obtained from the transesterification of fats (triglycerides). Such fats have traditionally been obtained from plant sources such as soy, jatropha and palm to name a few. The fatty acid profile of biodiesel so produced corresponds to that of its feedstock. Most common feedstock possess fatty acid profiles consisting mainly of five C16 and C18 fatty acids, namely, palmitic, stearic, oleic, linoleic and linolenic acids.

Biodiesel is similar to diesel in various properties such as caloric value and cetane number (the ignition delay time a fuel experiences upon injection into the combustion chamber of a diesel engine– higher the cetane number, lower the ignition delay and better the combustion
quality). However, biodiesel does not fare well in certain other characteristics of a fuel such as stability to oxidation and cold flow properties. But such problems can be overcome through the use of certain additives (Knothe 2009). Biodiesel obtained from plants faces criticism of dependence on arable land and displacement of food crops. However, it is still better than ethanol because of the following reasons. The net energy gain (as fraction of energy obtained from the fuel over and above the energy used for its production) from biodiesel is 93% compared to 25% for ethanol (Hill et al. 2006). Relative to the fossil fuels they displace, greenhouse gas emissions are reduced 12% by the production and combustion of ethanol and 41% by biodiesel. Biodiesel also releases less air pollutants per net energy gain than ethanol. These advantages of biodiesel over ethanol are due to lower agricultural inputs and more efficient conversion of feedstock to fuel.

However, as mentioned earlier, biodiesel production using plant oils is not sustainable and would become cost-prohibitive in the near future as the population grows and its food needs increase. In addition, the productivity of some of these plant-based biofuel technologies is quite low. Georgianna and Mayfield 2012 reports that 330% and 75% of the arable land in US would have to be contributed towards soy and jatropha production, respectively so as to replace all the US consumption of petroleum. Therefore, to ensure acceptance of biodiesel as a viable alternative to petroleum diesel, we need technologies which are efficient, cost-effective and do not rely on the requirement for resources such as arable land and clean water.

2.2. Electrofuels

In this study, we investigate microbial oil production and the FAMEs derived from it as alternative biofuels. The major challenge in the viability of the bioprocess is cost. Most of the cost (80%) of a fermentation process lies in the substrate (Fei, Chang, Shang & Choi 2011).
Thus, it is very important that we choose a microbial platform which can convert low-cost feedstock into oil. Previous attempts to produce biodiesel using oleaginous microbes have focused on the route as shown on the left half of Fig. 2.1. In this scenario, the carbon in CO$_2$ is fixed via photosynthesis into sugars. Photons from sunlight induce splitting of water molecules and the electrons released as a result of the process reduce CO$_2$ to glucose. Such sugars are then converted by oleaginous microbes such as *Y. lipolytica* into lipids. This has been the major approach to lipid production using *Y. lipolytica* in the past. However, the major drawback of this process is the dependence on sugars such as glucose. Glucose must be obtained from food crops grown on arable land. Increasing competition for land resources to meet food as well as fuel needs results in high costs of the feedstock. Since feedstock prices are an important factor in determining the price of the fuel, using glucose as a feedstock therefore is not a financially sustainable approach. Moreover, the percentage efficiency of the electron capture in this process is extremely low. It is reported that less than 1% of the energy from the sunlight is fixed in most crop plants as sugars (Govindjee, 1995). Thus, the process is also not suitable from an energetic standpoint.
The electrofuels approach altogether removes the dependence on arable land. It relies on the reduction of CO\(_2\) into carbon substrates using renewable sources of electrons (reducing power). Such substrates could be consumed by microbes to produce biofuels. Energy from sunlight can be converted into electricity using solar cells, which can in turn be used for the splitting of water to generate electrons. CO\(_2\) reduction using electrons (or reduced compounds such as H\(_2\)) can be carried out using acetogenic microbes (as opposed to plants) to produce compounds such as acetate. The acetate could then be fed to \(Y.\) lipolytica to produce lipids.
This strategy has two distinct advantages: a) There is no dependence on precious resources such as land for the production of acetate, which helps keep the price of acetate in check, b) The efficiency of electron capture (electrons are the carriers of energy; more reduced a compound, the more energy it contains) into acetate (analogous to electron capture from sunlight into sugars in plants) is much higher in this case (as high as 32% - product of the two efficiencies: 40% for solar cells from sunlight to electricity – (Luque 2011) and 80% from syngas to acetate – (Hu et al. 2013)). Now, the theoretical efficiency of the conversion of energy in glucose to lipids is slightly higher than that of acetate to lipids (2 g lipid/electron in acetate vs. 2.4 g lipid/ electron in glucose – these numbers are derived from the respective lipid yields, analysis shown in appendix A.1). However, due to the much higher energy efficiency of the electron capture in acetate as compared to glucose, the total energy efficiency of the process from sunlight to lipids is much higher in the case of the electrofuels approach as shown below in Fig. 2.2.

![Diagram](image)

Figure 2.2: Comparison of energy efficiencies of lipid production in the usual photosynthesis approach vs. the electrofuels approach. The numbers in brackets show the energy content in arbitrary units. Starting from 100 units of energy, the conventional approach would yield 2 g of lipids whereas electrofuels approach would yield 64 g of lipids. We are assuming a solar cell
efficiency of 40% and electron capture of 80% in the electrofuels approach calculation. The lipid yield calculations over glucose and acetate are discussed in further detail in chapter 3.

The process flow shown in Fig. 2.2 is representative of the two-stage biofuel production using the electrofuels approach. In this particular case, the electron donor is H₂. The first reactor would be an anaerobic reactor where CO₂ is reduced in the presence of H₂ into acetate. The acetate containing stream is diverted to another reactor where an oleaginous microbe converts it into lipids and stores it inside the cell body. The lipids are extracted from the cells and transesterified into biodiesel. Moreover, the cell mass left after the lipid extraction could be used as a source of nutrients as yeast extract for both the reactors. In addition, the CO₂ produced from the aerobic reactor could be reused in the anaerobic reactor. Hence, a major benefit of this process is that the by-products can be reused.

Figure 2.3: Process flowsheet for biodiesel production from CO₂ and H₂ using a two-stage approach involving an anaerobic process followed by use of an oleaginous microbe

2.3. Municipal Solid Waste as Feedstock

In addition to the electrofuels approach, there is another source of low-cost feedstock – municipal solid waste (MSW). The organic fraction of MSW can be degraded into a family of short chain organic acids called volatile fatty acids (VFAs) by the same group of acetogenic
microbes as were mentioned above. Such microbes degrade the long-chain organic compounds into simple two-carbon compounds like acetate through a series of reactions, before further decomposing it into methane and CO₂. However, from an efficiency standpoint, decomposing the carbon down to methane (to be used as biogas) leads to loss of half the carbon as CO₂, which does not have any fuel value. Moreover, the focus of the current study is to produce liquid fuel. Hence, our approach is based on stopping the degradation at the stage of the VFAs – where all the carbon has been retained, and then feed the VFAs to the second stage for lipid accumulation.

![Two stage fermentation diagram](image)

Figure 2.4: Schematic representation of the two-stage fermentation process to convert organic fraction of MSW to biodiesel

Due to the huge rise in the quantity of MSW generated in urban areas, MSW disposal has turned into a significant issue. Currently, in major cities, MSW collection and disposal costs as much as $100/ton. Hence, it can be deduced that MSW is a significant liability for governments and cities. Moreover, the cost of safe disposal can be expected to rise with rising estimates of MSW production (World Bank Report, 2012). This provides an opportunity to obtain MSW as a
feedstock for the biofuel production process at low prices. It might be even possible to obtain MSW in certain regions at potentially negative prices. In emerging countries, the quantity of waste generated has increased substantially in recent times, whereas the infrastructure required to handle it has not developed at a concomitant pace. As a result, the waste generated is littered on streets and is dumped at unauthorized places near human settlements (Annapu, 2012). Such waste becomes a draw for pathogenic microbes and vectors of diseases, which ultimately affects the health of the people living nearby. There have been instances in the past (Love canal landfill site, New York State) of groundwater contamination with toxic chemicals from the landfill site and it led to a variety of health concerns such as low birth weight and infant mortality (Rushton, 2003). Also, another common technique of waste disposal – incineration, has been linked to a variety of respiratory disorders and cancers (Dockery et al, 1994, Elliot et al, 1996). Thus, in addition to obtaining MSW at low prices to help bring down the cost of the final fuel, the proper disposal and management of MSW can have a great effect on improving the quality of life.

2.4. Microbe of Choice – *Yarrowia lipolytica*

2.4.1. Microbes associated with Biofuel Production

Microbes of different genera are capable of producing fuels. It would be most ideal to deal with triglycerides (fats) as it can be converted to biodiesel through transesterification. In the plant world, such fats have been obtained from jatropha and palm for the production of biodiesel. Bacteria are very ideal microbes on account of their high growth rates and ease of culturing. However, most bacterial species known to accumulate lipids such as *Arthrobacter, Rhodococcus, Acinetobacter* store only around 20-30% of their cell weight as lipids. Moreover, the bigger hurdle is that bacteria typically store polyhydroxyalkanoates such as polyhydroxybutyrates. Such molecules are chemically distinct from triacylglycerides (TAGs) and hence, bacteria are not
suitable candidates for biodiesel production (Meng et al., 2009, Alvarez et al., 2002). Algae are another class of microbes known to store lipids inside their cell body. The major advantage in working with algae is that it can directly fix CO₂ and store lipids and hence, no dependence on plant-based sugar feedstock. Moreover, they store lipids as TAGS and can accumulate as much as 50% or more of their mass as lipids. Algae are known to survive even on brackish or salt water. The major obstacle in algal biodiesel has been the cost of harvesting and dewatering among few others, which makes the process expensive (Georgianna et al., 2012). In addition, studies have also focused on heterotrophic microalgae who can feed on carbon sources such as acetate and store lipids. However, there are currently plenty of concerns with regard to growth characteristics, lipid productivity and bioreactor operating conditions, which need sufficient attention (Bumbak et al. 2011; Fan et al. 2012). Yeasts and fungi are the next group of microbes which contain a wide variety of oleaginous microbes – *Lipomyces starkeyi*, *Cryptococcus curvatus*, *Rhodotorula*, *Yarrowia lipolytica*, *Mortierella*. Most of these species are known to store lipids in the form of TAGs at lipid contents of 50-70% of dry cell weight. Moreover, the lipid profile is similar to the ones found in plant seed oils such as canola oil, palm oil, peanut oil – high percentage of unsaturated fatty acids, mostly C18.1, oleic acid. The table below shows the high similarity of *Yarrowia lipolytica*’s fatty acid profile generated from our experiments to those of the common plant seed oils used as biodiesel precursor as reported in Knothe 2008.

<table>
<thead>
<tr>
<th>Oil</th>
<th>C16</th>
<th>C18</th>
<th>C18.1</th>
<th>C18.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola oil</td>
<td>2-6%</td>
<td>4-6%</td>
<td>52-65%</td>
<td>18-25%</td>
</tr>
<tr>
<td>Palm oil</td>
<td>40-47%</td>
<td>3-6%</td>
<td>36-44%</td>
<td>6-12%</td>
</tr>
<tr>
<td>Peanut oil</td>
<td>6-14%</td>
<td>2-6%</td>
<td>36-67%</td>
<td>13-43%</td>
</tr>
</tbody>
</table>
Y. lipolytica

<table>
<thead>
<tr>
<th>Y. lipolytica</th>
<th>13%</th>
<th>14%</th>
<th>64%</th>
<th>5%</th>
</tr>
</thead>
</table>

* - The profile for Y. lipolytica has been reported from the reactor studies on acetate – minor amounts of C16:1 were also obtained – 4%

Table 2.1: Comparison of lipid profile of common plant oils used for biodiesel production and that of the fats stored by Y. lipolytica

2.4.2. Y. lipolytica – Target Microbe

*Yarrowia lipolytica*, previously known as *Candida lipolytica*, is an ascomycetous yeast which is known to accumulate lipids. It is an obligate aerobe readily found in dairy products, food media as well as in environmental areas rich in oils. It is non-pathogenic and is known to grow on glucose, alcohols, acetate and hydrophobic substrates like fatty acids or alkanes (Barth & Gaillardin 1997). It has been shown to be useful for the production of various metabolites like citric acid (Papanikolaou et al. 2002) and extracellular proteins (Fickers et al. 2009), apart from lipids. *Yarrowia lipolytica* has been used in applications such as detoxifying effluents like olive mill waste water to produce useful products like citric acid (Papanikolaou et al. 2008), valorizing raw glycerol to produce 1,3-propane diol (Papanikolaou & Aggelis 2003b), producing metabolites like mannitol and acetic acid (André et al. 2009) and production of single cell oil (SCO) for use as a cocoa butter substitute (Papanikolaou et al. 2003), to name a few.

*Y. lipolytica* typically grows in haploid state and it is also known to exhibit dimorphism, i.e., it shows both the hypha form as well as the regular spherical yeast form. Wild type strains are known to exhibit various colony shapes, ranging from smooth and glistening to highly convoluted and mat. It has been shown in studies (Botham & Ratledge 1979; Goma et al. 1992) that the accumulation of storage lipids from glucose or other similarly metabolized compounds is a secondary anabolic activity in oleaginous microbes, occurring after nitrogen exhaustion. Thus, a high carbon to nitrogen molar ratio (C/N) favors lipid accumulation. *Y. lipolytica* is an obligate
aerobe, necessitating the use of high agitation and aeration rates while growth in a bioreactor. According to the literature, the optimum pH for *Yarrowia* growth on glucose and glycerol ranges from 5 to 6. (Papanikolaou & Aggelis 2009; Papanikolaou & Aggelis 2002; Aggelis & Komaitis 1999) and the optimum temperature is 28-30 °C (Papanikolaou & Aggelis 2009; Papanikolaou & Aggelis 2002; Aggelis & Komaitis 1999; Beopoulos et al. 2008).

*Yarrowia lipolytica*’s genome has been sequenced and its physiology is well understood. It is considered one of the most intensively studied ‘non-conventional’ yeast. It is known to natively store lipids and is also relatively easy to genetically engineer the microbe. *Y. lipolytica* was thus considered the microbe of choice for oil accumulation.
Chapter 3

Theoretical Analysis of Lipid Accumulation onVFAs
3. THEORETICAL ANALYSIS OF LIPID ACCUMULATION ON VFAS

3.1. Biochemistry of Lipid Synthesis

The pathway for lipogenesis to produce TAGs in lipid bodies from glucose is shown in Fig. 3.1. Glucose is transported into the cytoplasm and through glycolysis, is broken down to pyruvate. Additionally, glycerol 3-phosphate is also formed through glycolysis. Pyruvate enters the mitochondria where it gets decarboxylated into acetyl CoA (ACA). ACA enters the TCA cycle and at various points in the TCA cycle, metabolites (such as alpha-keto glutarate) are diverted for biomass production. When nitrogen is limited, one of the key enzymes of the TCA cycle (Isocitrate Dehydrogenase) gets inhibited which leads to the accumulation of citrate in the mitochondria. The citrate crosses into the cytoplasm where it gets acted upon by the enzyme, ATP Citrate Lyase to form ACA. ACA then proceeds to enter the fatty acid synthesis cycle with the first committed step being the production of malonyl CoA. An n-carbon Acyl CoA is eventually produced which then combines with the glycerol 3-phosphate in the lipid bodies to form tri acyl glycerides through a series of reactions. Further details on each of the above pathways are provided in the section 3.2.
Figure 3.1: Metabolic pathways involved in the conversion of glucose to lipids
Figure 3.2: Pathways for lipid accumulation in *Yarrowia lipolytica* on glucose vs. acetate

Fig. 3.2 shows lipid accumulation on acetate vis-à-vis that on glucose. The pathway for lipid accumulation on acetate is quite similar. However, there are a few differences.

Cytosolic acetyl-CoA (ACA) is the starting metabolite for the lipid synthesis pathway. Acetate can directly be metabolized and converted to cytosolic ACA in one step, whereas glucose has to be metabolized into citrate through the tricarboxylic acid (TCA) cycle, and then lysed by the enzyme ATP-dependent citrate lyase (ACL) to form ACA. This difference in metabolism between the cases of glucose and acetate leads to a difference in the way the C/N (molar ratio of carbon to nitrogen) ratio affects lipid production in the two cases.

Nitrogen limitation inhibits the TCA cycle due to inactivation of the enzyme isocitrate dehydrogenase (IDH). As a result, citrate is accumulated and then transported into the cytosol.
As long as the extracellular nitrogen concentration is above 1 mM, the intracellular ammonium acts as an activator of ACL and causes the cleavage of citrate to acetyl CoA and oxaloacetate. However, when all of the intracellular nitrogen is depleted (external nitrogen concentration below 1 mM), ACL can no longer be activated. Excess citrate is secreted out from the cytosol into the extracellular medium and hence, high citrate production is observed at very high C/N (Ratledge and Wynn 2002, Cescut, 2009). Thus, lipid production on glucose requires an optimal nitrogen window.

![Diagram](image)

Figure 3.3: Extracellular nitrogen limitation causing a series of reactions leading to increased lipid biosynthesis (+ indicates increase in concentration/activator of pathway, - indicates decrease in concentration/deactivation of pathway)

However, on acetate, due to the direct conversion to ACA, it can be expected that lipid production would not depend on the medium nitrogen content. In the case of acetate, cytosolic ACA is produced through the action of the enzyme Acetyl CoA Synthetase (ACS) in one step.
from acetate. If sufficient pull for ACA is created like in the case of the ACCDGA strain discussed in Tai and Stephanopoulos, 2012, a high flux towards lipid synthesis is sustained without the need for acetate to go through the TCA cycle and form citrate. Thus, even at a high C/N ratio, citrate production need not be high as the acetate might be getting directly diverted to lipid synthesis thorough formation of ACA. For growth on acetate, therefore, nitrogen can be effectively used as a switch to modulate non-lipid (proteins, nucleic acids) production.

Acetate metabolism also differs from glucose metabolism in other ways. NADPH requirement for the fatty acid synthesis pathway is met via the pentose phosphate pathway on glucose vis-à-vis the malic enzyme pathway on acetate. The details of the pathway would be provided in subsequent sections.

It was observed in our own studies that during growth on acetate, there is a concomitant increase in the pH of the medium. During growth on glucose, the pH does not change much. On acetate though, it is observed that with increase in OD of the culture broth, the pH of the medium goes up in a proportional manner. Theories in literature are consistent with the above observation (Verduyn 1991; Casal et al. 2008). The papers report that monocarboxylic acids are transported across the yeast membrane using a proton symport as shown in Fig. 3.4. below.

![Figure 3.4: Monocarboxylates transported across the membrane via proton symport](image-url)
Studies on Candida species have determined the stoichiometry of the proton/monocarboxylate symport to be 1:1 (Cássio et al. 1987; Lefio & Uden 1986). Thus, it can be assumed that Y. lipolytica consumes organic acids like acetate in the neutral form (anion+H⁺) rather than the anion form. Thus, with continuous consumption of acetate from the surrounding medium, it can be expected that there is continuous depletion of the H⁺ ions and hence, the pH goes up. The high regression value (0.994) of the plot between the acetate consumed and acid pumped (to maintain pH control in response to pH rise) obtained in our experiments, shown in Fig. 3.5 corroborates the above hypothesis.

![Figure 3.5: Plots of cumulative acetic acid pumped in response to pH control and the cumulative acetate consumed](image)

### 3.2. Pathway Analysis

Lipids are produced by Y. lipolytica in the form of Tri-Acyl Glycerides (TAGs) such as tripalmitate (triglyceride ester of palmitic acid). The synthesis of lipids would first require the generation of its components, fatty acids and glycerol. Fatty acid synthesis has the following requirements:

a) Acetyl CoA, the starting carbon compound for fatty acid production
b) Reducing equivalents: NADPH

c) Energy: ATP

During glucose metabolism via glycolysis, glycerol is produced from dihydroxy acetone phosphate, an intermediate in the pathway. The exact pathway used by the cell for the production of acetyl CoA, NADPH, ATP and glycerol in lipid production differs depending upon the exact carbon substrate used by the microbe. The next subsections present the lipid production pathways used by the microbe while growth on glucose, which is one of the most preferred substrates, and on acetate, as well as other VFAs, which are the substrates under consideration in this research.

3.2.1. Glucose

Glucose is metabolized via glycolysis in the cytoplasm. Figure 3.6 shows the glycolytic pathway which begins with glucose and after a series of reactions, leads to the production of two molecules of pyruvate.
Figure 3.6: Glycolysis

This pathway serves three functions. The first function is to generate reducing equivalents in the form of NADPH via the pentose phosphate pathway (PPP), which yields 2 moles of NAPDH for every carbon oxidized as CO₂. The second function of glycolysis is to produce intermediates for glycerol production. Glyceraldehyde-3-phosphate, an intermediate in glycolysis isomerizes to dihydroxyacetone phosphate which goes through two additional steps to form glycerol. Third, pyruvate is generated as the end-product of glycolysis, which gets decarboxylated to form acetyl CoA. The acetyl CoA produced can be consumed in two ways; it can enter the mitochondria and be oxidized using the TCA cycle shown in Figure 3.7 or it can be used for fatty acid synthesis. The TCA cycle generates energy in the form of ATP. Thus, with acetyl CoA, NADPH and ATP
available, fatty acid synthesis can proceed to synthesize long chain acyl CoA using the fatty acid synthesis pathway shown in Figure 3.8. The pathway begins with the conversion of acetyl CoA to malonyl CoA. Malonyl ACP (the CoAs are substituted with ACP protein by the respective trasacylases) then reacts with another acetyl ACP to form aceto acetyl ACP along with the release of one CO$_2$ molecule. The four-carbon compound goes through a series of reactions to form butyryl ACP. The chain length increases by two in every cycle. In the subsequent cycle, butyryl ACP combines with another malonyl ACP (formed via acetyl CoA to malonyl CoA and the CoA-ACP swap) to generate hexyryl ACP and again, one CO$_2$ is lost. In this way, the cycle continues 7 or 8 times to synthesize palmityl ACP or stearyl ACP, respectively.

![Figure 3.7: Tricarboxylic acid (TCA) cycle](image-url)

Figure 3.7: Tricarboxylic acid (TCA) cycle
Finally the long chain acyl CoA molecules react with glycerol 3-phosphate in lipid vacuoles to yield TAGs as shown in Figure 3.9 using the Kennedy pathway.

Figure 3.9: TAG synthesis through Kennedy pathway
3.2.2. Acetate

Acetate is converted by Acetyl CoA Synthetase (ACS) into acetyl CoA in the cytoplasm. The acetyl CoA (ACA) is then used for fulfilling all the requirements of lipid synthesis. ACA can be transferred into the mitochondria via pyruvate and then get oxidized through the TCA cycle to produce ATP. Also, ACA can enter fatty acid synthesis but it would need reducing equivalents.

In the absence of glucose, glycolysis cannot operate and literature suggests that oleaginous microbes use the malic enzyme to generate NADPH (Ratledge & Wynn 2002). One molecule of NADPH is generated for each molecule of acetyl CoA that is transferred from the mitochondria to the cytoplasm as shown in Fig. 3.10.

Figure 3.10: Malic enzyme pathway (net reaction: NADH + ATP \( \rightarrow \) NADPH)

In addition, growth on acetate requires operation of an alternate pathway called gluconeogenesis to obtain glycerol 6-phosphate. In essence, gluconeogenesis is the reverse of
glycolysis as shown in Fig. 3.11. However, gluconeogenesis needs pyruvate or oxaloacetate as a starting point.

Figure 3.11: Gluconeogenesis (net reaction: 2 Pyruvate + 4 ATP + 2 GTP + 2 NADH → Glucose)

Acetyl CoA (two carbon) would need to produce oxaloacetate (four carbon) using an anaplerotic pathway. Glyoxylate pathway serves to provide an anaplerotic path in which two acetyl CoAs are used to create a four-carbon compound, malate. It is shown in Figure 3.12.
Figure 3.12: Glyoxalate cycle (net reaction: 2 Acetyl CoA $\rightarrow$ Succinate + NADH)

Malate then undergoes oxidation to produce oxaloacetate. Once oxaloacetate is produced, it goes up the gluconeogenesis pathway to produce glyceraldehyde-3-phosphate which ultimately provides glycerol. The various metabolic requirements of acetate for lipid production have been shown pictorially in Figure 3.13.
3.2.3. **Propionate**

On propionate, the microbe produces propionyl CoA, which enters the methyl citrate cycle (analogous to the glyoxalate cycle) to yield pyruvate as its final product. PCA combines with oxaloacetate to form 2-methyl citrate when gets converted further to 2-methyl isocitrate. Through the action of 2-methyl isocitrate lyase, succinate and pyruvate molecules are generated. Succinate continues onward in the methyl citrate cycle and gets finally converted to oxaloacetate. The pyruvate formed can directly feed into gluconeogenesis. It can be also decarboxylated to acetyl CoA which can feed into lipid synthesis and TCA cycle.
3.2.4. Butyrate

The first step of butyrate metabolism is the degradation of butyrate in the fatty acid degradation pathway to form two acetate moieties and the production of 1 NADH and 1 FADH$_2$ in the process. Once acetyl CoA is formed, the pathway to lipids is exactly similar to the case of acetate metabolism.
3.2.5. Pentanoate

Pentanoate (also known as valerate) enters the fatty acid degradation cycle and forms one molecule of acetyl CoA and one molecule of propionate. The analysis for lipid yield on valerate assumes a proportional amount of acetyl CoA and propionyl CoA to be present in the cell, which then follow the respective pathways for lipid production as shown in Fig. 3.13 and 3.14.
3.2.6. Hexanoate

The breakdown of hexanoate is similar to that of butyrate. Hexanoate enters the fatty acid degradation and forms three molecules of acetyl CoA and the corresponding NADH and FADH_2. The acetyl CoA form lipids using the same pathway as shown in 3.2.2.

3.3. Yield Calculations

In each of the following analysis, it has been assumed that the final lipid is tripalmitin, (triglyceride of palmitic acid). The substrate accounts for all the carbon needed for lipid synthesis as well as the substrate needed for the other requirements for lipid synthesis such as NADPD, NADH, and ATP.
The reactions to be considered are:

1. Fatty acid synthesis
   a. Glycolysis – Glucose to pyruvate
   b. Breakdown of pyruvate to acetyl CoA
   c. Acetyl CoA incorporation to form fatty acids

2. Glycerol production via glycolysis

3. Lipid production (tripalmitin) using Kennedy pathway

4. NADPH production using Pentose phosphate pathway

5. ATP production using TCA cycle

Analysing each of the above sets of reactions individually,

1. Fatty acid synthesis
   a. 12 Glucose $\rightarrow$ 24 Pyruvate + 24 ATP + 24 NADH (Stryer, Biochemistry, 6th edition)
   b. 24 Pyruvate $\rightarrow$ 24 ACA + 24 CO$_2$ + 24 NADH (Stryer, Biochemistry, 6th edition)
   c. 24 ACA + 18 NADPH + 24NADH + 69 ATP $\rightarrow$ 3 palmitic acid (van Milgen 2002)

   Final equation for fatty acid production from glucose:
   
   12 glucose + 18 NADPH + 45 ATP $\rightarrow$ 3 palmitic acid + 24 CO$_2$ + 24 NADH

1. Glycerol production via glycolysis
   Glucose + 2 ATP + NADPH $\rightarrow$ 2 Glycerol (Tai, 2012)

2. Lipid production using Kennedy Pathway
   Glycerol + 3 palmitic acid + ATP $\rightarrow$ Tripalmitin (Tai, 2012)
3. NADPH production using PPP

Glucose $\rightarrow$ 6 CO$_2$ + 12 NADPH (Stryer, Biochemistry, 6th edition)

4. ATP production using TCA cycle

Glucose $\rightarrow$ 6 CO$_2$ + 10 NADH + 2 FADH$_2$ + 2 ATP + 2 GTP

Assuming NADH=2.5 ATP, and FADH$_2$ =1.5 ATP and GTP=1 ATP, we obtain:

Glucose $\rightarrow$ 6CO$_2$ + 32 ATP

Thus, the above equations can now be further grouped into 3 categories:

a) Glucose to tripalmitin

12.5glucose + 47 ATP + 18.5 NADPH $\rightarrow$ 24 CO$_2$ + Tripalmitin + 24 NADH    - (1)

However, the excess NADH on the product side can be converted into ATP; in that case, there would be no ATP requirement.

b) Glucose to NADPH

Glucose $\rightarrow$ 6CO$_2$ + 12 NADPH - (2)

c) Glucose to ATP

Glucose $\rightarrow$ 6CO$_2$ + 32 ATP - (3)

To form the 19 NADPH for eqn. (1), the PPP would burn glucose so as to produce the requisite amount of NADPH.

19/12 Glucose $\rightarrow$ 9.5 CO$_2$ + 19 NADPH (multiplying (2) by 19/12)

Adding the above equation to the equation (1), would provide the final stoichiometric equation for lipid production from glucose

14.1 $C_6H_{12}O_6$ $\rightarrow$ 33.5 CO$_2$ + Tripalmitin
Yield of lipid over glucose = 806/(14.1*180) = 0.318 (Molecular weight of glucose, C$_6$H$_{12}$O$_6$ is 180 and that of tripalmitin, C$_{51}$H$_{98}$O$_6$ is 806)

The reactions involved in this process have been grouped into modules or sets for simplicity. The symbols (c) and (m) refer to whether the metabolite exists in the cytosol or in the mitochondria. The following modules of reactions have been assumed:

a. Acetate to Acetyl CoA (ACA)
b. ACA to oxaloacetate (OAA)
c. OAA to glycerol
d. ACA to C16 fatty acid.
e. (Fatty acid+Glycerol) forms lipid
f. Acetate to ATP
g. Acetate to NADPH

We will be analyzing the equations of each of the above modules.

Set a: Acetate + ATP $\rightarrow$ Acetyl CoA (Acetyl CoA Synthetase) -----(1)

This reaction proceeds through the action of the enzyme Acetyl CoA Synthetase (ACS) in the cytosol.

Set b: Gluconeogenesis occurs in the cytosol and hence, oxaloacetate needs to be present on the cytosol side in order to initiate gluconeogenesis. This process occurs through a number of successive reactions. First, the glyoxalate cycle operates and produces a succinate for every 2 acetyl CoA molecules. Next, the succinate produced in the cytosol permeates through the
mitochondrial membrane and enters the TCA cycle operating in the mitochondria. The succinate converts to malate, which permeates out into the cytosol. The cytosolic malate is acted upon by the malic enzyme to produce pyruvate, which ultimately is acted upon by the enzyme pyruvate carboxylase to form oxaloacetate in the cytosol. The reactions are shown below.

$$2 \text{ACA(c)} \rightarrow \text{Succinate (c)} + \text{NADHc (glyoxalate cycle)} \quad \text{(Stryer, Biochemistry, 6th edition)}$$

Succinate (c) $\rightarrow$ Succinate (m) (Succinate exit)

Succinate (m) $\rightarrow$ Malate (m) + FADH$_2$ (TCA cycle) (Stryer, Biochemistry, 6th edition)

Malate (m) $\rightarrow$ Malate (c) (Malate exit)

Malate (c) $\rightarrow$ Pyruvate (c) + NADPH$_c$ + CO$_2$ (Malic enzyme) (Stryer, Biochemistry, 6th edition)

Pyruvate (c) + CO$_2$ + ATP $\rightarrow$ OAA (c) (Pyruvate Carboxylase) (Stryer, Biochemistry, 6th edition)

The net reaction amounts to: $2 \text{ACA(c)} + \text{ATP} \rightarrow \text{OAA (c)} + \text{NADHc} + \text{FADH}_2 + \text{NADPH}_c$ ---- (2)

**Set c:** This accounts for the sum of reactions leading up to glycerol production from oxaloacetate via gluconeogenesis. (Stryer, Biochemistry, 6th edition)

OAA (c) + GTP + ATP + NADHc $\rightarrow$ Glycerol + CO$_2$ (gluconeogenesis) ----(3)

**Set d:** This accounts for the set of reactions leading up to palmitic acid production from acetyl CoA via the fatty acid synthesis pathway (van Milgen, 2002)

$24 \text{ACA(c)} + 18 \text{NADPH} + 24 \text{NADHc} + 69 \text{ATP} \rightarrow 3 \text{palmitic acid (fatty acid synthesis)}$ ---(4)
Set e: This accounts for the tripalmitin production via the Kennedy pathway. (Stryer, Biochemistry, 6th edition)

Glycerol (c) + 3 palmitic acid + ATP \rightarrow \text{Tripalmitin (Kennedy pathway)} --- (5)

Once the reaction modules have been analyzed to obtain a single reaction, the next step involves adding these to obtain our final equation. Adding sets b through e (reactions 2 to 5) would provide the following equation.

\[
2 \text{ACA}(c) + \text{ATP} \rightarrow \text{OAA}(c) + \text{NADH}_c + \text{FADH}_2 + \text{NADPH}_c --- (2)
\]

\[
\text{OAA} (c) + \text{GTP} + \text{ATP} + \text{NADH}_c \rightarrow \text{Glycerol + CO}_2 \text{ (gluconeogenesis)} ---- (3)
\]

\[
24 \text{ACA}(c) + 18 \text{NADPH} + 24 \text{NADH}_c + 69 \text{ATP} \rightarrow 3 \text{palmitic acid (fatty acid synthesis)} - (4)
\]

\[
\text{Glycerol (c) + 3 palmitic acid + ATP \rightarrow Tripalmitin (Kennedy pathway)} --- (5)
\]

The net equations being:

\[
26 \text{ACA}(c) + 17 \text{NADPH}_c + 72 \text{ATP} + \text{GTP} + 24 \text{NADH}_c \rightarrow \text{Tripalmitin + CO}_2 + \text{FADH}_2 ---- (6)
\]

At this point, the acetate required to provide the required NADPH, NADH and ATP needs to be accounted for.

We assume all of the NADPH to be provided through the action of the malic enzyme. The following equation accounts for NADPH production:

\[
\text{NADH}_c + \text{ATP} \rightarrow \text{NADPH}_c \text{ (Malic enzyme)} ---- (7) \text{ (Stryer, Biochemistry, 6th edition)}
\]
ATP production from cytosolic ACA is not as straightforward as ACA cannot cross the mitochondrial membrane to form mitochondrial ACA, which could then directly enter the TCA cycle. Thus, the glyoxalate cycle operates to form succinate, which eventually forms pyruvate. Pyruvate can permeate through the mitochondria and then decarboxylate to form acetyl CoA, which subsequently enters the TCA cycle. The equation for ATP/NADH production through the TCA cycle from cytosolic acetyl CoA proceeds through the following set of equations:

\[ 2 \text{ACA}(c) \rightarrow \text{Succinate}(c) + \text{NADH}_c \text{ (glyoxalate cycle)} \]

\[ \text{Succinate}(c) \rightarrow \text{Succinate}(m) \text{ (succinate exit)} \]

\[ \text{Succinate}(m) \rightarrow \text{Malate}(m) + \text{FADH}_2 \text{ (TCA cycle)} \]

\[ \text{Malate}(m) \rightarrow \text{Malate}(c) \text{ (Malate exit)} \]

\[ \text{Malate}(c) \rightarrow \text{Pyruvate}(c) + \text{NADPH}_c + \text{CO}_2 \text{ (Malic enzyme) (Stryer, Biochemistry, 6th edition)} \]

\[ \text{Pyruvate}(c) \rightarrow \text{Pyruvate}(m) \text{ (Pyruvate exit)} \]

\[ \text{Pyruvate}(m) \rightarrow 3 \text{CO}_2 + 4 \text{NADH}_m + \text{FADH}_2 + \text{GTP} \text{ (TCA cycle) (Stryer, Biochemistry, 6th edition)} \]

\[ 4 \text{NADH}_m \rightarrow 4 \text{NADH}_c \text{ (van Milgen, 2002)} \]

The net equation is: \[ 2 \text{ACA}(c) \rightarrow 4 \text{CO}_2 + 5 \text{NADH}_c + 2 \text{FADH}_2 + \text{GTP} + \text{NADPH}_c \text{ -- (8)} \]

However, equation (8) can also be used to produce more ATP through the conversion of the NADH and FADH\(_2\) in the electron transport chain. However, we are also assuming that the NADPH would not be oxidized because there is a high demand for NADPH in the cell during the
fatty acid synthesis as a reducing agent. (1 NADH = 2.5 ATP, 1 FADH$_2$ = 1.5 ATP, 1 GTP = 1 ATP; Stryer, Biochemistry, 6th edition)

The equation would become: $2 \text{ACA} (c) \rightarrow 4 \text{CO}_2 + 16.5 \text{ATP} + \text{NADPH} - (9)$

Equations 6, 7, 8 and 9 would add up together to arrive at the final equation for lipid production from acetate. The strategy employed is to assume the equations 7, 8 and 9 to be multiplied by unknowns x, y, and z and added to equation 6. The equations are to be added such that the coefficients of NADH, NADPH and ATP add up to 0. In this way, we can ensure that the equation accounts for the just the exact amount of acetate needed to arrive at 1 mol of tripalmitin. The details of the calculations are shown in the appendix A.2.

We obtain the final equation for conversion of acetate into lipids as the following:

$$50.11 \text{CH}_3\text{COOH} \rightarrow 49.22 \text{CO}_2 + \text{Tripalmitin}$$

Lipid yield on acetate = $806/(50.1*59) = 0.272$

The stoichiometric equation for the conversion of propionate to tripalmitin is:

$$29.2 \text{CH}_3\text{CH}_2\text{COOH} \rightarrow 36.7 \text{CO}_2 + \text{Tripalmitin}$$

Lipid yield on propionate = $806/(29.23*73) = 0.377$

The stoichiometric equation for the conversion of butyrate to tripalmitin is:

$$19.6 \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} \rightarrow 27.4 \text{CO}_2 + \text{Tripalmitin}$$
Lipid yield on butyrate = 806/(19.6*87) = 0.472

The stoichiometric equation for the conversion of pentanoate to tripalmitin is:

\[
15.3 \text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \rightarrow 25.4 \text{CO}_2 + \text{Tripalmitate}
\]

Lipid yield on valerate = 806/(15.3*101) = 0.521

The stoichiometric equation for the conversion of hexanoate to tripalmitin is:

\[
12.2 \text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \rightarrow 22.1 \text{CO}_2 + \text{Tripalmitin}
\]

Lipid yield on hexanoate = 806/(12.2*115) = 0.574

The table below summarizes the pathway analysis calculations for the substrates mentioned above:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Stoichiometric Equation</th>
<th>Lipid yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>14.1 Glucose → 33.5 CO₂ + Tripalmitin</td>
<td>0.32 g/g</td>
</tr>
<tr>
<td>Acetate</td>
<td>50.1 Acetate → 49.2 CO₂ + Tripalmitin</td>
<td>0.27 g/g</td>
</tr>
</tbody>
</table>
### Table 3.1 Summary of the stoichiometry of lipid production and lipid yield on glucose and VFAs

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Equation</th>
<th>Lipid Yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionate</td>
<td>$29.2 \text{ Propionate } \rightarrow 36.7 \text{ CO}_2 + \text{ Tripalmitin}$</td>
<td>0.38</td>
</tr>
<tr>
<td>Butyrate</td>
<td>$19.6 \text{ Butyrate } \rightarrow 27.4 \text{ CO}_2 + \text{ Tripalmitin}$</td>
<td>0.47</td>
</tr>
<tr>
<td>Pentanoate</td>
<td>$15.3 \text{ Pentanoate } \rightarrow 25.4 \text{ CO}_2 + \text{ Tripalmitin}$</td>
<td>0.52</td>
</tr>
<tr>
<td>Hexanoate</td>
<td>$12.2 \text{ Hexanoate } \rightarrow 22.1 \text{ CO}_2 + \text{ Tripalmitin}$</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Complete details of the yield analysis for propionate, butyrate, valerate and hexanoate have been provided in the appendix section (A.2)

### 3.4. Engineered ACC-DGA strain

The shake flask and bioreactor studies in this thesis have been performed with the lipid over-producing ACCDGA strain discussed in Tai and Stephanopoulos 2012. The following two-sub sections provide the rationale for the choice of the two target genes - ACC and DGA.

#### 3.4.1. Acetyl-CoA Carboxylase (ACC)

ACC enzyme is responsible for the conversion of acetyl CoA to malonyl CoA.
This step catalyzes the first committed step for fatty acid production and hence, overexpressing the gene encoding the Acetyl CoA Carboxylase enzyme would likely accelerate the overall rate of lipid production. An increased expression of ACC would divert higher amount of acetyl CoA into the fatty acid synthesis pathway and cause a ‘push’ on the pathway.

### 3.4.2. Diglycerol Acyltransferase (DGA)

DGA is responsible for the conversion of a diglyceride to a triglyceride through the incorporation of another acyl CoA molecule.

This reaction is the last step of lipid synthesis and the objective of overexpressing DGA is to accelerate this reaction. This reaction serves as a means to reduce the feedback inhibition of the fatty acid synthesis pathway by diglyceride due to its accumulation. Therefore, under increased flux through this pathway, the upstream pathway could continue to produce more diglyceride. This reaction thus serves as a ‘pull’ on the fatty acid synthesis cycle.

ACC and DGA overexpression together exert a push-pull effect on lipid synthesis, ensuring a high flux into and out of the fatty acid synthesis cycle leading to an overall high rate of lipid synthesis.
Chapter 4
Exploring High Cell Density Growth on Acetate
4. EXPLORING HIGH CELL DENSITY GROWTH ON ACETATE

4.1. Introduction

Dilute streams of acetate can be obtained at low cost from a variety of sources. Syngas fermentation processes can yield acetate at concentrations of 30 g/L using feedstocks such as CO₂ and H₂, which are expected to get cheaper with developments in CCS (carbon capture and sequestration) and renewable H₂ production technologies (Hu et al. 2013; Lyndon et al. 2013; Esposito et al. 2013; Volgusheva et al. 2013). Acetate is also generated in the water process of uranium bleaching and as an effluent from the Fischer-Tropsch reaction (Papanikolaou & Aggelis 2011). Such acetate containing streams cannot be reused or recycled back, and hence, it is possible that such streams could become available at low cost.

However, to take advantage of dilute sources of acetate for lipid production, the growth and lipid production of *Y. lipolytica* on dilute acetate must first be assessed. The use of dilute sources of acetate feedstock necessitates the introduction of efficient cell-recycling systems integrated with the bioreactor.

In prior studies, most high cell density systems of *Y. lipolytica* have been obtained using favorable substrates such as glucose or glycerol (Pan et al. 1986; Gill et al. 1977; Hall & Ratledge 1977; Papanikolaou & Aggelis 2002). There is minimal literature discussing growth and lipid production on acetate. One of the few papers discussing fed-batch systems on acetate is Fontanille et al. (2012). The lipid titer achieved with acetate as the sole carbon source was 2 g/L in 150 h. Another strategy involved using a two-stage feeding approach (glucose followed by acetate) in a large scale fermenter to obtain lipid concentrations of 12 g/L, a lipid content of 40% and a lipid productivity of 0.16 g/L/h. The paper does not mention utilization of dilute acetate.
Given the strong motivation for developing a biodiesel production platform based on dilute acetate, this study evaluates the growth of *Y. lipolytica* on acetate in the following ways: a) compare and contrast the growth on glucose versus that on acetate, b) evaluate the potential of high cell density growth and lipid production in a fed-batch system with concentrated acetic acid feed, and c) demonstrate the robustness of the system to generate similar lipid titer, yield and productivity on dilute acetate. Our results demonstrate the potential and promise of achieving high lipid titers, yields and productivity with the engineered ACCDGA strain of *Y. lipolytica* when grown on dilute acetate in a fed-batch reactor.

### 4.2. Materials and Methods

#### 4.2.1. Strains and Culture Conditions

The engineered ACC DGA strain was previously developed by overexpression of acetyl-CoA carboxylase 1 and diacylglyceride acyltransferase 1 (Tai and Stephanopoulos, 2012). A tube culture of the strain was grown overnight in YPD medium to an OD of 3-5 followed by streaking onto a YNB-Leu petridish. Selective YNB-Leu agar plates contained 1.7 g/L yeast nitrogen base (without amino acids), 0.69 g/L CSM (Leu-), 20 g/L glucose, and 15 g/L Bacto agar (Difco Laboratories, Detroit, MI). YPD medium was prepared with 20 g/L Bactopeptone (Difco Laboratories, Detroit, MI), 10 g/L yeast extract (Difco Laboratories, Detroit, MI), 10 g/L yeast extract (Difco Laboratories, Detroit, MI), 20 g/L glucose (Sigma-Aldrich, St.Louis, MO). YPA medium was similar to YPD other than the fact that it contained 20 g/L acetate (as 28 g/L sodium acetate – Macron Chemicals) instead of glucose.

Seed culture preparation involved the following procedure: from a selective plate, the initial pre-culture was inoculated into a tube culture of YPA (3 mL in 15 mL culture tube, 200 rpm, 28°C, 24 h). Cells from the tube at an OD of 3-5 were inoculated at a starting OD of 0.1 into Erlenmeyer flasks (50 ml YPA in 250 ml flasks, 200 rpm, 28°C, and 2 days). The bioreactor was
inoculated with exponentially growing cells from the seed culture to a final concentration of 5 % v/v.

4.2.2. **Bioreactor Run – Media and Process Parameters**

Bioreactor scale fermentation was carried out in a 2 L stirred-tank bioreactor (New Brunswick Scientific, Edison, NJ). The pH and dissolved oxygen (DO) levels during the course of the fermentations were monitored using a pH probe (Mettler-Toledo Ingold Inc., Bedford, MA) and a DO probe (Mettler-Toledo Ingold Inc., Bedford, MA). Three bioreactor runs are discussed in this chapter: first, a batch run with 36 g/L acetate, second, a fed batch run with feeding of 30% v/v acetic acid in response to pH control (growth on acetate is associated with rise in pH and hence, acetic acid feed was used to maintain pH at the desired set point) and third, a fed batch run with feeding of 3% v/v acetic acid in response to pH control. The acetic acid solutions were prepared using glacial acetic acid (Acros Chemicals, New Jersey).

The first reactor run (referred to henceforth as batch run) discussed is a batch run modeled on the glucose run discussed in Tai and Stephanopoulos, 2012. The run was conducted by Mitchell Tai, a previous student of the Stephanopoulos lab and the results of this run form the foundation for the rest of the study on acetate. The medium was composed of 50 g/L sodium acetate, 1 g/L yeast extract, 1.5 g/L yeast nitrogen base (without amino acids and ammonium sulfate, Amresco, Solon, Ohio) and 0.8 g/L ammonium sulfate (Macron Chemicals, Center Valley, PA) (molar ratio of carbon/nitrogen – 100). The pH set point was 8.0 (since growth on acetate only observed at basic pH) and there was no DO control (1 vvm air – working volume 2 L, 250 rpm agitation for the entire period of the run).

The fed batch run was performed using the same 2L bioreactor vessel. It will be referred to henceforth as the concentrated fed-batch run (CFB). The initial medium was composed of 30 g/L
sodium acetate, 2.5 g/L yeast extract, 4.2 g/L yeast nitrogen base (without amino acids and ammonium sulfate), and 2.4 g/L ammonium sulfate (C/N ratio of 20). The pH setpoint was 7.3 (shake flask experiments performed at a variety of initial pH values showed maximum initial growth at pH 7.3) and the DO setpoint was 20% maintained using cascade control. In the first 100 h of growth, the feed consisted of 30% acetic acid and 15 g/L ammonium sulfate so as to provide sufficient nitrogen for non-lipid generation. The feed consisted of only 30% acid for the rest of the run.

The third bioreactor run (referred to henceforth as dilute fed-batch - DFB) was exactly similar to the second run, except that the feed was more dilute. In this case, as expected, a large volume was being fed into the reactor and hence, a mechanism was needed to concentrate the broth and prevent the reactor from overflowing. To this end, a hollow fiber module (MiniKros® Sample Plus Filter Module, Spectrum Laboratories, Rancho Dominguez, CA) was installed which had a pore rating of 0.2 μm and surface area of 290 cm².

4.2.3. Principle of Action of Hollow Fiber Membrane

A hollow fiber membrane consists of a group of thin hollow fibers through which the cell broth is pumped. The walls of the fibers consist of a filter membrane whose pore size is smaller than the size of the microbes under investigation. A negative pressure is applied across the width of the fiber using a pump. As a result, the media constituents smaller than the mesh size pass through the pores in the walls and are collected as ‘permeate’. The concentrated cell broth is recycled back to the reactor. The working of the hollow fiber membrane is shown in Fig. 4.1. The operation of the hollow fiber membrane allows the culture to grow to high cell densities. Use of hollow fiber membrane has been reported earlier (David 1981; Cheryan and Mehaia, 1983; Parekh and Cheryan, 1994)
4.2.4. Measurements and Carbon Balance

Culture samples were collected every 24 h for the determination of OD600, biomass concentration, and lipid, acetate, citrate and ammonium titers. Samples were collected in 1-mL cuvettes, and the OD was measured at 600 nm using an Ultrospec 2100 pro UV/visible spectrophotometer (General Electric, Fairfield, CT). Acetate and citrate were analyzed in an HPLC (Waters Corporation, Milford, MA) using an Organic Acid Analysis Column (Aminex HPX-87H Ion exclusion column 300 mmx7.8mm). Ammonium concentration was measured using the YSI 2100 analyzer (YSI Life Sciences, Yellow Springs, Ohio). Analysis of gas
composition for CO₂ concentration measurement in the exit gas was conducted using a dual-channel Agilent micro-GC (Model 3000A) equipped with PLOT U and molecular sieve columns, and TCD detectors (Agilent Technology, Santa Clara, CA). Argon was used as the carrier gas. Biomass was determined gravimetrically using cellulose nitrate (Whatman GmbH, Germany) filters. The filters were dried initially at 60 °C for 2 h and pre-weighed. A certain volume of broth was poured into the holding reservoir fitted on the filter membrane. Vacuum was applied to pull the liquid through the membrane. The filters were dried for 24 h in the 60 °C oven and re-weighed.

4.2.5. **Lipid Extraction and Quantification**

Total lipids were measured and quantified using a modified version of a direct transesterification protocol adapted from US Patent 7932077 and Griffiths, van Hille, and Harrison 2010. Using a correlation between the OD and cell biomass, a certain volume of cell broth was aliquoted such that it would contain approximately 1 mg of cell biomass. After centrifugation and removal of the supernatant, the cell pellet was suspended in 100 µl of hexane containing the internal standard glyceryl triheptadecanoate (C17 Tri-Acyl Glyceride). Subsequently, 500 µl of 0.5 N sodium methoxide was added to the mixture and the cell suspension vortexed at room temperature for 60 min. Next, 40 µl of 98% (w/w) sulfuric acid was added followed by addition of 500 µl hexane. This entire mixture was vortexed for 30 min at room temperature so as to extract the FAMEs into the hexane. Finally, the mixture was centrifuged at 8000 rpm and the top hexane layer was transferred into vials for analysis through gas chromatography (GC). GC analysis of FAMEs was performed using a Bruker 450-GC instrument equipped with a flame-ionization detector and a capillary column HP-INNOWAX (30m x 0.25 mm) and the same oven conditions were used as discussed in Tai and Stephanopoulos, 2012. Peak areas were converted
into respective FAME concentrations using standard curves obtained using commercial FAME standards. The FAME concentrations were normalized to the internal standard concentrations (methyl C17) in the control tube with no cells. Total lipid content was calculated as the sum of total fatty methyl ester contents for five FAMEs: methyl palmitate (C16:0), methyl palmitoleate (C16:1), methyl stearate (C18:0), methyl oleate (C18:1), methyl linoleate (C18:2)

4.3. Results

4.3.1. Batch Fermentation using 50 g/L Sodium Acetate – Batch Run

The objective of this bioreactor study was to evaluate the engineered Y.lipolytica strain with respect to its potential to consume acetate and achieve similar lipid content as obtained on glucose (Tai and Stephanopoulos, 2012). To this end, a batch reactor on acetate was run at similar conditions to the run on glucose. Table 4.1 summarizes the results of the batch acetate run when compared to the glucose run. This run was significant as it demonstrated the capability of the engineered strain to achieve the same high lipid content (62%) on acetate as was obtained previously on glucose. It is important to note though, that the carbon fed to the reactor as acetate (36 g/L acetate = 1.2 carbon M) was much lower than the carbon fed as glucose (90 g/L glucose = 3 carbon M). Acetate could not be fed at similar concentrations as glucose because growth of Y. lipolytica was found to be inhibited at higher concentrations of acetate (data not shown). Hence, the total biomass and lipid titers produced on acetate were lower than those on glucose. The overall lipid productivity was also affected by the lower biomass concentration.

In this run, the starting C/N ratio was high (100) because the intention was to provide an environment which would encourage lipid production. However, the tradeoff was that there was not sufficient nitrogen (high C/N, so high C and low N) provided in the medium for the microbe to generate a high biomass. As a result, despite the high lipid content (62%), the final lipid titer
could not get higher than 5 g/L. Thus, in order to obtain higher lipid titers, it is imperative that more nitrogen be provided to the cells (to maintain a low C/N) in the initial periods of growth so as to develop significant non-lipid biomass. In the later stages, the supply of nitrogen can be restricted (creating a high C/N) to prevent any further non-lipid biomass generation and instead stimulate lipid production. In addition, to obtain higher lipid titers, it was also necessary to provide additional acetate to the system. Since acetate was observed to be inhibitory at higher concentrations, the reactor would have to be operated in fed-batch mode. We had observed a rise in pH concomitant with the growth and acetate consumption by the microbe. Hence, it was decided to use acetic acid for pH control. In doing so, one could both control the pH as well provide additional carbon to the cells to produce more biomass and lipids. Also, the concentrations of growth supplements such as yeast extract and yeast nitrogen base in the medium were increased (mentioned in the materials and methods section) to boost growth and reduce the lag phase.
### Table 4.1: Comparison of batch runs of engineered ACC DGA strain on glucose and acetate

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glucose (Tai and Stephanopoulos, 2012)</th>
<th>Acetate (Batch run)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final biomass (g/L)</td>
<td>28.5</td>
<td>9</td>
</tr>
<tr>
<td>Final lipid titer (g/L)</td>
<td>17.6</td>
<td>5.5</td>
</tr>
<tr>
<td>Final lipid content (% of dry cell weight)</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>Overall lipid productivity (g/L/h)</td>
<td>0.14</td>
<td>0.04</td>
</tr>
<tr>
<td>Overall lipid yield (g/g)</td>
<td>0.195</td>
<td>0.15</td>
</tr>
</tbody>
</table>

**4.3.2. Fed-batch Fermentation using Concentrated Acetate Feed (30% v/v acid feed) – Concentrated Fed Batch (CFB) Run**

The aim of this run was to test the hypothesis that providing a continuous supply of acetate to the reactor could help achieve high biomass and lipid concentrations. Growth had already been observed at a starting concentration of 50 g/L sodium acetate in the batch run. However, since acetate is known to be inhibitory to growth, the starting concentration was lowered to 30 g/L sodium acetate in the fed-batch run. Acetic acid was fed for pH control in the form of a 30% v/v aqueous solution.

The feeding of ammonium sulfate along with the acid at initial stages served as a means to limit the C/N ratio at a low value and allowed the cells to produce non-lipid biomass. The depletion of nitrogen at later stages allowed the C/N ratio to increase and prevented any further non-lipid production. This strategy has been used earlier in literature across a variety of oleaginous microbes: Fontanille et al., 2012 (*Yarrowia lipolytica*), Nicaud et al. 2011 (*Yarrowia*...
lipolytica), Eggink, Huijberts, and Meesters 1996 (Cryptococcus curvatus), Deo et al. 2012 (Cryptococcus curvatus).

The time courses of lipid titer, non-lipid titer, nitrogen levels, C/N ratio and citrate titer with time are shown in Fig. 4.2. As expected, once nitrogen feeding was stopped, the residual nitrogen was consumed after which the non-lipid biomass trend stopped increasing. Thus, after 120 h, all the biomass increase was accounted for by lipids. In spite of the high C/N ratio environment at the end of the run, the citrate concentration only reached approximately 3 g/L (reasons for low citrate production on acetate are discussed in chapter 3). This accounted for less than 2% of the total carbon fed to the system; thus by-product formation was minimal during the run.

A comparison of the results of the CFB run and batch run is shown in Table 4.2. It is interesting to note that the cells consumed 554 g of acetate through the fed-batch process compared to the 72 g consumed in the batch run. In addition to the increased carbon supply, the nitrogen fed in the early stages of growth led to increased biomass production and hence, the final lipid titer was much higher for the CFB run even though the lipid content is similar across the runs.

Furthermore, the high biomass levels as well as improved operating conditions led to improved productivity. The yield, being a characteristic of the strain, was constant between the runs. A high cell density culture of Y. lipolytica was thus obtained on acetate using a fed-batch strategy. However, the final objective was to use a feed which would be practical for a large-scale fermentation such as 3% (v/v) acetic acid.
4.3.3. **Fed-batch Fermentation using Dilute Acetate Feed (3% v/v acid feed) – Dilute Fed Batch (DFB) Run**

The final investigation of this study was conducted to test lipid accumulation in the engineered ACCDGA strain in a fed-batch system with a dilute feed (3% v/v) of acetic acid. Apart from the use of the hollow fiber membrane, all operational aspects of the DFB were very similar to those of the CFB. The schematic of the operation is shown in Fig. 4.3.
Ammonium sulfate was fed along with 3% (v/v) acetic acid until 70 h following which nitrogen feeding was stopped. Ammonium sulfate was fed for a shorter time interval than in the CFB run because in the CFB run, nitrogen was found to accumulate in the reactor after 72 h as per Fig. 4.2. This was not ideal as it was reducing the C/N ratio to 15 after its initial rise to a value of 40.

In this run, the acetate titer in the reactor was found to decrease rapidly, which meant that the acetic acid feeding (in response to pH control) was not able to compensate for all of the acetate being consumed. The acetate concentration decreased to 6 g/L after the first 48 h and hence, it required the external addition of sodium acetate solution every 24 h to prevent the acetate from getting completely depleted in the reactor. The time course of the acetate concentration in the DFB reactor is shown in Fig. 4.4.
Figure 4.4: Acetate titer in the bioreactor going through cycles of high and low concentration in the dilute acetate run. The acetate spike was created through bolus additions of concentrated sodium acetate (300 g/L).

Another reason for the depletion of acetate in the reactor was the operation of the hollow fiber membrane. The permeate stream generated during the cell recycle operation contained acetate lost over the course of the run. The total permeate collected was found to contain 10 g/L acetate and represented 16% of the total acetate fed into the reactor.

The traces of lipid, non-lipid, nitrogen, and citrate were very similar to those of the CFB run as shown in Fig. 4.5. A comparison of the results of the batch, CFB and DFB run has been shown in Table 4.2. First, it can be concluded that the lipid results from the reactor on concentrated acetic acid feed (CFB run) have been replicated in the reactor on dilute feed (DFB run) and second, high lipid titers and productivity have been achieved in both the runs as compared to the batch run.
Figure 4.5: Bioreactor trends of the DFB run.

Table 4.2: Comparison of bioreactor runs - batch, CFB and DFB runs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Batch</th>
<th>Fed-batch with concentrated feed (CFB)</th>
<th>Fed-batch with dilute feed (DFB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final lipid titer (g/L)</td>
<td>5.5</td>
<td>51</td>
<td>46</td>
</tr>
<tr>
<td>Final lipid content (%) of dry cell weight</td>
<td>62</td>
<td>61</td>
<td>59</td>
</tr>
<tr>
<td>Overall lipid productivity (g/L/h)</td>
<td>0.04</td>
<td>0.26</td>
<td>0.27</td>
</tr>
<tr>
<td>Overall lipid yield (g/g)</td>
<td>0.15</td>
<td>0.15</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table 4.2: Comparison of bioreactor runs - batch, CFB and DFB runs
4.3.4. Lipid Profile

The lipid profile of the fats stored by the microbe during the bioreactor run is one of the major factors in evaluating the quality of the resulting biodiesel. The lipid profile of the microbial lipids should be as close as possible to that of current plant biodiesel, to facilitate the transition to the use of microbial biodiesel in existing infrastructure. It is interesting to note that the lipid profile was similar across the CFB and DFB runs as the operating conditions used were very similar (in terms of DO and media composition), as opposed to the batch run. The average of the final lipid profile generated in the CFB and DFB runs was the following (variation less than 3%): 64% C18.1 (oleate), 14% C18 (stearate), 13% C16 (palmitate), 5% C18.2 (linoleate), 4% C16.1 (palmitoleate). This profile as shown in Fig. 4.6 is very similar to that of canola oil biodiesel (Knothe 2009). Such a lipid distribution is very desirable as higher fractions of unsaturated fats increase the cetane number of the fuel and decrease the cloud point of the resulting biodiesel. However, there are also certain disadvantages to this such as low oxidative stability. But such limitations can be overcome through use of various additives. In conclusion, the lipid profile is very highly suitable for use as a potential biodiesel.

![Lipid Profile Chart](image)

Figure 4.6: Lipid profile of the fats stored by *Y. lipolytica* during the bioreactor studies
4.3.5. **Carbon Balance**

The carbon balance was performed to account the flow of carbon across metabolites during the bioreactor run. Acetate served as the primary carbon substrate in the bioreactor, which was consumed by the microbe and converted either into CO$_2$, biomass (lipids and non-lipids) or by-products (citrate). A cumulative carbon content trend for the DFB run is shown in Fig. 4.7. The cumulative carbon balance is met within a difference of 5%.

![Carbon Balance Graph](image)

**Figure 4.7:** Carbon balance of the DFB run showing carbon in the output (lipid, non-lipid, by-products, CO$_2$) stacked up cumulatively against the input carbon as acetate (linear trend).

It was interesting to note in the carbon distribution pie chart shown in Fig. 4.8 that CO$_2$ accounted for 54% of the total carbon. Since we would want to maximize the recovery of carbon in lipids (29.8%), it was worth studying if a theoretically higher recovery of carbon in the lipids could be achieved.
The approach involved building a model of the microbial metabolism to determine the stoichiometry of lipid and non-lipid production on acetate. The stoichiometric equations would provide a basis to calculate the theoretical amounts of acetate required to produce the experimentally observed amounts of lipids and non-lipids. At the same time, it would also help calculate the estimated amounts of CO₂ that would have to be produced as per the stoichiometry. Comparing the theoretical estimates and the experimental observations would yield information which would be useful in developing strategies to increase the recovery of carbon in lipids in future runs.

The equation for lipid production on acetate is shown in equation 1 (obtained during the lipid yield analysis in chapter 3). It describes the stoichiometry of the pathway for lipid production from acetate and yields information about the moles of carbon lost as CO₂ in the process. This equation does not represent the only source of CO₂ production though. Additional carbon is lost as CO₂ in the production of non-lipids (proteins, nucleic acids). Non-lipid biomass of average yeasts has a chemical formula of C₅H₈O₂.₇N₀.₇ (von Stockar & Liu 1999). Using the analysis
shown in Fei et al, 2011, a stoichiometric equation for non-lipid biomass production was obtained relating the moles of acetate consumed to moles of CO₂ produced and non-lipid biomass produced (equation 2). Further details of the equation derivation have been provided in the appendix (A.3).

\[ 50.1CH\_3COOH \rightarrow 49.2CO\_2 + C\_{51}H_{98}O_6 (Tripalmitin) - eqn.1 \]
\[ 6.3CH\_3COOH \rightarrow 7.7CO\_2 + C\_{5}H_{83}O_{27}N_{0.3} (Biomass) - eqn.2 \]

With the total amount of lipid and non-lipid produced in the run known, the theoretical amounts of acetate needed and CO₂ produced were estimated. The theoretical numbers were matched with the actual measurements and are shown in the last two columns of Table 4.3. The values are reasonably close to each other (within 10%). The difference can be attributed to errors in measurements as well as the simplistic nature of the metabolism model. The closeness of the values signifies that the strain is producing lipids at its maximum possible theoretical yield (recovery of carbon in lipids is maximal) and that further improvements in lipid recovery (and hence lipid content) would require additional genetic engineering of the microbe.

<table>
<thead>
<tr>
<th>Metabolite under study</th>
<th>Theoretical calculations</th>
<th>Experimentally observed numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid production of 46 g using equation 1 (a)</td>
<td>Non-lipid production of 32 g using equation 2 (b)</td>
<td>Total theoretical requirements (a+b)</td>
</tr>
</tbody>
</table>
Table 4.3: Comparison of theoretical predictions (calculated using equations discussed in section 4.3.5) and experimental measurements for CO₂ produced and acetate required in the bioreactor run to produce 46 g of lipids and 32 g of non-lipids (obtained in DFB run)

<table>
<thead>
<tr>
<th></th>
<th>124 g</th>
<th>90 g</th>
<th>214 g</th>
<th>237 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂ production</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>169 g</td>
<td>98 g</td>
<td>267 g</td>
<td>288 g</td>
</tr>
</tbody>
</table>

4.4. Discussion

Single cell oil production using oleaginous microbes is a relatively old concept (Ratledge 1989), and there have been numerous studies on high cell density cultures of *Y. lipolytica* in the past (Hall & Ratledge 1977; Gill et al. 1977; Pan et al. 1986). Such studies have focused on a broad range of applications such as protein production, citric acid production, alpha-keto-glutaric acid production, lipase production, and erythritol production to name a few (Du et al. 2012; Rymowicz et al. 2009; Fickers et al. 2009; Rywinska et al. 2010; Nicaud et al. 2002). Oil production for use as a diesel substitute has been a promising topic of research considering the similarity of the lipid profile of oleaginous microbes such as *Y. lipolytica* to that of current plant oils used for biodiesel production (Meng et al. 2009). However most of these studies on oil production in *Y. lipolytica* have used glucose as the substrate for growth. A recent study (Fei, Chang, Shang, Choi, et al. 2011) estimates that the biodiesel obtained from glucose using microbial fermentations would be available at $3.8/L, assuming a glucose price of $500/ton (the 2011 price), whereas diesel price in US as of October 2013 is around $1/L as per US Energy...
Information Administration (US EIA, 2013). The choice for the right substrate is crucial to facilitate a cost-effective bioprocess, as according to the economic analysis of biodiesel production by Fei et al, 2011, the feedstock accounts for more than 60% of the total production costs in a fermentation process. Taking the above into consideration, it seems likely that using a cheaper feedstock will be instrumental in realizing the full potential of *Y. lipolytica* in the renewable fuels sector.

Nonetheless, very few groups have focused on alternative feedstock such as acetate and other volatile fatty acids (VFAs). Apart from syngas fermentation and waste water streams, acetate can also be obtained from municipal solid waste. Studies have shown that MSW can be decomposed via anaerobic digestion to obtain acetate amongst other volatile fatty acids (VFAs) at concentrations of 15-20 g/L (Bassetti et al. 1995; Traverso et al. 2001; Jiang et al. 2005). Municipal solid waste (MSW) is currently disposed of in landfills or through incineration. With a steep rise in MSW generation projected in the near future (World Bank Report, 2012), it is expected that MSW would be available as a low-cost feedstock to enable a two-part bioprocess – MSW to acetate and then acetate to biodiesel. The challenge is to thus demonstrate robust growth and lipid accumulation of *Y. lipolytica* on acetate. Our results are some of the first in literature documenting the high cell-density growth and lipid production of *Y. lipolytica* on acetate; and dilute streams of acetate at that. We were successful in accounting for all the carbon flow by closing the carbon balance. In a similar study in literature of growth of *Y. lipolytica* on VFAs (Fontanille et al, 2012), the cells were first grown on glucose to develop non-lipid biomass, after which they were grown on acetate for lipid production. Glucose is a much more preferred substrate over acetate according to their results as well as other literature (Barth and Gaillardin, 1997). Thus, parameters such as lipid productivity are expected to be higher for such a run.
compared to a sole acetate run. On the contrary, our results are comparable to theirs in almost all parameters such as yield and titer as well as productivity. The high lipid content achieved in our run (60%) can be attributed to the fact that our experiments have been performed with a lipid over-producing ACC DGA strain.

High lipid titers in excess of 30 g/L have been obtained in high cell density fed-batch fermentations across a wide variety of oleaginous microbes in literature. The results are shown in Table 4.4. The lipid productivity is quite high for few runs due to factors such as use of favorable substrates such as glucose and glycerol, and strategies such as starting from a very high inoculum volume (50% v/v) or feeding salt solutions at regular intervals (Pan et al., 1986; Meesters et al., 1996). The lipid titers and yields achieved in our bioreactor runs are comparable to or slightly lower than those achieved in some of these studies. In part, this was expected as acetate is inhibitory to growth. Furthermore, it is to be noted that we used acetate as the sole substrate in our run and did not employ any of the strategies mentioned above. It is however, to be seen whether the effects of inhibition can be diminished through further research. Factors such as hypha production can slow down lipid accumulation and impact productivity, and lipase production could also affect the final lipid content. Some preliminary extracellular lipase measurements indicate that there is indeed detectable amount of lipase in the extracellular broth (details provided in appendix section A.4); however, further investigation is required to examine the effect of these lipases on the plateauing of the lipid content curve.
Table 4.4: Literature on high cell density runs across a variety of oleaginous microbes on different substrates. N/A – Information not provided in the paper

<table>
<thead>
<tr>
<th>Oleaginous microbe</th>
<th>Substrate</th>
<th>Lipid titer – g/L (lipid content - % of dry cell weight)</th>
<th>Lipid productivity (g/L/h)</th>
<th>Lipid yield (g/g)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhodotorula glutinis</em></td>
<td>Glucose</td>
<td>75 (40%)</td>
<td>0.90</td>
<td>N/A</td>
<td>(Pan et al., 1986)</td>
</tr>
<tr>
<td><em>Crytococcus curvatus</em></td>
<td>Glucose</td>
<td>37 (53%)</td>
<td>0.21</td>
<td>0.14</td>
<td>(Hassan et al., 1996)</td>
</tr>
<tr>
<td><em>Crytococcus curvatus</em></td>
<td>Glycerol</td>
<td>30 (25%)</td>
<td>0.59</td>
<td>0.11</td>
<td>(Meesters et al., 1996)</td>
</tr>
<tr>
<td><em>Rhodosporidium toruloides</em></td>
<td>Glucose</td>
<td>73 (67%)</td>
<td>0.54</td>
<td>0.23</td>
<td>(Li et al., 2007)</td>
</tr>
<tr>
<td><em>Yarrowia lipolytica</em></td>
<td>Dilute acetate</td>
<td>46 (59%)</td>
<td>0.27</td>
<td>0.16</td>
<td>This study</td>
</tr>
</tbody>
</table>

There are a few shortcomings in our current bioprocess, such as the requirement to externally add sodium acetate solution to the dilute feed reactor. This feeding was however, not necessary for the case of the concentrated feed reactor. The choice for 30% (v/v) as the concentration of the feed acid for the CFB run was made such that the volume added in response to pH control could compensate the acetate consumption, to ensure no other acetate addition would be required during the course of the run. However, while working with the dilute 3% (v/v) feed, such a
balance could not be established. Being a weak acid, the pH of acetic acid solutions does not correlate proportionally with the concentration. The pH of the dilute acid solution (pH of 2.6) was not much higher than that of the concentrated acid solution (pH of 2.1), and hence the volume fed in response to pH control was not high enough for the acid feed to balance acetate consumption. Moreover, 16% of the total acetate fed was lost in the permeate stream. As a result, the acetate concentrations in the reactor decreased very quickly, necessitating the external addition of sodium acetate. Another shortcoming was the need for the manual controlling of the permeate pump flow rate. The solution to both these issues is to model the acetic acid feeding and acetate consumption profiles of the system so as to determine a continuous feed rate for sodium acetate solution as well as a flow rate for the permeate pump. In such a scenario, the model could enable the maintenance of the acetate concentration at a low and steady level in the reactor, which might also help reduce the loss of acetate in the permeate.

4.5. Conclusions

The goal of this study was to answer the questions enumerated in the introduction section. First, we observed that the microbe can achieve the same lipid content on acetate as on glucose, which proves that the cell does not discriminate between sources of carbon as far as lipid production is concerned. However, we also noticed that the microbe grew much more slowly on acetate than on glucose. We overcame this challenge to a large extent by using a fed-batch reactor setup in which nitrogen was fed in the early stages of growth to promote biomass generation. Acetic acid was fed continuously during the course of the experiment to provide carbon for non-lipid and lipid production, whilst maintaining reasonably low levels of acetate in the culture broth. We observed a lipid titer of 50 g/L at an overall lipid productivity of 0.25 g/L/h, almost ten-fold and seven-fold higher than the respective values in the batch experiment. Thus, we concluded that
indeed high cell density cultures of *Y. lipolytica* with 60% lipid content could be achieved on acetate. Finally, we were able to successfully achieve similar levels of lipid titer, yield and productivity with a dilute 30 g/L acetate stream using a hollow-fiber membrane for the purpose of cell recycle. This demonstrated the robustness of the system and moreover, showcased the practicality of the system as upstream processes would yield acetate at 20-30 g/L.

Further developments in strain engineering would enable the cells to achieve a higher final lipid content in the bioreactors, which in turn would help improve overall lipid yield. Also, adopting better strategies for bioreactor operation would help decrease losses in the permeate stream and streamline the bioprocess.
Chapter 5

Investigating VFAs as an Alternative Substrate for Oil Production
5. INVESTIGATING VFAS AS AN ALTERNATIVE SUBSTRATE FOR OIL PRODUCTION

5.1. Introduction

In the previous chapter, acetate was identified as an alternative substrate to glucose and it was found to sustain growth of our engineered oleaginous microbe. We were successful in obtaining high cell density cultures in fed-batch bioreactors using dilute (30 g/L) streams of acetate. Lipid titer of around 50 g/L at overall lipid productivity of 0.27 g/L/h and yield of 0.16 g/g was attained. One of the sources of acetate discussed in the study was syngas fermentation which involves fixing CO₂ in the presence of electron donors like H₂ and CO (Hu et al. 2013). This method of obtaining acetate, although effective, relies on the fact that CO₂ and H₂ would be available in plenty and at low prices in the near future. Research and development efforts to obtain CO₂ and H₂ through cost-effective means are ongoing and it is reasonable to assume a certain development phase before such technologies are scaled up to commercial levels.

Hence, it might be useful to turn our attention to sources of acetate which can be utilized at present. Municipal solid waste (MSW) represents a source of carbon which can be degraded by anaerobic bacteria to produce a mixture of short-chain fatty acids, one of them being acetate. As described in Chapter 2, MSW represents an ideal source as the volume of MSW generated poses issues in its disposal and hence, MSW can be potentially obtained at low-cost. The short-chain fatty acids are collectively referred to as volatile fatty acids (VFAs). There are a couple of challenges though associated with using VFAs as substrates for microbial growth. First, like acetate, all other VFAs are known to be toxic and inhibitory to growth (Fontanille et al., 2012). Second, MSW degradation yields a mixture of dilute VFAs and the composition of the VFA
stream may vary from one batch to another depending on the MSW composition and operating conditions of the digester.

Hence, the following studies were designed to assess the feasibility of a lipid-producing bioprocess which would depend on an upstream MSW degradation process to yield VFAs as its feedstock. The first goal was to test growth of *Y. lipolytica* on VFAs other than acetate, individually at varying concentrations. In addition, this study would give an idea of the rate of incorporation of other VFAs in comparison to acetate. Second, it was to be examined if *Y. lipolytica* can metabolize a mixture of VFAs at a shake-flask scale and the growth characteristics were to be determined. Finally, the conclusions drawn from the previous experiment as well the bioreactor run described in chapter 4 were used to guide the design and operation of a bioreactor to investigate high cell density growth on VFAs using a fed-batch setup with cell recycle. Moreover, the response of the system to perturbations, such as fluctuations in the VFA feed composition, was tested.

5.2. Materials and Methods

5.2.1. Strains and Culture Conditions

The engineered ACC DGA strain was previously developed by the overexpression of acetyl-CoA carboxylase 1 and diacylglyceride acyltransferase 1 (Tai and Stephanopoulos, 2012). Maintenance of the ACCDGA cultures was done as discussed in chapter 4. YPD medium was prepared with 20 g/L Bactopeptone (Difco Laboratories, Detroit, MI), 10 g/L yeast extract (Difco Laboratories, Detroit, MI), 20 g/L glucose (Sigma-Aldrich, St.Louis, MO). Acetic acid was obtained from Macros Chemicals. Sodium propionate, sodium butyrate, valeric acid and hexanoic acid were obtained from Sigma-Aldrich (St. Louis, MO). Seed culture preparation
involved the following procedure. From a selective plate, the initial pre-culture was inoculated into a tube culture of YPD (3 mL in 15 mL culture tube, 200 rpm, 28°C, 24 h). Cells at an OD of 3-5 from the tube were inoculated at a starting OD of 0.1 into Erlenmeyer flasks (50 ml YPD in 250 ml flasks, 200 rpm, 28°C, 2 days). Exponentially growing cells from the seed culture were transferred into shake flasks at a starting OD of 0.1. The bioreactors were inoculated with 5% (v/v) of seed culture.

5.2.2. **Shake Flask Experiments – Media and Process Parameters**

The shake flask experiments were performed in 250 ml Erlenmeyer flasks. The starting OD in the shake flasks was 0.1 in 50 ml of media with an initial pH of 6.3. The flasks were maintained at 28°C and incubated at rotary shakers at 200 rpm.

In the experiments with individual VFAs, two sets of shake flasks for each VFA were prepared— one with 5 g/L and other at 10 g/L of the respective anion. Apart from the VFA, the media composed of 0.5 g/L of YE, 1.7 g/L of YNB (without amino acids and ammonium sulfate) and ammonium sulfate so as to start with a C/N ratio of 20. The experiment was run until there was no residual VFA left in the flask. Samples were taken every 24 h for OD and HPLC (to determine VFA titers). Biomass and lipid measurements were performed for the final time point.

In the experiment with mixture of VFAs, three different starting ratios of VFAs were chosen and for each such ratio, three different total VFA concentrations were used. The VFAs under study were acetate, propionate, butyrate and valerate. The ratios were chosen such that one other VFA would be present in the same carbon molar as acetate and the rest two would be present in lower amounts. This enabled us to compare the rate of each of the other VFA’s consumption in comparison to acetate and to also determine if any particular VFA had a particularly large
inhibitory effect. The initial concentrations were varied to determine the maximum concentration of VFA that the cells could tolerate. The three ratios (of respective carbon M) used were: 4:4:2:1, 4:1:4:2 and 4:2:1:4 of acetate, propionate, butyrate and valerate. The three starting concentrations were 0.4, 0.6 and 0.8 carbon M (1 carbon M of acetate: 59/2 = 29.5 g/L, 1 carbon M of propionate: 73/3 = 24.3 g/L and so on). The other constituents in the media as well as the sampling strategy were similar to that in the experiment with individual VFAs. The naming convention used for denoting the flasks was the following: flasks containing the same carbon M of a VFA as that of acetate in the mixture would be named after that VFA. Hence, the flasks with 4:4:2:1 carbon M of C2 (acetate), C3 (propionate), C4 (butyrate), and C5 (valerate) is referred to as p (after propionate). The three flasks in the ‘p’ group are designated as p-0.4, p-0.6 and p-0.8 according to the respective total VFA concentrations. The nine shake flasks would henceforth be referred to as p-0.4, p-0.6, p-0.8, b-0.4, b-0.6, b-0.8, v-0.4, v-0.6 and v-0.8.

5.2.3. Bioreactor Run with Mixed VFA Feed – Media and Process Parameters

Bioreactor scale fermentation was carried out using the BioFlo 115 bioreactor (New Brunswick Scientific Co., Edison, NJ) with 2L working volumes. The pH and dissolved oxygen (DO) levels were monitored using a pH probe (Mettler-Toledo Ingold Inc., Bedford, MA) and a DO probe (Mettler-Toledo Ingold Inc., Bedford, MA). The bioreactor run discussed here is modeled after the dilute acetate (3% v/v) run discussed in chapter 4.

The initial media composition was chosen based on the results of the shake flask study with mixture of VFAs. The media contained: 4:1:4:2 carbon M ratios of acetate, propionate, butyrate and valerate at a total carbon M concentration of 0.8 carbon M. The rest of the media components include 2.5 g/L yeast extract, 4.2 g/L yeast nitrogen base (without amino acids and ammonium sulfate), and 2.64 g/L ammonium sulfate (C/N ratio of 20). The pH setpoint was 7.3
and DO setpoint was 20% maintained using cascade control. The acid feed for pH control was prepared using a 1:1 carbon M ratio mixture of acetic and valeric acids with total carbon concentration of 1 M (15 g/L acetic acid + 10 g/L valeric acid). The first 72h of the run also included a nitrogen feed of 1.5 g/L ammonium sulfate along with the acid feeding (C/N of feed was 45) to promote non-lipid accumulation. The next phase of acid feeding contained 0.38 g/L ammonium sulfate (C/N of feed = 180). The nitrogen-limited environment would prevent any further non-lipid accumulation and instead, promote lipid accumulation.

In the dilute acetate run discussed in chapter 4, in spite of the acetic acid feeding, the acetate titer in the bioreactor decreased within the first 48 h to around 6 g/L. Hence, external addition of sodium acetate was required every 24 h to prevent the titer from going to zero and inhibiting the growth of the cells. The acetate titer therefore, went through cycles of high and low concentrations as shown in Fig. 5.1. In the current dilute VFA run, a similar requirement for manual addition was expected. To avoid manual intervention, a feed of sodium salts of VFAs (25 g/L of 2:2:1 carbon M ratio of sodium acetate, sodium butyrate and sodium propionate) was constantly pumped into the reactor after 48 h.
Figure 5.1: Acetate titer in the bioreactor going through cycles of high and low concentration in the dilute acetate run. The acetate spike was created through bolus additions of concentrated sodium acetate (300 g/L).

In this case too, because of the dilute nature of the feed, a hollow fiber membrane operation was required so as to get rid of excess volume and maintain a stable working volume.

5.2.4. Bioreactor Run with Perturbed VFA Feed – Media and Process Parameters

The bioreactor run discussed in this section was similar to the above run except that there were two major changes: a) Implementation of a model to determine the feed and permeate flow rates for controlling the VFA titer at a low and stable value and b) The system was perturbed through sudden changes in the VFA feed composition. The following changes were made in the concentrations of the feed in the run:

<table>
<thead>
<tr>
<th>Duration of bioreactor run</th>
<th>Ratio of C2:C3:C4:C5 in acid feed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-58h</td>
<td>1:0:0:1 (no C3 or C4)</td>
</tr>
<tr>
<td>58-119h</td>
<td>3:3:3:1</td>
</tr>
<tr>
<td>119-h-168h</td>
<td>0:1:2:2 (no C2)</td>
</tr>
</tbody>
</table>

Table 5.1: Composition of the VFA acid feed during the bioreactor run (perturbed feed)
The model to be discussed in the next section was useful in automating the flow rate of the salt feed as well as the permeate stream. The flow rates determined for every 12 hour period $F_2$ and $F_3$ were used to help maintain the VFA titer at a low value and hence, reduce the permeate losses.

5.2.5. Model for Fed-batch Reactor with Cell Recycle

![Diagram](5.2: Modeling the dilute VFA bioreactor system with cell recycle)

Description of the system: A microbial culture grows in a bioreactor which has a substrate concentration $S(t)$ and a working volume $V(t)$. An acid feed and a salt feed are the input flows to the reactor. The concentration of the substrate carbon in the acid feed is $S_1$ and that in the salt feed is $S_2$. $F_1$ and $F_2$ are the flow rates of the acid and salt feeds, respectively. The cell recycle operation produces a permeate stream with the same substrate concentration, $S$ as in the bioreactor. The permeate flow rate is $F_3$. Of these three flow rates, $F_1$ for any time period is determined by the system so as to maintain pH control in the reactor. The flow rates $F_2$ and $F_3$ can be externally controlled. Once selected at any time point $t_n$, $F_2$ and $F_3$ are not varied until $t_{n+1}$, the next sampling point. These flow rates are chosen such that the substrate concentration in
the reactor reaches a stable titer $S_0$ and the working volume reaches a stable volume $V_0$. $S_0$ is chosen such as to minimize the substrate concentration in the reactor, which, in turn, helps decrease the loss in the permeate stream. The working volume $V_0$ is maximized subject to the constraint that the system should not overflow with slight perturbations.

Objective: The aim of this exercise is to determine the flow rates $F_2$ and $F_3$ for the time period $t_n$ to $t_{n+1}$ such that the substrate concentration $S$ in the reactor becomes $S_0$ and working volume becomes $V_0$ at $t_{n+1}$.

Measurements: The time points $t_{n-1}$, $t_n$, $t_{n+1}$, etc. denote the times at which the bioreactor is sampled. At any time point $t_n$, the following measurements are made: substrate concentration in the bioreactor $S_n$, working volume of the reactor $V_n$, and acid flow rate from $t_{n-1}$ to $t_n$, $F_{1n}$. Using this information as well as the flow rates $F_{2n}$ and $F_{3n}$ from $t_{n-1}$ to $t_n$, one can calculate $dQ_n / dt$, the substrate consumption rate in the bioreactor. Given this information, we aim to analyze the system using mass and volume balances to determine the flow rates $F_{2n+1}$ and $F_{3n+1}$.

Analysis:

1. Volume balance:

$$\frac{dV}{dt} = F_1 + F_2 - F_3 \quad (1)$$

$F_1$, $F_2$ and $F_3$ refer to the flow rates from $t_n$ to $t_{n+1}$

Integrating the above equation between the last time point $t_n$ and a time $t$ ($t_n < t < t_{n+1}$) yields:

$$V = V_n + (F_1 + F_2 - F_3) \times (t - t_n) \quad (2)$$
Moreover, integrating the equation between \( t_n \) and \( t_{n+1} \), when the volume would reach \( V_0 \), we obtain:

\[
V_0 = V_n + (F_1 + F_2 - F_3) \times (t_{n+1} - t_n) \quad (3)
\]

2. Substrate balance:

\[
\frac{d(S \cdot V)}{dt} = (F_1 \times S_1) + (F_2 \times S_2) - (F_3 \times S) - \frac{dQ}{dt} \quad (4)
\]

Expanding the LHS,

\[
V \frac{dS}{dt} + S \frac{dV}{dt} = F_1 \times S_1 + F_2 \times S_2 - F_3 \times S - \frac{dQ}{dt} \quad (5)
\]

Substituting the expressions for \( \frac{dV}{dt} \) and \( V \) from equations (1) and (2), respectively, we get,

\[
[V_n + (F_1 + F_2 - F_3) \times (t - t_n)] \frac{dS}{dt} + (F_1 + F_2 - F_3)S = F_1 \times S_1 + F_2 \times S_2 - F_3 \times S - \frac{dQ}{dt} \quad (6)
\]

On simplifying and rearranging, we get

\[
\frac{dS}{dt} = \frac{dt}{V_n + (F_1 + F_2 - F_3) \times (t - t_n)} \quad (7)
\]
The above equation has been shown in a form, \[ \frac{dS}{f(S)} = \frac{dt}{g(t)} \] (as all the rest of the parameters are either known or are constants)

Integrating (7) from the time \( t_n \) to \( t_{n+1} \), and substrate concentration from \( S_n \) to \( S_0 \), we obtain

\[
\frac{1}{F_1 + F_2} \log \left( \frac{F_1 \times S_1 + F_2 \times S_2 - \frac{dQ}{dt} - (F_1 + F_2)S_0}{F_1 \times S_1 + F_2 \times S_2 - \frac{dQ}{dt} - (F_1 + F_2)S_n} \right) = \\
\frac{1}{(F_1 + F_2 - F_3)} \log \left( \frac{V_n + (F_1 + F_2 - F_3)(t_{n+1} - t_n)}{V_n} \right)
\]

3. Proton balance

The proton balance is represented by the following expression:

\[
\frac{dH^+}{dt} = F_1[H^+]_{\text{acidfeed}} + F_2[H^+]_{\text{saltfeed}} - F_3[H^+]_{\text{medium}} - k_1(\frac{dQ}{dt}) + k_2(\frac{d[NH_4^+]_{\text{consumed}}}{dt})
\]

The first three terms on the RHS represent the proton flow in and out of the reactor due to the respective feed/permeate streams. The acid stream has a proton concentration of \([H^+]_{\text{acidfeed}}\), the salt feed stream has a proton concentration of \([H^+]_{\text{saltfeed}}\) and the permeate stream has the same proton concentration as the medium \([H^+]_{\text{medium}}\). The next term represents the decrease in the protons in the medium due to the consumption of VFA. As described earlier, there is a proportional relationship between the acid pumped in response to pH control and the VFA consumption. The underlying reason is the mechanism of VFA uptake as described in literature (Casal et al, 1986; Verduyn, 1991). The uptake of short chain fatty acids, such as VFAs, proceeds via proton symport. Thus, with the uptake of the carboxylate anions, there is a concomitant uptake of H+ ions from the medium. The last term represents the increase of protons
in the medium due to the uptake of ammonium. Ammonium is taken up as ammonia (the electroneutral molecule) and hence, for every ammonium ion consumed as ammonia, there will be a proportional increase in the proton concentration in the medium.

In our bioreactor studies, the acid stream, apart from providing additional carbon to the microbe, performs the function of pH control. As a result, the pH is maintained at a particular setpoint. Hence, the LHS of equation (9) is 0.

In this case, the actual values of the pH of the feeds can be used to further simplify the equation.

\[
[H^+]_{\text{acidfeed}} = 10^{-2.6} \text{ for the acid stream of 25 g/L VFA}
\]

\[
[H^+]_{\text{saltfeed}} = 10^{-8.1} \text{ for the salt feed stream of 25 g/L VFA salts}
\]

\[
[H^+]_{\text{medium}} = 10^{-7.3} \text{ for the medium pH}
\]

Since the proton concentration of the salt feed and the medium is much lower (by \(10^{-5}\) times) than that of the acid feed, the contribution of the salt feed and permeate terms to the proton balance can be neglected. We thus arrive at the following equation:

\[
F1 \times \left[H^+\right]_{\text{acidfeed}} = k1 \left(\frac{dQ}{dt}\right) - k2 \frac{d[NH4^+]_{\text{consumed}}}{dt} \quad (10)
\]

Moreover, the model is implemented after the initial 36 hours of growth. After the initial growth phase (upto 36h), the medium is maintained at a nitrogen-deficient state as shown in Fig. 5.3. The rate of non-lipid increase is insignificant and hence, it can be inferred that the rate of ammonium consumption is also very low as there is no other source of nitrogen in the medium after the first 24-30 hours.
Figure 5.3: Ammonium titer in the bioreactor during the course of the run. After the first 24 h, the nitrogen concentration is below 1 mM. (nitrogen-starved environment)

Hence, the equation (10) can be further simplified to the following form,

\[ F1 \times [H^+]_{\text{acidotic}} = k \times \frac{dQ}{dt} \]  \hspace{1cm} (11)

The three equations obtained in our analysis relating the known parameters to the unknowns (F1, F2, F3 and dQ/dt) are:

\[ V_0 = V_n + (F1 + F2 - F3) \times (t_{n+1} - t_n) \] \hspace{1cm} (3)
\[ 1 \frac{1}{(F_1 + F_2)} \log \left( \frac{F_1 \times S_1 + F_2 \times S_2 - \frac{dQ}{dt} - (F_1 + F_2) S_0}{F_1 \times S_1 + F_2 \times S_2 - \frac{dQ}{dt} - (F_1 + F_2) S_n} \right) = \]

\[ 1 \frac{1}{(F_1 + F_2 - F_3)} \log \left( \frac{V_n + (F_1 + F_2 - F_3) (t_{n+1} - t_n)}{V_n} \right) \]

\[ F_1 \times [H^+]_{\text{acids}} = k \times \frac{dQ}{dt} \]

With 4 variables and 3 equations, we would need to specify one of the variables so as to solve this set of equations.

The consumption rate, \( \frac{dQ}{dt} \) for the next time period (\( t_n \) to \( t_{n+1} \)) is estimated using the measurements made at \( t_n \).

Our objective is to obtain an expression for the consumption rate for the time period \( t_n \) to \( t_{n+1} \) in the bioreactor run. The measurements available at \( t_n \) are: biomass concentration, \( X_n \), substrate concentration, \( S_n \), and the flow rates for the acid and salt feed. Since the biomass concentration cannot be obtained instantly (measurement procedure limitations), we use OD\(_n\) as a proxy to estimate biomass \( X_n \).

1. The first task is to obtain the specific growth rate, \( \mu \) for the time period \( t_{n-1} \) to \( t_n \).

\[ \mu_n = \left( \frac{dX}{dt} \right) \times \left( \frac{1}{X} \right) \]

\( \mu_n \) is calculated as:

\[ \mu_n = \frac{\log(OD_n / OD_{n-1})}{t_n - t_{n-1}} \]
2. Due to the lack of a model for \( \mu \), we assume that \( \mu \) in the previous time period would be similar to the \( \mu \) in the next time period, i.e. \( \mu_n = \mu_{n+1} \). This assumption would be valid if the time interval between the measurements is sufficiently small.

3. We then project the rate of biomass increase at \( t_n \), \( \frac{dX}{dt} \) for the following period

\[
\frac{dX}{dt} = \mu_{n+1} X_n
\]

4. The biomass yield on substrate consumed, \( Y_{s/s} \) is similar across time periods. We thus assume the biomass yield for the last period (calculated by dividing the biomass produced in the latest period over the substrate consumed in the same period) to be the same for the subsequent period.

\[
Y_{s/s,n} = Y_{s/s,n+1}
\]

5. We finally obtain \( \frac{dQ}{dt} \) as

\[
\frac{dQ}{dt} = (\frac{dX}{dt}) \times (\frac{1}{Y_{s/s,n+1}})
\]

Thus, we determine the projected consumption rate for the period \( t_n \) to \( t_{n+1} \). Next, using equation (11), we determine the projected acid flow rate \( F1 \). At this point, we can determine the unknowns \( F2_{n+1} \) and \( F3_{n+1} \) by solving the equations (3) and (8). Solving the two equations requires the use of a non-linear solver. The MATLAB function ‘fsolve’ was used to determine \( F2_{n+1} \) and \( F3_{n+1} \). The MATLAB code has been provided in the appendix section A.4.

Note: Using the above flow rates, ideally, by the time point \( t_{n+1} \), the substrate concentration in the reactor should be \( S_0 \). However, the above analysis projects the consumption rate and hence, an acid flow rate for the ensuing time period. This would not be necessarily true depending on
how well our assumptions hold and hence, the substrate concentration would be different from So. Reducing the time period between successive measurements would be the recommended way to ensure the drift from So is identified quickly enough to take corrective action (changing the flow rates) and prevent S from deviating too far from So. The algorithm for determining the flow rates F2 and F3 after every time point using the model is shown below in Fig. 5.4:

Figure 5.4: Schematic representation of the model algorithm for determination of the salt feed and permeate flow rates

5.2.6. Measurements

The bioreactor cultures were collected every 24 h in order to measure the OD600, total biomass, VFA titer in reactor, ammonium titer, and lipid titer. The protocol for these measurements has been discussed earlier in chapter 4. The lipid measurement protocol was exactly similar except that in this case, C14 TAG was used as the control instead of C17 TAG. On odd chain VFAs, Y.
lipolytica was found to produce odd chain fatty acids such as C17 and C17.1. Hence, glyceryl trimyristate (C14 TAG) was used as the control.

5.3. Results

5.3.1. Shake Flask Experiment – Individual VFAs

We had demonstrated growth on acetate in fed-batch reactors at starting concentrations as high as 20 g/L in chapter 4. However, literature suggests that VFAs are inhibitory to growth of Y. lipolytica at concentrations above 5 g/L (Fontanille et al., 2012). Therefore, our first objective was to determine if the engineered strain could grow effectively on VFAs apart from acetate at concentrations higher than 5 g/L. Two initial concentrations of 5 and 10 g/L were chosen for the shake flask studies to test growth on each of the five VFAs individually from C2 (acetate) to C6 (hexanoate). It was also to be determined if Y. lipolytica could effectively produce lipids on VFAs other than acetate.

Y. lipolytica was found to grow on all VFAs other than hexanoate. The absence of growth of Y. lipolytica on hexanoate has also been reported elsewhere in literature (Michely et al. 2013). In the case of each of the other VFAs, no difference was observed in the initial OD trends between the 5 and 10 g/L shake flasks indicating that there are no major effects of inhibition up to concentrations of 10 g/L. Moreover, all of the VFAs were consumed within 72 h. Lipid and total biomass measurements for the final time point have been provided in Table 5.2. It was very encouraging to note that the lipid content obtained on butyrate was comparable to that on acetate. Lower lipid content was observed in the shake flasks on propionate and valerate compared to the other VFAs, which could suggest that propionate and valerate are not as preferred a substrate as acetate and butyrate.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Biomass (g/L) at the end of 72 h</th>
<th>Lipid titer (g/L) at the end of 72 h</th>
<th>Lipid content (% of DCW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate, C2</td>
<td>2.2 g/L</td>
<td>0.5 g/L</td>
<td>23%</td>
</tr>
<tr>
<td>Propionate, C3</td>
<td>3.1 g/L</td>
<td>0.5 g/L</td>
<td>15%</td>
</tr>
<tr>
<td>Butyrate, C4</td>
<td>4.0 g/L</td>
<td>0.8 g/L</td>
<td>22%</td>
</tr>
<tr>
<td>Valerate, C5</td>
<td>4.1 g/L</td>
<td>0.6 g/L</td>
<td>16%</td>
</tr>
</tbody>
</table>

Table 5.2: Results of the shake flask study on individual VFAs

5.3.2. **Lipid Profile while Growth on Individual VFAs – Production of Odd Chain Fatty Acids**

The fatty acid profile of the propionate and valerate flasks indicates the production of odd chain fatty acids, chiefly C17 and C17.1, as shown in Fig. 5.5. The fraction of the odd chain fatty acids, especially C17.1 is significant (~40%) for the propionate flask. This was expected according to the literature reported on the production of odd-chain fatty acids during growth on propionate (Ingram et al. 1977; Emmanuel 1978).
Figure 5.5: Lipid profile on the acetate and propionate shake flasks
Figure 5.6: Difference in fatty acid synthesis products in the cases of acetate and propionate

The mechanism of fatty acid synthesis and elongation gives some insight into the possibility of formation of odd or even chain fatty acids. In the case of acetate, the first step is the production of malonyl CoA from acetyl CoA. The elongation proceeds in the following way: a) to an initial acetyl ACP molecule (2 carbon), a malonyl ACP (3 carbon) gets added (the CoA gets swapped for ACP through a transacylation reaction) b) The reaction produces acetoacetyl ACP (4 carbon) and one CO₂ is lost c) After a series of reactions, acetoacetyl ACP finally gets converted to butyryl ACP (4 carbon) d) Next, another malonyl ACP gets added to butyryl ACP, however, one CO₂ is lost again in the reaction, and the final product is a 6-carbon compound. This cycle keeps continuing until the elongation process stops. Hence, one always obtains even chain fatty acids on acetate (and by the similar logic, on butyrate).

The case for propionate is slightly different. First, propionate can be metabolized through the methyl citrate cycle to form pyruvate which can get converted to acetyl CoA. Hence, in this case, both acetyl CoA and propionyl CoA can act as primers. Moreover, the addition of carbon can
occur through both malonyl CoA (formed from acetate) or through methyl malonyl CoA (formed from propionate). The cases where odd chain fatty acids can be formed are the following: a) propionate is the primer and malonyl CoA gets added odd or even number of times b) propionate is the primer and methyl malonyl CoA gets added an even number of times) c) acetate is the primer and methyl malonyl CoA gets added an odd number of times. Of course, even chain fatty acids would be formed in the rest of the cases. As a result, one obtains a mixture of odd and even fatty acids on propionate and valerate. In the case of valerate though, the percentage abundance of (C17+C17.1) is lower than in the case of propionate (17% as opposed to 43%). 1 carbon mole of valerate produces 0.4 carbon mole of acetate and 0.6 mole of propionate through the fatty acid degradation. Thus, the fraction of propionate available from 1 carbon mole of valerate is lower than that from 1 carbon mole of propionate. Hence, the percentages of odd chain fatty acids are lower for valerate as compared to propionate.

**Butyrate**

<table>
<thead>
<tr>
<th>Butyrate</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>c16</td>
<td>9%</td>
</tr>
<tr>
<td>c16.1</td>
<td>5%</td>
</tr>
<tr>
<td>c17</td>
<td>0%</td>
</tr>
<tr>
<td>c17.1</td>
<td>0%</td>
</tr>
<tr>
<td>c18</td>
<td>11%</td>
</tr>
<tr>
<td>c18.1</td>
<td>69%</td>
</tr>
<tr>
<td>c18.2</td>
<td>6%</td>
</tr>
</tbody>
</table>
5.3.3. Shake Flask Experiment – Mixture of VFAs

The anaerobic degradation of MSW yields a mixture of VFAs as described in Sans et al., 1995. Therefore, if MSW is to be considered a potential source for VFA production, we would have to demonstrate growth on mixture of VFAs. Having established the growth of *Y. lipolytica* on individual VFAs other than hexanoate, the next step was to test growth on a mixture of VFAs. MSW degradation does not usually produce hexanoate and therefore, the lack of growth on hexanoate was not a matter of concern anymore (Sans et al., 1995, Traverso et al., 2000). Before conducting a bioreactor study on a mixture of VFAs, it was considered prudent to run shake flask experiments with varying ratios of VFA and total VFA concentration. The results of the shake flask experiment would be useful in guiding the medium composition and feeding strategy for the subsequent bioreactor study.

The OD trends of all nine flasks suggest that *Y. lipolytica* does not demonstrate any diauxic growth phenomenon on a mixture of VFAs. The OD trends of the ‘b’ flasks are shown in Fig.5.8.
Thus, it can be concluded that the microbe does not differentiate between the VFAs during growth. The VFA consumption trends of six representative flasks are shown in Fig. 5.9 and Fig 5.10. According to the naming convention being followed, the 'p' set of flasks had the same starting concentration of acetate and propionate. It can be clearly inferred from Fig. 5.9 that propionate is consumed at a much slower rate than acetate and moreover, the nature of the trends remains the same irrespective of the total VFA starting concentration. Fig. 5.10 displays the consumption profiles for the flasks with the same starting concentration of 0.4 carbon M. The plots provide an estimate of the relative rates of consumption of other VFAs with respect to acetate. It can be concluded from the plots that the rates of VFA consumption with respect to acetate follows the order: butyrate>valerate>propionate.

![B flasks](image)

**Figure 5.8:** OD trends in the mixed shake flask study - 'b' flasks (4:1:4:2 of C2:C3:C4:C5)
Figure 5.9: VFA consumption profiles in the ‘p’ set of flasks

Figure 5.10: VFA consumption profile for the flasks with an initial VFA concentration of 0.4 carbon M

The results of the lipid analysis performed for the final time point of each of the nine flasks is shown in Table 5.3. In conclusion, *Y. lipolytica* was able to metabolize up to 0.8 carbon M of
VFAs (equivalent to 20 g/L of an individual VFA) and produce lipids in each of the p, b and v flasks. The ‘b’ set of flasks seemed to display higher lipid content values and the highest lipid content of 33% among the nine flasks was obtained in the b-0.8 flask.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lipid titer (g/L)</th>
<th>Lipid content (%)</th>
<th>Overall lipid yield (g lipid/ carbon mol in VFA)</th>
<th>Overall lipid yield (g lipid/ g VFA) assuming 0.4 carbon M= 10 g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-0.4</td>
<td>0.59 g/L</td>
<td>15%</td>
<td>1.76 g/mol</td>
<td>0.07 g/g</td>
</tr>
<tr>
<td>b-0.4</td>
<td>0.85 g/L</td>
<td>20%</td>
<td>2.42 g/mol</td>
<td>0.10 g/g</td>
</tr>
<tr>
<td>v-0.4</td>
<td>0.61 g/L</td>
<td>15%</td>
<td>1.73 g/mol</td>
<td>0.07 g/g</td>
</tr>
<tr>
<td>p-0.6</td>
<td>1.12 g/L</td>
<td>23%</td>
<td>2.08 g/mol</td>
<td>0.08 g/g</td>
</tr>
<tr>
<td>b-0.6</td>
<td>1.43 g/L</td>
<td>26%</td>
<td>2.71 g/mol</td>
<td>0.11 g/g</td>
</tr>
<tr>
<td>v-0.6</td>
<td>1.15 g/L</td>
<td>21%</td>
<td>2.11 g/mol</td>
<td>0.08 g/g</td>
</tr>
<tr>
<td>p-0.8</td>
<td>1.84 g/L</td>
<td>31%</td>
<td>2.52 g/mol</td>
<td>0.10 g/g</td>
</tr>
<tr>
<td>b-0.8</td>
<td>2.17 g/L</td>
<td>33%</td>
<td>2.99 g/mol</td>
<td>0.12 g/g</td>
</tr>
<tr>
<td>v-0.8</td>
<td>2.06 g/L</td>
<td>31%</td>
<td>2.78 g/mol</td>
<td>0.11 g/g</td>
</tr>
</tbody>
</table>

Table 5.3: Lipid analysis results of the mixed VFA shake flask study

5.3.4. Bioreactor Run on Dilute Mixed VFA Feed

The goal of the bioreactor study was to evaluate growth and lipid production on dilute mixed VFAs and compare the results with those obtained in the dilute acetate run discussed in chapter 4. In this run, similar to the dilute acetate run, an acid feed was used for pH control. A 1 carbon M solution of 1:1 acetic and valeric acid was used as acid feed. The results obtained have been shown in Table 5.4. It was very encouraging to note that the lipid titer, content and productivity
were very similar across the dilute acetate and dilute mixed VFA runs. The strain behavior was thus reproduced on a mixture of VFAs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dilute acetic acid feed (30 g/L)</th>
<th>Mixed VFA feed (25 g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final lipid titer (g/L)</td>
<td>46</td>
<td>41</td>
</tr>
<tr>
<td>Final lipid content (%)</td>
<td>59</td>
<td>61</td>
</tr>
<tr>
<td>Overall lipid productivity (g/L/h)</td>
<td>0.27</td>
<td>0.24</td>
</tr>
<tr>
<td>Overall lipid yield (g/g VFA)</td>
<td>0.16</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Table 5.4: Comparison of the results of bioreactor runs on dilute acetate and dilute mixed VFAs

Figure 5.11: Bioreactor trends of the dilute mixed VFA run

The plot of lipid content, C/N ratio, and the nitrogen concentration in the reactor is shown in Fig. 5.11. The C/N ratio increases initially which is desirable from the point of view of lipid accumulation. However, after the 72h mark, the nitrogen is maintained at a constant level which
helps keep the C/N ratio stable between 70 and 100. This can be attributed to the fact that a non-zero nitrogen concentration was maintained in the second stage of feed. This was purposefully done so as to prevent the C/N ratio in the reactor from going too high (>200) and in turn prevent citrate accumulation as extremely high C/N ratios are known to encourage citrate accumulation in the reactor (Ratledge and Wynn, 2002). Not surprisingly, less than 0.5 g/L of citrate was detected in the reactor in this run.

5.3.5. Bioreactor Run on Perturbed Mixed VFA Feed

The goal of this study was the following: a) control the VFA titer in the reactor through the implementation of the flow rates as determined by the model b) create perturbations in the VFA composition in the reactor and observe changes if any, in the final biomass and lipid production.

As shown in Fig. 5.12., the VFA concentration in the reactor (shown in carbon M, e.g. 1 acetate M = 2 carbon M) decreased initially from 0.8 carbon M to 0.3 carbon M in the first 24 hours. From the 34 hour mark, the model was implemented in maintaining the VFA titer in the reactor at a constant and low level of 0.06 carbon M. However, in the period from 40 to 120h, the model was not as successful in controlling the VFA titer at 0.06 carbon M and it often exceeded 0.1 carbon M. This could be attributed to limitations of the model and the fact that measurements could not be taken more frequently. After 120h, the VFA titer was successfully maintained at 0.06 carbon M.
Figure 5.12: VFA titer in the bioreactor (perturbed VFA feed) over the course of the run

Fig. 5.13 shows the trend of the percentage composition of various VFAs over the course of the bioreactor run. As intended, the percentage compositions of all VFAs show significant variations during the run. After 58 h, when the feed composition was first changed, we observe decrease in the acetate and valerate percentages and increase in propionate and butyrate. Similarly, after 120 h, another perturbation in the feed caused propionate to decrease and valerate to increase. The actual concentrations in the acid feed are provided below in Table 5.5.

<table>
<thead>
<tr>
<th>Duration</th>
<th>C2 concentration in feed (carbon M)</th>
<th>C3 concentration in feed (carbon M)</th>
<th>C4 concentration in feed (carbon M)</th>
<th>C5 concentration in feed (carbon M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-58h</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>58-119h</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>119-168h</td>
<td>0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 5.5: VFA concentrations of the acid feed in the perturbed VFA feed run
Despite the fluctuations in VFA composition, the biomass trend obtained in the perturbed VFA feed run was approximately similar to the biomass trend in the previous run with no perturbations, as shown in Fig. 5.14. In addition, lipid content of 60% was obtained with an overall lipid productivity of 0.21 g/L/h.
Figure 5.14: Comparison of the biomass trends over the two bioreactor studies on dilute mixed VFAs

5.3.6. Model in Action and Limitations of the Model

The model was successful in controlling the VFA titer in the bioreactor for the most part at a value below 0.1 carbon M (equivalent to 2.5 g/L). However, the titer was found to oscillate and did not always converge to the desired value. This limitation of the model can be better explained by analyzing the two cases shown in Table 5.6 where the predicted flow rate of acid stream (output from the model) and the observed flow rate have been listed for two specific time intervals.

Depending on the information fed to the model such as the specific growth rate and the OD measurement at $t_n$, the model predicts the acid flow rate $F_1$ for the future time interval and this value helps in determining the salt feed and permeate flow rates, $F_2$ and $F_3$. 
Time duration | Predicted flow rate, $F_1$ | Observed flow rate, $F_1$
--- | --- | ---
34-46h | 98 mL/h | 84 mL/h
143-151h | 48 mL/h | 49 mL/h

Table 5.6: Comparison of the predicted and observed acid feed flow rate

As discussed earlier, prediction of $F_1$ involves projecting the consumption rate for the future time interval, which in turn depends on the specific growth rate being used for the calculation. We assume the specific growth rate to stay constant over the next interval and this assumption is very critical for the precise estimation of $F_1$. As shown in Table 5.7, the observed specific growth rate varied much more between the 34h and 46h measurement than between the 143h and 151h measurement.

<table>
<thead>
<tr>
<th>Time of measurement</th>
<th>34h</th>
<th>46h</th>
<th>143h</th>
<th>151h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific growth rate, $\mu$</td>
<td>0.019</td>
<td>0.012</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 5.7: Specific growth rate of the culture at specific points during the bioreactor run

As a result, the model’s prediction of the acid flow rate $F_1$ is much farther off the actual flow rate in the 34-46h period as compared to the 143-151h period. Hence, it can be concluded that accurate estimation of the specific growth rate is vital for the model to operate effectively, which in turn would help minimize drift and lead to better control of the VFA titer.

Currently, the model suffers from the following limitations in its operation:

- The assumption that $\mu_n = \mu_{n+1}$ is not valid for time intervals as long as 12h. A solution would be to use a lower time interval to make the assumption more realistic and hence, reduce deviations.
• Lack of a model for the specific growth rate, $\mu$ of $Y.\ lipolytica$ on VFAs. Ability to accurately determine the specific growth rate for the future time interval would help better the estimates of the model.

• The biomass measurements cannot be obtained on a very short time scale (such as half hour). As a result, OD measurements were used as a proxy.

• There is a small but finite delay between the measurement process and the updating of the flow rates. The goal should be to lower the delay as much as possible.

5.3.7. Permeate Loss and Improved VFA Titer Control in Perturbed VFA Feed Run

The hollow fiber membrane was used in a similar fashion as in the case of the dilute acetate run for the purpose of cell recycle. In the perturbed VFA feed run, the VFA concentration in the reactor was maintained at around 2 g/L by setting the pump flow rates at the values determined by the model. This ensured that the permeate stream contained a very low concentration of VFAs. As a result, the loss of VFA through the permeate was brought down to 8%.

5.4. Discussion

VFAs have generated considerable interest in recent times as a potential substrate for oleaginous microbes. There have been reports on growth of oleaginous microbes such as $Y.\ lipolytica$, Cryptococcus on VFAs in literature (Fei, Chang, Shang, Choi, et al. 2011; Fei, Chang, Shang & Choi 2011; Fontanille et al. 2012). An economic analysis to estimate the price of biodiesel obtained using VFAs as a feedstock vis-à-vis glucose has been shown in Fei et al., 2011. Using glucose, biodiesel could be obtained at $3.80/L$ whereas using VFAs as substrate, biodiesel could be obtained for as cheap as $0.30/L$ assuming VFAs are available at a negative cost. Such analysis highlights the importance of studying and evaluating the potential of VFAs as substrates for microbial lipid production.
The bioreactor run on mixture of dilute VFAs was modeled on the dilute acetate run discussed in chapter 4. The goal was to achieve comparable growth and lipid production on VFAs as was obtained using dilute acetate. The results are indeed alike with respect to parameters such as overall lipid yield, overall lipid productivity as well as lipid content. Moreover, there were certain aspects of the bioprocess which have been improved upon in the current system. In the dilute acetate run, the sodium acetate addition was not automated and furthermore, the permeate flow rate was controlled manually. Such handling of the system caused the acetate concentration to oscillate between concentrations of 2-10 g/L, which was not desirable from the point of view of reducing losses in the permeate. To resolve the above shortcomings, we developed a model of the bioprocess which was based on the premise that the VFA concentration in the reactor would be maintained at a known, low concentration. The calculations performed using the model provided a continuous feeding rate of a salt solution of VFAs as well as the corresponding permeate pump flow rate. By implementing the feeding and flow rates generated by the model, we were successful in removing any manual intervention and in addition, were able to maintain the VFA titer in the reactor at a constant level of 2 g/L. The low VFA titer in the reactor had a direct impact on lowering the losses in the permeate.

In the current study, the loss of carbon through permeate was brought down to as 8% - much lower compared to the 16% obtained in the study using dilute acetate. However, in this run, there was yet another source of carbon loss. It is speculated that the loss could be associated with the stripping of the VFAs, especially valerate, from the reactor due to the high aeration rate. Maximizing the agitation whilst minimizing the aeration rate could be adopted as a useful strategy to minimize such loss.
In the shake flask study with individual VFAs as well as mixture of VFAs, it was observed that growth on propionate was impaired. One of the possible reasons could be the production of methyl citrate, which is one of the metabolites generated during propionate metabolism. It is reported to be an inhibitor of TCA cycle enzymes such as citrate synthase, aconitase and isocitrate dehydrogenase (Cheema-Dhadli et al. 1975). The TCA cycle generates precursors for biomass production as well as is involved in the production of energy equivalents in the form of NADH, for growth and lipid production. Hence, it is quite expected that the inhibition of the TCA cycle by propionate would affect growth adversely.

Our run is perhaps one of the only high cell density bioreactor studies on *Y. lipolytica* performed exclusively on a mixture of VFAs. It is important, though, to evaluate the characteristics of the run in comparison to other experiments in literature on VFAs. One of the few papers in literature describing lipid accumulation in *Y. lipolytica* on VFAs is Fontanille et al., 2012. The experiments in the paper study growth of *Y. lipolytica* on individual VFAs in small scale bioreactors, and also on glucose + mixed VFAs in a two-stage feeding approach. On individual VFAs in 500 ml bioreactors, consumption of 8 or 12 g/L of VFAs was reported and lipid titers of 1-2 g/L were obtained at a lipid content of 30%. We have obtained similar results in 250 ml shake flask studies, where the growth conditions are far less regulated than in a bioreactor.

The two-stage glucose + mixed VFA run was performed in a 7L fermenter in which the microbes are first grown on glucose to develop non-lipid biomass and the substrate is then switched to mixed VFAs to test lipid production. In spite of utilizing exclusively a mixture of VFAs, we have achieved significantly higher lipid titer (41 g/L compared to 16 g/L) and comparable results with regard to lipid yield and productivity in our bioreactor study. The use of
an engineered strain enabled us to attain much higher lipid content (60%) compared to their study (40%). Fei et al., in 2011 reported growth of *Cryptococcus albidus*, another oleaginous microbe on mixture of VFAs. In shake flask studies, the lipid content and lipid yield obtained are comparable. A similar study to Fontanille et al., 2012 involving a two-stage feeding of glucose and mixed VFA was also performed. The yield and lipid content attained are comparable to our results; however, the lipid productivity obtained in their study is much lower (0.1 g/L/h) than in ours (0.25 g/L/h).

To entertain the scheme of a two-step biodiesel production bioprocess with MSW as the primary feedstock, we would have to test the robustness and flexibility of *Y. lipolytica* in real-world environments. In the current study, the VFA feed was prepared using pure VFAs and hence, the feed was expected to be free of impurities. In addition, the feed was modified by adding nitrogen so as to promote cell growth. Information on the composition of real VFA streams obtained from acidogenesis of MSW would be vital as it would help decide whether the streams would need any processing, such as addition of nitrogen, before it can be fed to the bioreactor. Moreover, such streams might also contain various inhibitory chemicals which might negatively affect growth. Literature suggests that *Y. lipolytica* is quite resistant to inhibitory compounds to the point that it has been used for bio-remediation purposes – olive mill waste water streams containing high fractions of phenols which are known to be highly inhibitory to microbial growth (Lanciotti et al. 2005). In addition, such streams contain phosphates, metals and the BOD and COD concentrations are as much as 200 times higher than conventional municipal sewage (Cossu et al. 1993). The ability of *Y. lipolytica* to grown in such harsh conditions reveals the resilience of *Y. lipolytica* to harmful compounds. Hence, it can be speculated that with some basic processing, such streams could be considered suitable as a feed
to the bioreactor. Therefore, in order to better simulate real-world situations, it will be worthwhile to consider additional bioreactor studies testing various other perturbation schemes in the feed.

It is to be noted that even with the VFA concentration maintained around 2 g/L in the current bioreactor study, similar results with respect to growth and lipid accumulation were obtained as compared to the dilute acetate run where the acetate concentrations were much higher. Hence, we can conclude that the system is flexible enough to deal with low substrate concentrations without inhibiting growth. By sampling the bioreactor more frequently, one could better estimate the desired flow rates using the model and in turn, maintain the VFA levels reliably at even lower levels which would eventually decrease the losses through the permeate stream.

Finally, it is vital to perform a thorough analysis of the economics of the two-stage bioreactor system to evaluate the commercial feasibility of the system. The economic analysis shown by Fei et al., 2011 investigated only the second stage of the system – VFA to biodiesel. Moreover, the analysis was performed in the context of South Korea. We are in the process of a comprehensive evaluation of a two-stage system which converts MSW to biodiesel in the setting of developed nations such as US and Germany as well as emerging nations such as India. With further improvements in lipid yield obtained through genetic engineering of the microbe, and bioprocess engineering, one may be able to bring down the costs of biodiesel production to enable its sale at competitive or even lower prices than petroleum diesel. Moreover, with MSW landfills being the third-largest source of anthropogenic methane emissions in US, diverting MSW for biodiesel production would significantly aid in lowering methane emissions (US EPA LMOP, 2012). Thus, in addition to being low-cost, the biofuel production process would provide environmental benefits and prevent precious land to be wasted off as landfills.
5.5. Conclusions

The objective of the study was to explore and investigate VFAs as potential substrates for biodiesel production in an engineered strain of \textit{Y. lipolytica}. We had demonstrated growth and lipid production on acetate in chapter 4. The results of the previous study served as a basis for comparison. First, it was examined if growth can be sustained on various VFAs in shake flask experiments. Apart from hexanoate, growth was observed on all VFAs. In addition, the lipid content on butyrate was found to be comparable to that on acetate. The VFA consumption trends while growth on mixtures of VFAs seem to indicate that butyrate is a more preferred substrate compared to valerate and propionate. Propionate was found to impair growth both in the case of individual VFA study as well as on a mixture of VFAs. Using the dilute acetate run of the previous study as a model, a high cell density fed-batch bioreactor culture of \textit{Y. lipolytica} was successfully obtained using a mixture of dilute VFAs as the feed. A lipid titer of 41 g/L was obtained at an overall lipid productivity of 0.25 g/L/h and an overall lipid yield of 0.17 g/g. The growth and lipid results obtained on dilute acetate were essentially replicated in the current study. Moreover, variations were tested out with perturbations in the feed composition and similar results were obtained. More importantly, a model was implemented to help maintain the VFA titer at a low level of 2 g/L which in turn, helped bring down the permeate losses to as low as 8%. It is one of the first studies of a high cell density fed batch system of \textit{Yarrowia lipolytica} exclusively on VFAs. However, further runs on varying feed composition and simulating real-world acidogenic streams in the feed, as well as a comprehensive economic analysis are necessary to determine the commercial viability of a two stage bioprocess from MSW to biodiesel.
Chapter 6

Conclusions and Recommendations
6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Introduction

We have been successful in fulfilling the thesis objectives as outlined in Chapter 1. The field of exploring microbial growth on VFAs for biofuel production is fairly new and therefore, merited investigation, especially given that VFAs could be obtained at a low cost from sources such as municipal solid waste (MSW). Assessing the potential of the engineered microbe to attain high cell densities on VFAs is the first step to realizing a two-step biodiesel production process on MSW. The studies on bioprocess engineering have yielded some preliminary insight into the feasibility of this project. We have been successful in developing a fed-batch process which has the ability to utilize dilute streams of VFAs and generate high lipid titers. We have been able to establish that the microbe can indeed tolerate such toxic compounds to reasonably high concentrations (20 g/L) and moreover, can withstand sudden changes in the feed composition. The results of the current work create a lot of scope for future work directed towards further refining and scaling the bioprocess to make it suitable for consideration on a commercial scale.
6.2. Summary of Results

Baseline run
- 60% lipid content on acetate

Fed-batch run using concentrated acetic acid (CFB)
- Lipid results - titer of 51 g/L at productivity of 0.26 g/L/h; final lipid content - 61%

Fed-batch run using dilute acetic acid (DFB)
- Lipid results - similar to CFB
- Hollow-fiber used for cell recycling
- Permeate loss of 16% due to relatively higher concentration of acetate in bioreactor
- Carbon balance met within 10%

Shake flasks – VFAs (individual and mixture)
- Potential substrate for lipid accumulation (other than hexanoate)
- Can tolerate upto 0.8 carbon M – propionate is most inhibitory

Fed-batch run using dilute mixed VFAs
- Lipid results – similar to DFB

Fed-batch run using dilute mixed VFAs with perturbation
- Model implementation helped maintain VFA titer at 2 g/L
- Permeate losses brought down from 16 to 8%
- Lipid content of 60%, productivity lower than mixed VFAs run
6.3. Recommendations for Future Work

Molecular Biology
- Better lipid accumulating strains which can accumulate lipids to higher lipid content
- Improving lipid productivity

Bioprocess development
- Model improvements – modeling $\mu$
- Testing other perturbations – establish robustness of system

Two stage integration
- MSW to VFA production - upstream bioreactor
- Evaluate the commercial feasibility of the project and develop a two-stage working prototype
Chapter 7

7. CAPSTONE STUDY

7.1. Introduction

The Capstone study aims to understand and analyze options for organic waste management through the lens of commercial viability. Waste management is an issue which bothers governments and municipalities both in the emerging and developed countries. There have been attempts to solve the crisis by creating processes which create value out of trash. Non-biodegradable waste components (plastics, clothes and other non-fermentable material) are being recycled and reused in various forms, one of them being refuse derived fuel (RDF) where plastics are compressed and used alongside traditional sources of fuel in coal power plants and cement kiln industry.

![Image of RDF bricks](image.jpg)

Figure 7.1: RDF (Refuse Derived Fuel) bricks obtained from compaction of recycled plastics

Organic waste refers to fermentable (or biodegradable) fraction of trash; this includes food waste, kitchen waste, agricultural waste, and hotel and canteen waste. Management of organics has traditionally centered on composting or biogas production. Both these processes are established, well-studied and widely implemented\(^2\). Nonetheless, there are other novel approaches to organics recovery which might be worth exploring. The ultimate objective of this study is to estimate the value created through all approaches for organics recovery — new and old, and determine the optimal approach for trash-to-cash. One of the novel approaches for value
creation from organics follows from the previous chapters in this thesis – a two-stage organics-to-biodiesel system where the first stage would involve the decomposition of food waste (and other biodegradable waste) into VFAs (volatile fatty acids), and the second stage would involve conversion of the dilute streams of VFAs into concentrated oil droplets within an oleaginous platform, *Y. lipolytica*. However, the previous chapters do not dissect this process from a commercial viewpoint.

For the purposes of this report, we focus on organics management methods which convert organics to fuels or valuable chemicals. We would be analyzing three approaches for waste valorization in this Capstone study – biogas production, biodiesel production, and lactic acid production. Moreover, it is vital to understand the characteristics of the waste generation and collection process in different countries to better comment on the feasibility of the approach.

### 7.2. Characteristic of Organics Generation in India and US

Developed and emerging markets differ highly in their municipal solid waste (MSW) attributes such as percentage of organics in total MSW and per capita MSW generation\(^4\). This difference can be attributed to the socio-economic differences, such as average income level and urbanization\(^4\). Therefore, in order to better implement a particular organics management strategy at a location, it is useful to understand the features of organics generation in those respective environments. We chose US and India to represent the developed and emerging markets respectively.

US produced 250 million tons of MSW in 2011 of which close to 15% was food waste – 35 million tons\(^5\). US’s MSW generation has declined lately though and is at best estimated to grow by under 10% until 2025. After MSW recovery through recycling, 164 million tons of MSW
were discarded in 2011 of which the largest component was food waste\(^5\). So we can expect that a significant amount of carbon (in food waste) is currently being left to decay in landfills, and generate harmful greenhouse gases such as methane which is 20 times more potent than carbon dioxide on weight-to-weight basis\(^6\). Given the low recovery of food waste, it is therefore not surprising that landfill generated methane was the third largest source (18%) of methane emissions in the USA in 2012\(^6\). Moreover, tipping fees associated with waste hauling have been rising and average at $44/ton in USA, with states such as Massachusetts having as high a tipping fee as $72/ton\(^7\). Thus, landfilling waste is not only harmful from an environmental perspective but is also expensive. However, with recent interest in effective waste management and recycling, there have been mandates passed in several states and cities to divert food waste from landfills. As an example, effective October 1, 2014, as per the directive from the MassDEP (Department of Environmental Protection), no institution or business in Massachusetts generating more than 1 ton of food waste per week can direct the waste to a landfill and would instead have to process the waste through green strategies such as composting, or biogas production\(^8\). This and several such mandates in states such as California, Vermont, and Washington\(^9\) are increasingly driving the need for effective and cheap waste management solutions.

India produces a different story. The total MSW generated in urban India in 2012 was 100,000 tons per day, much lower than the figure for US; however, the amount is estimated to triple by 2025\(^4\). Moreover, the percentage of organics in MSW is much higher in India – 35%, as shown in the figure below.
Despite the Indian government’s policies such as MSW Rules enacted in 2000 to implement better waste management practices, there has been very little done to address the waste disposal issue. The problem has escalated to the point of unrest and protests in various parts of the country over the dumping of waste in landfills close to residential areas shown below in the form of news clips.

Figure 7.3: Newsclips demonstrating the protests of residents in Pune and Bangalore (major cities in India) over waste disposal in landfills close to their homes (The Times of India)
MSW collection efficiency ranges between 50-70% with the rest of the waste being left on roadsides\(^1\). Moreover, the organic fraction of the MSW left on roadsides becomes a source for infectious vectors and the situation ultimately leads to a variety of disease and illness\(^{10}\).

It is thus interesting that the US and India have very different and unique environments to each other, yet both countries need sustainable, eco-friendly and commercially viable approaches for organics management. The next section investigates the options available for organics management in detail.

7.3. Approaches for Waste Valorization

7.3.1. Biogas Production

7.3.1.1. Introduction

Biogas is produced through the process of anaerobic digestion. Anaerobic digestion refers to the biological decomposition of organic matter under anaerobic (no oxygen) condition which leads to the production of methane (\(\text{CH}_4\)) and other gases such as carbon dioxide (\(\text{CO}_2\))\(^{13}\). The anaerobic digestion of organic material is accomplished by a consortium of microorganisms working synergistically. The methane content of biogas is typically 60% (by volume). The digestion occurs in cylindrical structures built above ground or even below ground. The final biogas produced can be used as a fuel for cooking and heating purposes. The fuel can also be used to generate electricity through steam-powered turbines. The digestate left at the end of the digestion is effective as a fertilizer owing to the presence of high amounts of nitrogen, phosphorus, and potassium\(^{11}\). The figure below shows the schematic of the biogas generation process.
The process of biogas production proceeds via a four-step conversion\(^1\) – in the first step, complex organics in food waste (carbohydrates, proteins, fats) are hydrolyzed (by hydrolytic microbes or through bio-chemical means such as extracellular enzymes) into simpler forms (sugars, amino acids, fatty acids) which can be metabolized by the microbes in the next stage; the second step involves acidogens (a class of bacteria) which convert the simpler organics into organic acids such as lactic acid and volatile fatty acids such as acetic acid, propionic acid and butyric acid. The third step involves acetogens which convert the higher chain organic acids into acetic acid and finally, another group of bacteria called methanogens feed on acetic acid and produce methane.

Anaerobic digestion has gained traction in various countries and it is operated at various scales (50kg-100 ton per day)\(^2,13\). The global market for biogas was $17.3 billion in 2011 and is projected to double by 2022\(^14\). Thus, there is an established market for biogas. A typical 5 ton per day digester in India is shown in the figure below.
7.3.1.2. **Material Flow**

To understand the value created through this process, we need to first know the stoichiometry of the conversion of organics to biogas. The typical stoichiometry of biogas production from organics reported by biogas installing companies both in India as well as in the developed world is – 1 ton of organics sludge (with 20% solids content) yields $70 \text{ m}^3$ of biogas\textsuperscript{15,16}. The by-product in the process is the digestate which is used as a fertilizer. The biogas produced is 60% methane and the rest is CO$_2$.

![Diagram](image_url)

*Figure 7.7: Input and Output streams in biogas production*
7.3.1.3. Economic Value

The economic value from the process can be estimated by computing the value created from the two product streams – biogas and compost. We calculate the amount of methane available in biogas, and then price it according to CNG prices\textsuperscript{17} to determine the value of the biogas as a fuel (details provided in appendix). We used typical prices of manure\textsuperscript{18} to determine the value of the digestate stream.

<table>
<thead>
<tr>
<th>Product Stream</th>
<th>Amount produced (kg)</th>
<th>Pricing ($/ton)</th>
<th>Value created</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biogas</td>
<td>70m\textsuperscript{3} of biogas (60% methane) = 27 kg of methane at STP\textsuperscript{*}</td>
<td>$222</td>
<td>$6</td>
<td>Fuel</td>
</tr>
<tr>
<td>Digestate</td>
<td>90 kg</td>
<td>$50/ton</td>
<td>$4.5</td>
<td>Fertilizer</td>
</tr>
</tbody>
</table>

Table 7.1: Breakdown of the value created from the respective product streams during biogas production (* - Standard Temperature and Pressure)

Thus, the total value created from this process is $10.5/ton of wet organic waste (20% solids).

7.3.2. Biodiesel Production

7.3.2.1. Introduction

Fuel production from organic waste extends beyond the purview of gaseous fuels to include liquid fuels such as biodiesel, which can substitute diesel in applications ranging from transportation to operation of cell phone towers and farm operations\textsuperscript{19}. The concept of waste-to-biodiesel relies on a two stage fermentation process where in the first stage the fermentable material is converted into volatile fatty acids through anaerobic digestion, and in the second stage, volatile fatty acids are converted into lipids through an aerobic fermentation process using a lipid-accumulating microbial platform. The schematic of the process is shown below.
Organics to VFAs has been reported in literature\textsuperscript{20,21} and it is similar to the biogas production process where instead of digesting the organics completely to methane, the process is stalled at the volatile fatty acids stage. The research area of converting VFAs-to-lipids is relatively novel and there have been studies reported in recent literature\textsuperscript{22}. Apart from lipids, the system also produces the following by-products: manure from the anaerobic digestion step, and animal feed from the aerobic reactor (the microbial cell mass remaining after the lipids are extracted). The overarching goal of a waste-to-biodiesel system is to allow liquid fuel production from low-cost feedstock such as municipal solid waste and avoid dependence on feedstock such as soy and palm. Biodiesel has gained popularity in a number of regions in the world such as Europe and US, but in order to further increase its use and reduce dependence on ever-volatile petroleum diesel, there is a need to move away from feedstock such as soy and palm, and yet have a system with high conversion efficiency. Therefore, the microbial conversion of organics to lipids is a particularly interesting process.

The global biodiesel market is expected to grow from $8.6 billion in 2009 to $12.6 billion in 2014\textsuperscript{23}. The biodiesel market specifically offers immense opportunities in countries such as U.K., India, and China, as these regions have high diesel fuel prices and a large number of diesel fueled vehicles. Moreover, market growth is primarily dependent on the availability, quality, and yield of feedstock, as it accounts for 65\% to 70\% of the cost of biodiesel production. Thus, an abundant and low-cost feedstock such as organic waste would play a significant role in increasing the cost-competitiveness of biodiesel with petroleum diesel.
7.3.2.2. **Material Flow**

The stoichiometry of the biodiesel production process is obtained by combining the yield information of the two steps in the process. Lactic acid is assumed as the intermediate metabolite in this process. The yield for conversion of organics to lactic acid is 0.5 g VFA/(g solids in organics) (data not published) and the lactic acid to lipids step has been assumed to have a yield of 0.19 g/g. Since there was no literature data on the lipid yield on lactate, we used a correlation between the ratios of the observed and theoretical lipid yields on acetate and lactate to solve for the observed lipid yield on lactate – 0.19 g/g \((\text{observed/theoretical})_{\text{lactate}} = (\text{observed/theoretical})_{\text{acetate}}\). The experimentally determined lipid yield on acetic acid is 0.17 g/g (results in chapter 4 of thesis). The theoretical yield calculation for acetate (0.27 g/g) and lactate (0.3 g/g) are provided in the appendix of this thesis. Manure is assumed to be produced at similar yields as in the biogas process. A lipid content of 60% (obtained as per the results described in chapter 4 of thesis) was assumed to determine the non-lipid cell mass that would be produced concomitantly in the process and would be used as animal feed.

![Flowchart](Image)

**Figure 7.9:** Input and Output streams in lipid production
7.3.2.3. Economic Value

In this scenario, we have three distinct revenue streams – lipids, compost and animal feed. We assume all the lipids would be converted to biodiesel with efficiency close to 1, and then price the biodiesel using diesel prices so as to factor in the cost-competitiveness with diesel (details provided in appendix). The animal feed is priced using literature data – it sells for $360/ton.

<table>
<thead>
<tr>
<th>Product Stream</th>
<th>Amount produced (kg)</th>
<th>Pricing ($/ton)</th>
<th>Value created ($)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipids</td>
<td>19 kg</td>
<td>$950/ton</td>
<td>$18.1</td>
<td>Fuel</td>
</tr>
<tr>
<td>Non-lipid cell mass</td>
<td>13 kg</td>
<td>$360/ton</td>
<td>$4.7</td>
<td>Animal Feed</td>
</tr>
<tr>
<td>Digestate</td>
<td>90 kg</td>
<td>$50/ton</td>
<td>$4.5</td>
<td>Fertilizer</td>
</tr>
</tbody>
</table>

Table 7.2: Breakdown of the value created from the respective product streams during lipid production

Thus, the total value created through the biodiesel process is $27.3 per ton of wet organics (20% solids).

7.3.3. Lactic Acid Production

7.3.3.1. Introduction

The two approaches mentioned above were concerned with the conversion of organics into biofuels. This section investigates the conversion of organics into a high value chemical - lactic acid. Lactic acid, C₂H₄OHCOOH, is a weak organic acid which is a metabolite obtained during the anaerobic digestion of organic waste – typically its production precedes the production of VFAs in the broth. The following figure shows the schematic of lactic acid production from organic waste:
Lactic acid is a high value chemical with price varying from $1300-$2300/ton\textsuperscript{28}. The U.S. is the largest consumer of lactic acid in the world, accounting for 45% of the global $22 billion per year market\textsuperscript{29}. Lactic acid has applications in the food industry as an acidity regulator, preservative and flavoring agent, and in other industries such as pharmaceuticals, consumer goods, and recently in biodegradable plastics using its polymeric form – Poly Lactic Acid (PLA)\textsuperscript{30}. Due to its amazing array of uses, the lactic acid market is estimated to grow at an annual growth rate of 20% until 2019\textsuperscript{31}. Currently, lactic acid is produced on an industrial scale via fermentation of sugars. Dependence on sugars is undesirable as it is an expensive feedstock ($500/ton) compared to organic municipal solid waste which might be available at a zero or even negative cost\textsuperscript{7}.

7.3.3.2. Material Flow

A lactic acid yield of 0.5 g/g-of-dry-organics (or 0.1 g/(g wet waste with 20% solids)) has been assumed (data not published) for the analysis shown below. In this case too, a concomitant manure stream is produced with the lactic acid stream. The process flow would therefore look like the following:
7.3.3.3. **Economic Value**

Given that lactic acid is being derived from municipal solid waste, we assume a lower estimate of the price of lactic acid – $1300/ton. The manure stream is valued in the same way as shown before for the biogas and biodiesel case.

<table>
<thead>
<tr>
<th>Product Stream</th>
<th>Amount produced (kg)</th>
<th>Pricing ($/ton)</th>
<th>Value created ($)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic Acid</td>
<td>100 kg</td>
<td>$1300/ton</td>
<td>$130</td>
<td>Chemical</td>
</tr>
<tr>
<td>Digestate</td>
<td>90 kg</td>
<td>$50/ton</td>
<td>$4.5</td>
<td>Fertilizer</td>
</tr>
</tbody>
</table>

Table 7.3 Breakdown of the value created from the respective product streams during lactic acid production

The total value created from the lactic acid process would be $134.5 per ton of wet organics (20% solids).

**7.4. Comparison of the Value Created**

Thus, with the three alternatives discussed in the previous section for organics management, it is now useful to compare the value created from the three processes to determine the optimal approach to organics management.
The following graph compares the total value created from all revenue streams for the three cases under consideration:

Figure 7.12: Comparison of value created ($/ton of organics) from the three routes under analysis

As can be clearly seen, the lactic acid approach creates the highest value per ton of organics. For the purposes of this study, we do not delve into the cost side of each of the three options mentioned above. But with the knowledge of the process schematic, one can estimate the capital expenditure associated with the three processes. The biodiesel production process involves two reactors, and aerobic reactors typically consume more energy (owing to aeration and agitation requirements) during its operation than anaerobic reactors\(^3\)\(^2\)– hence, we can assume that the biodiesel approach would certainly be costlier to build and operate than the biogas and lactic acid approach. The other two approaches have very similar requirements from a capital standpoint as they both need anaerobic digestion chambers. It is to be noted, however, that the biodiesel approach might be optimal in certain environments where fuel is in great scarcity and a rather large economic incentive is given to production of alternative liquid fuels which could tilt the balance towards making the biodiesel process more valuable than the lactic acid process.
The lactic acid route apart from creating most value of the three routes also provides added flexibility because one could build and operate a lactic acid producing digester, and then in the future, depending on favorable policy incentives and fuel prices, decide to build the aerobic reactor next to the digester so as to switch the final product from chemical to fuel. The next section is dedicated to examining the case of lactic acid production from organics in a detailed manner.

7.5. Organics-to-Lactic Acid – Navi-Chem

We explore in detail the idea of organics conversion to lactic acid, and we would be referring to the technology as Navi-Chem. Navi means ‘new’ in Hindi (major language in India) and Chem refers to ‘chemical’. A novel approach is being pursued to produce a high value chemical; hence the business idea has been named as Navi-Chem.

7.5.1. Value Proposition

The objective of Navi-Chem is to extract the most value out of organic waste by converting the organics into a high-value chemical such as lactic acid. There is a growing demand for lactic acid fueled by the rise in use of bioplastics such as PLA (polylactic acid). Moreover, it is currently being produced from sugars – an expensive feedstock at $500/ton. Our technology would allow the production of lactic acid in a cost-effective and sustainable manner to meet this growing demand due to the low-cost nature of the feedstock. The following chart demonstrates the opportunity in the lactic acid industry:
Figure 7.13: Opportunity in the fast growing lactic acid market\textsuperscript{29,31}

Moreover, the value proposition goes beyond the economics to address two other concerns – environment and policy. The WARM Model provided on the US EPA\textsuperscript{33} website was used to determine the greenhouse gas emissions offset by to the diversion of organics from landfills. It is estimated that 755 tons of CO\textsubscript{2} equivalent are offset for every 1000 tons of food waste processed through our technology – in another way, 1 million tons of food waste processed would offset yearly CO\textsubscript{2} emissions from 150,000 cars. Now, Massachusetts alone produces nearly 1.1 million tons of food waste annually\textsuperscript{34}. Thus, Navi-Chem has the potential to produce a significant impact on the environment.
The other dimension of our value proposition is facilitating states such as Massachusetts to meet their landfill diversion mandates. Other states are following suit in implementing mandates for landfill diversion in the light of rising tipping fees and negative effects of landfill-generated methane\(^3\). Hence, this technology would enable state governments and municipalities to resolve their waste disposal challenges, and in the process, this could ensure Navi-Chem a reliable and steady supply of organics.

The issue of waste disposal is especially acute in developing countries such as India, which has poor waste management practices. The current systems in place in India will be further challenged in the future since waste generation rate is estimated to be almost three times the growth rate of that of the average nation in the world\(^4\). Moreover, the percentage of organics in the waste is also estimated to be very high in developing countries as shown in the section 1.1, and thus, Navi-Chem’s business will have great applications in an emerging country such as India.
Additionally, Navi-Chem can diversify into other essential commodities such as biodiesel which can be produced biochemically as an extension of the lactic acid production process (as shown in section 1.3.2). Alternative fuels are very much the need of the hour in emerging countries such as India which imports 80% of its oil\textsuperscript{36}. As an example, just from the 15 tons per month of waste produced at MIT, 315 liters of fuel a month can be produced (yields of 0.1 g lactic acid/g organics, and 0.19 g lipid/g lactic acid, biodiesel density of 0.9 g/ml\textsuperscript{37}), at a value of $220 ($2.8 per gallon\textsuperscript{38}) or 300 miles (mileage of 3.7 miles per gallon\textsuperscript{39}) for a city bus. A whole fleet of buses could be fueled by increasing the volumes of waste processed in Navi-Chem’s waste-to-fuel system. In short, Navi-Chem is a robust and complete solution to the organics disposal problem and it targets the whole portfolio of high-value chemicals and fuels.

7.5.2. Competitive Analysis

Navi-Chem has successfully tested the organics-to-lactic acid conversion process on a bench scale (2L reactors) in the laboratory of Prof. Greg Stephanopoulos in MIT Chemical Engineering. Current production of lactic acid starts with sugar, which can cost over $500/ton\textsuperscript{40}. When compared to the technology employed by Navi-Chem, where the feedstock costs are much lower and can be potentially negative, the advantage is very clear. The following table shows the production parameters such as titer (amount of lactic acid in unit volume of the reactor) and productivity (amount of lactic acid produced in unit time in unit volume of the reactor) of Navi-Chem in comparison to the traditional route of lactic acid involving glucose\textsuperscript{41}:
Table 7.4: Comparison of lactic acid titer (g/L) and productivity (g/L/h) between Navi-Chem’s technology and current sugar-based fermentation technology

The technology underlying Navi-Chem is currently the subject of a pending patent application filed by the MIT Technology Licensing Office. From a competition standpoint to the current lactic acid producers, Navi-Chem’s technology allows production of lactic acid at lower costs (by as much as $300/ton of lactic acid to traditional technologies\(^{42}\)) due to low-cost nature of the feedstock. The cost advantage would enable sale of lactic acid at competitive or even lower prices whilst staying profitable. From a process standpoint, the lactic acid produced in the bioreactor can be easily separated from the broth and purified to required levels using well-known membrane-based separation and purification technologies\(^{29}\). Therefore, any concerns of product purity with regards to competitiveness with traditional lactic acid producers can be mitigated.

7.5.3. Development Plans

The initial goal will be the construction and testing of the 500 kg/day pilot scale facility at MIT. Navi-Chem is currently working with the MIT Office of Sustainability to identify a suitable location to house the equipment. MIT produces 15 tons a month (0.5 ton per day) of food waste\(^{43}\); thus, this plant could process all of MIT’s food waste. This would be a significant step towards scale up and also would test the robustness of the technology. Thus, the first plan of
action involves raising capital in the summer of 2015 to build this plant, and then operating the plant in Fall of 2015.

Figure 7.14: Development Plans of Navi-Chem (Summer 2015 – Spring 2016)

The operation of the pilot plant at MIT would be followed by another round of capital raising to build the minimal viable business product. This plant would process organics at the scale of 2 tons per day – sufficient to produce enough lactic acid to meet Astro Chemicals’ minimum batch size requirement of 50 barrels a month (personal communication with C.J. Diamond). Astro Chemicals is a chemicals distributor in the New England area which distributes various chemicals including lactic acid with annual sales of $60 million. C.J. Diamond, the son of the president mentioned that he was interested in buying lactic acid at the scale mentioned above. He mentioned the desired purity levels too. Apart from securing an end customer for the lactic acid produced, this association with Astro Chemicals would help Navi-Chem receive useful feedback about product quality and gain information about the lactic acid industry. The further steps also include operating a plant at this scale at IIT-Bombay, a leading university in India through collaboration with the Tata Center of Technology and Design at MIT.
7.5.4. Financial Analysis

The business model being pursued has the following steps:

![Diagram of steps in Navi-Chem's operations](image)

Figure 7.15: Steps in Navi-Chem’s operations

Access to organic waste in our business model can be achieved through one of two possible approaches:

1. **Individual organizations generating organic waste:** Institutions such as MIT, Harvard University, Boston University, Hotel Lenox are known to have implemented systems for disposing the tons of food and organic waste generated. Navi-Chem could enter into a contract with many such institutions to source the organics required to operate the plants.

2. **Large Organics Haulers:** The alternative is to directly contact organics haulers such as Casella organics, who would serve as a one-stop source for all the organics that would be needed. Most importantly, since organizations like Casella are in the waste collection and hauling business, a lot of the logistical and transportation concerns would have already been addressed through their business model.

The initial treatment, the digester operation and solids-liquid separation steps are very similar to the biogas industry and hence, Navi-Chem does not anticipate much risk. Moreover, Navi-
Chem hopes to use lactic acid separation methods currently used in the lactic acid industry which again reduces risks associated with plant operation. The compost stream undergoes some minimal treatment (drying) before it is ready to be transported to the respective customer. Meanwhile, the lactic acid stream after concentration to the desired purity level is also transported to a lactic acid buyer.

There are three distinct revenue streams – first, the tipping fees associated with the organics collection; second, the sale of lactic acid and finally, the sale of compost. The economics of Navi-Chem’s operations was determined through the financial modeling of a large scale facility which could convert organic waste into lactic acid. A typical lactic acid production facility of 10 tons per day was assumed for these calculations. The following list of assumptions was employed as shown in the table below:

<table>
<thead>
<tr>
<th>Assumptions</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Plant Capacity</td>
<td>10</td>
<td>Tons per day (TPD)</td>
</tr>
<tr>
<td>2 Number of working days in year</td>
<td>350</td>
<td>Days</td>
</tr>
<tr>
<td>3 Yield of organics to lactic acid (LA)</td>
<td>0.1</td>
<td>g/g (shown in section 1.3.3)</td>
</tr>
<tr>
<td>4 Organics required</td>
<td>100</td>
<td>TPD</td>
</tr>
<tr>
<td>5 1 ton of LA is associated with manure production of</td>
<td>0.904</td>
<td>Tons 15</td>
</tr>
<tr>
<td>6 Price of LA</td>
<td>1300</td>
<td>$/ton 27</td>
</tr>
<tr>
<td>7 Price of Manure</td>
<td>50</td>
<td>$/ton 17</td>
</tr>
<tr>
<td>9 Digestor CAPEX cost ($ million) where x organics processed (thousand tons per year) 44</td>
<td>( y = 1.7171x^{0.5581} )</td>
<td></td>
</tr>
<tr>
<td>10 Digestor OPEX cost ($/ton) where x is organics (thousand tons per year) 44</td>
<td>( y = 315.62x^{-0.617} )</td>
<td></td>
</tr>
<tr>
<td>12 LA Separation unit CAPEX ($million) where x (LA TPY) 45</td>
<td>( y = 0.1375 \frac{x^{0.6}}{15} )</td>
<td></td>
</tr>
<tr>
<td>13 LA Separation unit OPEX ($million/ton LA) where x (LA TPY) 45</td>
<td>( y = 0.0025 \frac{x^{0.6}}{15} )</td>
<td></td>
</tr>
<tr>
<td>14 Transportation Cost (Collection + 75 miles transport) (Range)</td>
<td>16.23</td>
<td>$/ton 46</td>
</tr>
</tbody>
</table>
Plant operates at 95% capacity

Table 7.5: Assumptions used for the economic modeling of a 10 ton per day lactic acid plant

The following figure shows the revenue and cost streams associated with the operation of the 10 ton per day lactic acid plant. The numbers are all shown as per ton of lactic acid:

Figure 7.16: Business Model of Navi-Chem in terms of revenue and cost streams

The above costs and revenue streams were incorporated to arrive at the final IRR calculation for the plant shown in the table below. The detailed calculations are shown in the appendix.

<table>
<thead>
<tr>
<th>CAPEX (million $)</th>
<th>OPEX (S/ton)</th>
<th>Price (S/ton)</th>
<th>IRR</th>
<th>Avg. Cost of Production over 15 yrs (S/ton)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.5</td>
<td>600</td>
<td>1300</td>
<td>20%</td>
<td>700</td>
</tr>
</tbody>
</table>

Table 7.6: Major financial features of Navi-Chem’s 10 ton per day lactic acid plant

It is to be noted that the above costs of production are assumed for a 100 ton per day organics processing facility which is only 3% of the total daily food waste generated in MA. Moreover, the tipping fee used for the above calculation is $25/ton of food waste whereas it could potentially be $44/ton since the average tipping fee in US is $44/ton. Thus, through economies
of scale as well as through access to food waste at further negative cost, Navi-Chem can potentially produce lactic acid at much lower costs than $700/ton and higher IRRs than 20%.

7.6. Conclusions

The Capstone study examined the current and novel options for organics management from the lens of commercial viability. Despite differences in waste generation features between emerging and developed regions of the world, there is an urgent need in both regions for sustainable solutions to successfully divert food and other organic waste from landfills.

It was shown conclusively that of the three approaches studied in this report, lactic acid production created most value and that owing to the great demand for lactic acid, a waste-to-lactic acid bioprocess would be a very lucrative venture. Most importantly, this novel approach could enable lactic acid production at lower prices than current lactic acid manufacturers who depend on resources such as sugars. The low-cost nature of the feedstock and dependence on the fairly established science of anaerobic digestion would greatly derisk the venture and enable IRRs of 20%.

7.7. Acknowledgments

The author would like express gratitude to Prof. Greg Stephanopoulos for advice and support during the Capstone Study. The author acknowledges support from the Tata Center for Technology and Design, and the Legatum Center for Development and Entrepreneurship for their respective fellowships and for funding travel to India. The author would like to thank Devin Currie and all members in team Navi-Chem: Shamel Merchant, Sivaraman Ramaswamy and Dolonchampa Maji. Also, special thanks to Prof. Sanjay Mahajani at IIT-Bombay, and his staff and graduate students for help and advice during visits to IIT-Bombay.
Appendix

Price of Diesel and Natural Gas

We used the commodities pricing portal, Indexmundi to obtain the price of diesel and natural gas that we would be using for pricing the biodiesel and methane produced through the respective routes.

Natural Gas

Price as found on Indexmundi: $3.9/mmBTU. 1mmBTU = 1055 MJ, and Calorific value of CNG = 53.6 MJ/kg. Therefore, we obtain a price of $0.2/kg, or $222/ton.

Diesel

Price as found on Indexmundi - $0.76/liter. Using the density of diesel of 0.8 kg/liter, we obtain a price of $950/ton.

References


43. Ruth Davis, Manager, MIT Dept. Of Facilities - personal communication

10 ton per day lactic acid plant financial projections sheet
<table>
<thead>
<tr>
<th>Plant Operation Capacity</th>
<th>Year 0</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
<th>Year 4</th>
<th>Year 5</th>
<th>Year 6</th>
<th>Year 7</th>
<th>Year 8</th>
<th>Year 9</th>
<th>Year 10</th>
<th>Year 11</th>
<th>Year 12</th>
<th>Year 13</th>
<th>Year 14</th>
<th>Year 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total amount of lactic acid (tons)</td>
<td>2460</td>
<td>3150</td>
<td>3325</td>
<td>3325</td>
<td>3325</td>
<td>3325</td>
<td>3325</td>
<td>3325</td>
<td>3325</td>
<td>3325</td>
<td>3325</td>
<td>3325</td>
<td>3325</td>
<td>3325</td>
<td>3325</td>
<td>3325</td>
</tr>
<tr>
<td>Total amount of manure (tons)</td>
<td>7214.8</td>
<td>7847.8</td>
<td>8053.8</td>
<td>8053.8</td>
<td>8053.8</td>
<td>8053.8</td>
<td>8053.8</td>
<td>8053.8</td>
<td>8053.8</td>
<td>8053.8</td>
<td>8053.8</td>
<td>8053.8</td>
<td>8053.8</td>
<td>8053.8</td>
<td>8053.8</td>
<td>8053.8</td>
</tr>
<tr>
<td>Price of LA ($/ton)</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
</tr>
<tr>
<td>Price of Manure ($/ton)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Revenue from lactic acid ($)</td>
<td>3185000</td>
<td>4095000</td>
<td>4322500</td>
<td>4322500</td>
<td>4322500</td>
<td>4322500</td>
<td>4322500</td>
<td>4322500</td>
<td>4322500</td>
<td>4322500</td>
<td>4322500</td>
<td>4322500</td>
<td>4322500</td>
<td>4322500</td>
<td>4322500</td>
<td>4322500</td>
</tr>
<tr>
<td>Revenue from manure ($)</td>
<td>130740</td>
<td>142380</td>
<td>150290</td>
<td>150290</td>
<td>150290</td>
<td>150290</td>
<td>150290</td>
<td>150290</td>
<td>150290</td>
<td>150290</td>
<td>150290</td>
<td>150290</td>
<td>150290</td>
<td>150290</td>
<td>150290</td>
<td>150290</td>
</tr>
<tr>
<td>Total revenue from sale of products ($)</td>
<td>3227740</td>
<td>4237380</td>
<td>4472790</td>
<td>4472790</td>
<td>4472790</td>
<td>4472790</td>
<td>4472790</td>
<td>4472790</td>
<td>4472790</td>
<td>4472790</td>
<td>4472790</td>
<td>4472790</td>
<td>4472790</td>
<td>4472790</td>
<td>4472790</td>
<td>4472790</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cost Structure</th>
<th>Year 0</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
<th>Year 4</th>
<th>Year 5</th>
<th>Year 6</th>
<th>Year 7</th>
<th>Year 8</th>
<th>Year 9</th>
<th>Year 10</th>
<th>Year 11</th>
<th>Year 12</th>
<th>Year 13</th>
<th>Year 14</th>
<th>Year 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organics Access Cost ($/ton of organics)</td>
<td>12785309.9</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Transportation Cost ($/ton of organics)</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
<td>602250</td>
</tr>
<tr>
<td>Anerobic digestor CAPEX ($)</td>
<td>371554.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total CAPEX ($)</td>
<td>16500863.9</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total OPEX ($)</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
<td>2191699.8</td>
</tr>
<tr>
<td>Total Cost ($)</td>
<td>17710063.7</td>
<td>12791998.8</td>
<td>12791998.8</td>
<td>12791998.8</td>
<td>12791998.8</td>
<td>12791998.8</td>
<td>12791998.8</td>
<td>12791998.8</td>
<td>12791998.8</td>
<td>12791998.8</td>
<td>12791998.8</td>
<td>12791998.8</td>
<td>12791998.8</td>
<td>12791998.8</td>
<td>12791998.8</td>
<td>12791998.8</td>
</tr>
<tr>
<td>Yearly net profit ($)</td>
<td>-14464021.7</td>
<td>2958180.2</td>
<td>3139350.2</td>
<td>3139350.2</td>
<td>3139350.2</td>
<td>3139350.2</td>
<td>3139350.2</td>
<td>3139350.2</td>
<td>3139350.2</td>
<td>3139350.2</td>
<td>3139350.2</td>
<td>3139350.2</td>
<td>3139350.2</td>
<td>3139350.2</td>
<td>3139350.2</td>
<td>3139350.2</td>
</tr>
<tr>
<td>IRR</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
</tr>
</tbody>
</table>

Total cost over 15 yrs | 36966060.1 | 36966060.1 | 36966060.1 | 36966060.1 | 36966060.1 | 36966060.1 | 36966060.1 | 36966060.1 | 36966060.1 | 36966060.1 | 36966060.1 | 36966060.1 | 36966060.1 | 36966060.1 | 36966060.1 | 36966060.1 |

Cost of LA ($/ton) | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |

CAPEX (million $) | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 |

OPEX ($/ton of LA) | 600.5 | 600.5 | 600.5 | 600.5 | 600.5 | 600.5 | 600.5 | 600.5 | 600.5 | 600.5 | 600.5 | 600.5 | 600.5 | 600.5 | 600.5 | 600.5 |
APPENDIX

A.1. Lipid yield from glucose and acetate per reducing equivalent

On glucose, the lipid yield = 0.318 g/g glucose

1 g of glucose contains 1/180 moles of glucose.

1 mole of glucose has 24 moles of electrons $C_6H_{12}O_6$ (degree of reduction – 4*6+12*1-6*2)

Thus, the lipid yield of glucose per reducing equivalent from glucose = 0.318/ (24/180) = 2.4

Similarly, lipid yield on acetate = 0.272 g/g acetate = 0.267 g/g acetic acid

1 g of acetic acid contains 1/60 moles of acetic acid.

1 mole of acetic acid has 8 moles of electrons $CH_3COOH$ (degree of reduction – 2*4+4*1-2*2)

Thus, the lipid yield of acetic acid per reducing equivalent from acetic acid = 0.267/ (8/60) = 2

A.2. Lipid yield calculations

A.2.1. Glucose

The main idea of this analysis is to arrive at a final stoichiometric equation which describes the number of moles of acetate needed to produce1 mole of lipid and any by-product, if any. Lipids (triglycerides) are formed using the Kennedy pathway through the reaction of fatty acid and glycerol. The fatty acid production occurs through the fatty acid synthesis pathway, which apart from the carbon (in this case, acetate), needs NADPH as a reducing agent and ATP to serve the energy requirements. Glycerol is produced as a side pathway in the glycolysis.
The pathway from glucose to lipids is relatively straightforward compared to the VFAs. The reactions to be considered are:

1. Fatty acid synthesis
   a. Glycolysis – Glucose to pyruvate
   b. Breakdown of pyruvate to acetyl CoA
   c. Acetyl CoA incorporation to form lipids
2. Glycerol production via glycolysis
3. Lipid production using Kennedy pathway
4. NADPH production using Pentose phosphate pathway
5. ATP production using TCA cycle

Analysing each of the above sets of reactions individually,

1. Fatty acid synthesis
   a. $12 \text{ Glucose} \rightarrow 24 \text{ pyruvate} + 24 \text{ ATP} + 24 \text{ NADH}$ (ref - Stryer, Biochemistry, 6th edition)
   b. $24 \text{ pyruvate} \rightarrow 24 \text{ ACA} + 24 \text{ CO}_2 + 24 \text{ NADH}$ (ref - Stryer, Biochemistry, 6th edition)
   c. $24 \text{ ACA} + 18 \text{ NADPH} + 24 \text{ NADH} + 69 \text{ ATP} \rightarrow 3 \text{ palmitic acid}$ (ref – Milgen, 2002)

   Final equation for fatty acid production from glucose:
   $13 \text{ glucose} + 18 \text{ NADPH} + 45 \text{ ATP} \rightarrow 3 \text{ palmitic acid} + 24 \text{ CO}_2 + 24 \text{ NADH}$

2. Glycerol production via glycolysis
   Glucose + 2 ATP + NADPH $\rightarrow$ 2 Glycerol (ref – Tai, 2012)

3. Lipid production using Kennedy Pathway
Glycerol + 3 palmitic acid + ATP \rightarrow \text{Tripalmitin} \text{ (ref – Tai. 2012)}

4. NADPH production using PPP

Glucose \rightarrow 6 \text{CO}_2 + 12 \text{NADPH} \text{ (ref - Stryer, Biochemistry, 6}^{\text{th}} \text{ edition)}

5. ATP production using TCA cycle

Glucose \rightarrow 6 \text{CO}_2 + 10 \text{NADH} + 2 \text{FADH}_2 + 2 \text{ATP} + 2 \text{GTP}

Assuming \text{NADH} = 2.5 \text{ ATP}, and \text{FADH}_2 = 1.5 \text{ ATP} and \text{GTP} = 1 \text{ ATP}, we obtain:

Glucose \rightarrow 6\text{CO}_2 + 32 \text{ ATP}

Thus, the above equations can now be further grouped into 3 categories:

a) Glucose to tripalmitin

12.5 \text{Glucose} + 47 \text{ATP} + 18.5 \text{NADPH} \rightarrow 24 \text{CO}_2 + \text{Tripalmitin} + 24 \text{NADH} \quad - (1)

However, the excess \text{NADH} on the product side can be converted into ATP; in that case, there would be no ATP requirement.

b) Glucose to NADPH

\text{Glucose} \rightarrow 6\text{CO}_2 + 12 \text{NADPH} \quad - (2)

c) Glucose to ATP

\text{Glucose} \rightarrow 6\text{CO}_2 + 32 \text{ ATP} \quad - (3)

To form the 19 \text{NADPH} for eqn. (1), the PPP would burn glucose so as to produce the requisite amount of \text{NADPH}.

19/12 \text{Glucose} \rightarrow 9.5 \text{CO}_2 + 19 \text{NADPH} \text{ (multiplying (2) by 19/12)}

Adding the above equation to the equation (1), would provide the final stoichiometric equation for lipid production from glucose
14.1 Glucose $\rightarrow$ 33.5 CO$_2$ + Tripalmitin

Yield of lipid over glucose = 806/(14.1*180) = 0.318 (Molecular weight of glucose, C$_6$H$_{12}$O$_6$ is 180 and that of tripalmitin, C$_{51}$H$_{98}$O$_6$ is 806)

A.2.2. Acetate

In the case of VFAs, the reverse pathway, gluconeogenesis has to be operational in order to get to glycerol. Then again, the starting substrate for gluconeogenesis needs to be a four carbon compound (oxaloacetate/pyruvate), whereas the VFAs are all metabolized into acetate, a two-carbon compound or, propionate, a three-carbon compound. The cell therefore, operates anaplerotic pathways such as the glyoxalate cycle or the methyl-citrate cycle, in the case of acetate or propionate metabolism, respectively to obtain four carbon compounds. All the above pathways are taken into consideration to arrive at the final equation for acetate to lipids.

The reactions involved in this process have been grouped into modules or sets for simplicity. Most references choose tripalmitate as the lipid of interest while modeling lipid synthesis. Hence, we have also assumed tripalmitate (triglyceride of palmitic acid) as our target lipid molecule. The symbols (c) and (m) refer to whether the metabolite exists in the cytosol or in the mitochondria. The following modules of reactions have been assumed:

a. Acetate to Acetyl CoA (ACA)

b. ACA to oxaloacetate (OAA)

c. OAA to glycerol

d. ACA to C16 fatty acid.

e. (Fatty acid+Glycerol) forms lipid
We will be analyzing the equations of each of the above modules.

**Set a:** Acetate + ATP $\rightarrow$ Acetyl CoA (Acetyl CoA Synthetase) \(\text{Acetyl CoA Synthetase}\) \((1)\)

This reaction proceeds through the action of the enzyme Acetyl CoA Synthetase (ACS) in the cytosol.

**Set b:** Gluconeogenesis occurs in the cytosol and hence, oxaloacetate needs to be present on the cytosol side in order to initiate gluconeogenesis. This process occurs through a number of successive reactions. First, the glyoxalate cycle operates and produces a succinate for every 2 acetyl CoA molecules. Next, the succinate produced in the cytosol permeates through the mitochondrial membrane and enters the TCA cycle operating in the mitochondria. The succinate converts to malate, which permeates out into the cytosol. The cytosolic malate is acted upon by the malic enzyme to produce pyruvate, which ultimately is acted upon by the enzyme pyruvate carboxylase to form oxaloacetate in the cytosol. The reactions are shown below.

\[2 \text{ ACA(c) } \rightarrow \text{ Succinate (c) + NADH}_c \text{ (glyoxalate cycle)} \text{ (ref- Stryer, Biochemistry, 6}\text{th edition)}\]

\[\text{ Succinate (c) } \rightarrow \text{ Succinate (m) (Succinate exit)}\]

\[\text{ Succinate (m) } \rightarrow \text{ Malate (m) + FADH}_2 \text{ (TCA cycle) (ref – Stryer, Biochemistry, 6}\text{th edition)}\]

\[\text{ Malate (m) } \rightarrow \text{ Malate (c) (Malate exit)}\]

\[\text{ Malate (c) } \rightarrow \text{ Pyruvate (c) + NADPH}_c + \text{ CO}_2 \text{ (Malic enzyme) (ref – Stryer, Biochemistry, 6}\text{th edition)}\]

\[\text{ Pyruvate (c) } + \text{ CO}_2 + \text{ ATP } \rightarrow \text{ OAA (c) (Pyruvate Carboxylase) (ref – Stryer, Biochemistry, 6}\text{th edition)}\]
The net reaction amounts to: 2 ACA(c) + ATP → OAA (c) + NADHc + FADH₂ + NADPHc ---(2)

**Set c:** This accounts for the sum of reactions leading up to glycerol production from oxaloacetate via gluconeogenesis. (Stryer, Biochemistry, 6th edition)

\[ \text{OAA (c) + GTP + ATP + NADHc} \rightarrow \text{Glycerol + CO₂ (gluconeogenesis)} \] ----(3)

**Set d:** This accounts for the set of reactions leading up to palmitic acid production from acetyl CoA via the fatty acid synthesis pathway (ref - Milgen, 2002)

\[ 24 \text{ACA(c) + 18 NADPH + 24 NADHc + 69 ATP} \rightarrow 3 \text{palmitic acid (fatty acid synthesis)} \] –(4)

**Set e:** This accounts for the tripalmitin production via the Kennedy pathway. (ref - Stryer, Biochemistry, 6th edition)

\[ \text{Glycerol (c) + 3 palmitic acid + ATP} \rightarrow \text{Tripalmitin (Kennedy pathway)} \] ---(5)

Once the reaction modules have been analyzed to obtain a single reaction, the next step involves adding these to obtain our final equation. Adding sets b through e (reactions 2 to 5) would provide the following equation.

\[ 2 \text{ACA(c) + ATP} \rightarrow \text{OAA (c) + NADHc + FADH₂ + NADPHc} \] ---(2)

\[ \text{OAA (c) + GTP + ATP + NADHc} \rightarrow \text{Glycerol + CO₂ (gluconeogenesis)} \] ----(3)

\[ 24 \text{ACA(c) + 18 NADPH + 24 NADHc + 69 ATP} \rightarrow 3 \text{palmitic acid (fatty acid synthesis)} \] –(4)

\[ \text{Glycerol (c) + 3 palmitic acid + ATP} \rightarrow \text{Tripalmitin (Kennedy pathway)} \] --- (5)
The net equations being: 

$$26 \text{ACA}(c) + 17 \text{NADPH}_c + 72 \text{ATP} + \text{GTP} + 24 \text{NADH}_c \rightarrow \text{Tripalmitin} + \text{CO}_2 + \text{FADH}_2 \text{ (6)}$$

At this point, the acetate required to provide the required NADPH, NADH and ATP needs to be accounted for.

We assume all of the NADPH to be provided through the action of the malic enzyme. The following equation accounts for NADPH production:

$$\text{NADH}_c + \text{ATP} \rightarrow \text{NADPH}_c \text{ (Malic enzyme) (7) (Stryer)}$$

ATP production from cytosolic ACA is not as straightforward as ACA cannot cross the mitochondrial membrane to form mitochondrial ACA, which could then directly enter the TCA cycle. Thus, the glyoxalate cycle operates to form succinate, which eventually forms pyruvate. Pyruvate can permeate through the mitochondria and then decarboxylate to form acetyl CoA, which subsequently enters the TCA cycle. The equation for ATP/NADH production through the TCA cycle from cytosolic acetyl CoA proceeds through the following set of equations:

$$2 \text{ACA (c)} \rightarrow \text{Succinate (c)} + \text{NADH}_c \text{ (glyoxalate cycle)}$$

$$\text{Succinate (c)} \rightarrow \text{Succinate (m)} \text{ (succinate exit)}$$

$$\text{Succinate (m)} \rightarrow \text{malate (m)} + \text{FADH}_2 \text{ (TCA cycle)}$$

$$\text{Malate (m)} \rightarrow \text{Malate (c)} \text{ (Malate exit)}$$

$$\text{Malate (c)} \rightarrow \text{Pyruvate (c)} + \text{NADPH}_c + \text{CO}_2 \text{ (Malic enzyme) (ref - Stryer, Biochemistry, 6th edition)}$$

$$\text{Pyruvate (c)} \rightarrow \text{Pyruvate (m)} \text{ (Pyruvate exit)}$$
Pyruvate (m) → 3 CO₂ + 4 NADHm + FADH₂ + GTP (TCA cycle) (ref- Stryer, Biochemistry, 6th edition)

4 NADHm → 4 NADHc (ref – Milgen, 2002)

The net equation is: 2 ACA(c) → 4 CO₂ + 5 NADHc + 2 FADH₂ + GTP + NADPHc  -- (8)

Equation (8) can also be used to produce more ATP through the conversion of the NADH and FADH₂ in the electron transport chain. However, we are also assuming that the NADPH would not be oxidized because there is a high demand for NADPH in the cell during the fatty acid synthesis as a reducing agent.(1 NADH = 2.5 ATP, 1 FADH₂ =1.5 ATP, 1 GTP =1ATP; ref – Stryer, Biochemistry, 6th edition)

The equation would become: 2 ACA (c) → 4 CO₂ + 16.5 ATP + NADPHc – (9)

Equations 6, 7, 8 and 9 would add up together to arrive at the final equation for lipid production from acetate. The strategy employed is to assume the equations 7,8 and 9 to be multiplied by unknowns x.y. and z and added to equation 6. The equations are to be added such that the coefficients of NADH, NADPH and ATP add upto 0. In this way, we can ensure that the equation accounts for the just the exact amount of acetate needed to arrive at 1 mol of tripalmitin.

26 ACA(c) + 17 NADPHc + 72 ATP + 1GTP + 24 NADHc → Tripalmitin + CO₂ + FADH₂ ---- (6)

xNADHc +x ATP → xNADPHc -----Multiplying (7) by the unknown x

2y ACA(c) → 4y CO₂ + 5y NADHc + 2y FADH₂ + y GTP + y NADPHc ----- Multiplying (8) by the unknown y
\[ 2z \text{ACA (c)} \rightarrow 4z \text{CO}_2 + 16.5z \text{ATP} + z\text{NADPHc} \]

Multiplying (9) by the unknown \(z\)

They add up to: 
\[ (26+2y+2z) \text{ACA} + (17-x-y-z) \text{NADPH} + (71.5+x-4y-16.5z) \text{ATP} + (24 +x -5y) \text{NADH} \]

\[ \rightarrow (4y+4z+1) \text{CO}_2 + \text{Tripalmitin} \] (Converting the FADH2 and GTP into ATP) ----(10)

However, the substrate is acetate and hence, multiplying equation (1) by \((26+2y+2z)\) and adding to (10) would substitute all the ACA by acetate.

\[ (26+2y+2z) \text{Acetate} + (17-x-y-z) \text{NADPH} + (71.5+x-4y-16.5z+26+2y+2z) \text{ATP} + (24 +x-5y) \text{NADH} \]

\[ \rightarrow (4y+4z+1) \text{CO}_2 + \text{Tripalmitin} \] –(11)

Assigning the coefficients of \(\text{NADPH, NADH and ATP}\) to be 0, we obtain a system of 3 equations in \(x, y\) and \(z\):

\[ 17-x-y-z = 0 \ - \text{coefficient of NADPH} \]

\[ 24+x-5y=0 \ - \text{coefficient of NADH} \]

\[ 97.5+x-2y-14.5z = 0 \ - \text{coefficient of ATP} \]

Solving for the above 3 equations, we obtain \(x = 4.944, y = 5.788, \) and \(z = 6.267.\)

Putting the above values of \(x, y\) and \(z\) into equation (11), we obtain the final equation for conversion of acetate into lipids as shown in section 3.3.

**50.1.1 Acetate \rightarrow 49.22 \text{CO}_2 + \text{Tripalmitin}**

The yield of lipid over acetate is \(806/(50.1*59) = 0.272\)

**A.2.3. Lactate**
We follow the similar framework as was used for acetate to obtain the lipid yield. We will be first finding the equation relating the moles of lactate required to produce 1 mole of lipid (tripalmiting) using the modules a to e. Next, we will account for the NADPH and ATP requirement similar to the case for acetate.

**Set a:** Lactate to Acetyl CoA - Lactate gets converted into pyruvate in the cytoplasm through the following equation:

\[ \text{Lactate} \rightarrow \text{Pyruvate} + \text{NADH} \]  

(1)

The pyruvate formed gets converted into acetyl CoA (ACA) using pyruvate decarboxylase:

\[ \text{Pyruvate} \rightarrow \text{ACA} + 2\text{NADH} + \text{CO}_2 \]  

(2)

We add (1) and (2) to get the reaction which produces acetyl CoA from lactate:

\[ \text{Lactate} \rightarrow \text{ACA} + 2\text{NADH} + \text{CO}_2 \]  

(3)

**Set b:** Lactate to Oxaloacetate. The following equations are used for the conversion:

\[ \text{Lactate} \rightarrow \text{Pyruvate} + \text{NADH} \]  

(1)

\[ \text{Pyruvate} + \text{CO}_2 + \text{ATP} \rightarrow \text{OAA} \]  

(2)

Adding (1) and (2), we obtain,

\[ \text{Lactate} + \text{CO}_2 + \text{ATP} \rightarrow \text{OAA} + \text{NADH} \]  

(4)

**Set c:** Oxaloacetate to Glycerol.

\[ \text{OAA} + \text{GTP} + \text{ATP} + \text{NADH} \rightarrow \text{Glycerol} + \text{CO}_2 \]  

(5)
**Set d:** Lactate to C16 fatty acid. This is obtained through the following equations:

\[
\text{Lactate} \rightarrow \text{ACA} + 2\text{NADH} + \text{CO}_2 - (3)
\]

\[
24\text{ACA} + 18\text{NADPH} + 24\text{NADH} + 69\text{ATP} \rightarrow 3\text{ Palmitic acid} - (6)
\]

Multiplying (3) by 24, and adding with (6), we get

\[
24 \text{ Lactate} + 18 \text{ NADPH} + 69 \text{ ATP} \rightarrow 3 \text{ Palmitic acid} + 24 \text{ NADH} + 24 \text{ CO}_2 - (7)
\]

**Set e:** Glycerol and fatty acid to tripalmitin.

\[
\text{Glycerol} + 3 \text{ palmitic acid} + \text{ATP} \rightarrow \text{Tripalmitin} - (8)
\]

Adding (4), (5), (7) and (8), we get

\[
25 \text{ Lactate} + 73 \text{ ATP} + 18 \text{ NADPH} \rightarrow \text{Tripalmitin} + 24 \text{ NADH} + 24 \text{ CO}_2 - (9)
\]

Now, we need to account for the lactate required to produce the ATP and NADPH.

NADPH was assumed to be produced using the malic enzyme:

\[
\text{NADH} + \text{ATP} \rightarrow \text{NADPH} - (10)
\]

The equation for the oxidation of ACA through the TCA cycle was shown in the acetate section:

\[
2 \text{ ACA} \rightarrow 4 \text{ CO}_2 + 5 \text{ NADH} + 2 \text{ FADH}_2 + \text{GTP} + \text{NADPH} - (11)
\]

Multiplying (3) by 2 and adding to (11), we get the equation for NADH generation:

\[
2 \text{ Lactate} \rightarrow 6 \text{ CO}_2 + 9 \text{ NADH} + 2 \text{ FADH}_2 + \text{GTP} + \text{NADPH} - (12)
\]

If we were to use all the lactate towards ATP production, then the resulting equation would be

\[
(\text{NADH} = 2.5 \text{ ATP}, \text{FADH}_2 = 1.5 \text{ ATP}, \text{GTP} = 1 \text{ ATP})
\]
2 Lactate → 6 CO₂ + 26.5 ATP + NADPH – (13)

Therefore, now the equations under consideration are (9) – lipid production from lactate, (10) – NADPH generation, (12) – NADH generation and (13) – ATP generation.

25 Lactate + 73 ATP + 18 NADPH → Tripalmitin + 24 NADH + 24 CO₂ – (9)

NADH + ATP → NADPH – (10)

2 Lactate → 6 CO₂ + 9 NADH + 4 ATP + NADPH – (12)

2 Lactate → 6 CO₂ + 26.5 ATP + NADPH – (13)

We use the similar methodology as used in the case for acetate – we multiply (10) by x, multiply (12) by y, (13) by z and add all to (9). The resulting equation is:

\[(25+2y+2z)\text{ Lactate} \rightarrow (73+x-4y-26.5z)\text{ ATP} + (18-x-y-z)\text{ NADPH} + (x-9y-24)\text{ NADH} \rightarrow (24+6y+6z)\text{ CO}_2 \rightarrow \text{Tripalmitin}\]

Now, there are 3 unknowns (x, y and z). We would want to solve for x, y and z, such that the coefficients of ATP, NADPH and NADH are 0.

The equations are:

\[x-9y = 24\]

\[26.5z -x + 4y = 73\]

\[x+y+z=18\]

Solving the above set of equations, we obtain y<0, which implies that the NADH produced from the lipid production step is sufficient to sustain the NADPH production from equation (10).
Thus, as per equation (9), 18 moles of NADH would be required to produce the 18 moles of NADPH required for the lipid generation step. Thus using up the NADH required for NADPH production, the lipid generation step would have the form:

25 Lactate + 91 ATP \rightarrow Tripalmitin + 6 NADH + 24 CO_2 – (14)

The 6 moles of NADH would oxidixe through the electron transport chain to yield \((6 \times 2.5) = 15\) moles of ATP. Equation (14) would further simplify to:

25 Lactate + 76 ATP \rightarrow Tripalmitin + 24 CO_2 – (14)

Equation 13 provides the stoichiometry for ATP production from lactate. In addition to the 26.5 moles of ATP available, the extra mole of NADPH would also be oxidized (since all the NADPH requirement is met) to produce more ATP as per equation (15) below

2 Lactate \rightarrow 6 CO_2 + 29 ATP – (15)

To obtain the 76 moles of ATP for equation (14), we thus need \((2 \times 76/29) = 5.24\) moles of lactate

5.24 Lactate \rightarrow 15.72 CO_2 + 76 ATP – (15)

Adding (15) to (14), we get

30.2 Lactate \rightarrow Tripalmitin + 39.7 CO_2 – (14)

Thus, the final equation for tripalmitin production from lactate is:

**30.2 Lactate \rightarrow Tripalmitin + 39.7 CO_2**

The lipid yield is \((806/(30.2 \times 90)) = 0.297\) g/g (molecular weight of tripalmitin = 806, molecular weight of lactic acid = 90)
A.2.4. Propionate

Propionate gets converted into Propionyl CoA in the presence of a synthetase.

Propionate + ATP → Propionyl CoA ---(1)

Like in the case with acetate, propionate cannot enter gluconeogenesis without first being converted into a four carbon compound. Propionyl CoA (PCA) thus enters the methyl citrate cycle and forms pyruvate.

**Set a:** PCA → Pyruvate + NADH + FADH₂ ---(1)

Pyruvate (c) + CO₂ + ATP → OAA (c) (Pyruvate Carboxylase) (ref – Stryer, Biochemistry, 6th edition) --- (2)

Adding the above equations, we get PCA + ATP + CO₂ → OAA (c) + NADH + FADH₂ – (3)

**Set b:** OAA forms glycerol as per equation shown in above section:

OAA (c) + GTP + ATP + NADHc → Glycerol + CO₂ (gluconeogenesis) ---- (4)

To proceed towards lipid synthesis, PCA has to first form Acetyl CoA.

Pyruvate forms ACA as per: Pyruvate → ACA + CO₂ + NADH—(5)

On adding (1) and (5), we get: PCA → ACA + CO₂ + 2 NADH + FADH₂—(6)

The equation for fatty acid synthesis from ACA is: 24 ACA(c) + 18 NADPH + 24 NADHc + 69 ATP → 3 palmitic acid (fatty acid synthesis) ---(7)

Thus, equation from PCA to fatty acid (using eqns. 6 and 7) would be: 24 PCA + 18 NADPH + 69 ATP → 24 CO₂ + 3 palmitic acid + 24 NADH + 24 FADH₂—(8)
Equations that would be required for lipid synthesis from PCA:

\[ \text{PCA} + \text{ATP} + \text{CO}_2 \rightarrow \text{OAA (c)} + \text{NADH} + \text{FADH}_2 \] (3)

\[ \text{OAA (c)} + \text{GTP} + \text{ATP} + \text{NADHc} \rightarrow \text{Glycerol} + \text{CO}_2 \text{ (gluconeogenesis)} \] ----(4)

\[ 24 \text{ PCA} + 18 \text{ NADPH} + 69 \text{ ATP} \rightarrow 24 \text{ CO}_2 + 3 \text{ palmitic acid} + 24 \text{ NADH} + 24 \text{ FADH}_2 \] ----(8)

Glycerol + 3 palmitic acid + ATP \rightarrow \text{Tripalmitin} ----(9)

Adding the above equations, we obtain

\[ 25 \text{ PCA} + 71.5 \text{ ATP} + 18 \text{ NADPH} \rightarrow \text{Tripalmitin} + 24 \text{ CO}_2 + 24 \text{ NADH} + 24 \text{ FADH}_2 \] \(\) (10)

Equation for NADPH requirement: \(\text{NADH} + \text{ATP} \rightarrow \text{NADPH} \) \(\) (11)

Equation for NADH generation through TCA cycle from ACA:

\[ 2 \text{ ACA(c)} \rightarrow 4 \text{ CO}_2 + 5 \text{ NADHc} + 2 \text{ FADH}_2 + \text{GTP} + \text{NADPHc} \] --(12)

By equation (6), \(\text{PCA} \rightarrow \text{ACA} + \text{CO}_2 + 2 \text{ NADH} + \text{FADH}_2 \) \(\) (6)

Adding (12) and \( \times 2 \) \((6) \), we get: \( 2 \text{ PCA} \rightarrow 6 \text{ CO}_2 + 9 \text{ NADH} + 4\text{FADH}_2 + \text{NADPHc} + \text{GTP} \) \(\) (13)

Similarly, equation for ATP requirement, \( 2 \text{ PCA} \rightarrow 6 \text{ CO}_2 + 29.5 \text{ ATP} + \text{NADPHc} \) \(\) (14)

Thus, the equations under consideration are (10), (13) and (14).

\[ 25 \text{ PCA} + 71.5 \text{ ATP} + 18 \text{ NADPH} \rightarrow \text{Tripalmitin} + 24 \text{ CO}_2 + 24 \text{ NADH} + 24 \text{ FADH}_2 \] \(\) \(\) (10)

\(\text{NADH} + \text{ATP} \rightarrow \text{NADPH} \) \(\) (11) \(\) (multiplying by \( x \))

\[ 2 \text{ PCA} \rightarrow 6 \text{ CO}_2 + 9 \text{ NADH} + 4\text{FADH}_2 + \text{NADPHc} + \text{GTP} \] \(\) \(\) (13) \(\) (multiplying by \( y \))

165
2 \text{ PCA} \rightarrow 6 \text{ CO}_2 + 29.5 \text{ ATP} + \text{NADPHc} - (14) \text{ (multiplying by z)}

There would be ATP requirement for the conversion of propionate to \text{PCA}. \text{(equation 1)}

A similar treatment as shown in section A.1.2. would lead to the equation:

\begin{align*}
(25+2y+2z) \text{ propionate} &+ (71.5+x-36-7y-29.5z+25+2y+2z) \text{ ATP} + (18-x-y-z) \text{ NADPH} \\
\text{Tripalmitin} &+ (24-x+9y) \text{ NADH} + (24+6y+6z) \text{ CO}_2 - (15)
\end{align*}

Solving for the three variables \( x, y, \) and \( z, \) using the balances for \text{NADH}, \text{NADPH} and \text{ATP}, we get

\begin{align*}
18-x-y-z &= 0 \\
24-x+9y &= 0 \\
71.5+x-36-7y-29.5z+25+2y+2z &= 0
\end{align*}

\( y \) turns out to be negative, which means that the cell does not need additional \text{NADH}. So we assume \( y=0 \) and \( z=0 \) for now.

In that case, equation becomes: \( 25 \text{ propionate} + 78.5 \text{ ATP} \rightarrow \text{Tripalmitin} + 6 \text{ NADH} + 24 \text{ CO}_2 \)

\text{(16)}

6 \text{ NADH} would yield 15 \text{ ATP}. We would have to assume the rest of \text{ATP} to be obtained using the oxidation of propionate in the \text{TCA} cycle.

\( 2 \text{ PCA} \rightarrow 6 \text{ CO}_2 + 29.5 \text{ ATP} + \text{NADPHc} \) is the equation obtained. Even oxidizing the \text{NADPH} and accounting for the \text{ATP} required for \text{PCA} formation would lead to:

\( 2 \text{ propionate} \rightarrow 6 \text{ CO}_2 + 30 \text{ ATP}---(17) \)
The required ATP is (78.5-15) = 63.5 ATP.

So, according to equation (17), to obtain 63.5 ATP, the equation would need to be multiplied by 63.5/30

4.23 propionate $\rightarrow$ 12.69 CO$_2$ + 63.5 ATP --- (18)

Adding equation (18) to (16), we get

**29.23 Propionate $\rightarrow$ 36.7 CO$_2$ + Tripalmitin**

The lipid yield: $\frac{806}{(29.23*73)} = 0.377$

**A.2.5. Butyrate**

Butyrate metabolism is very similar to acetate metabolism. The first step is the conversion of butyrate to butyryl CoA through the action of the synthetase,

Butyrate + ATP $\rightarrow$ Butyryl CoA (BCA) ----(1)

Next, BCA enters the fatty acid degradation step through which it splits into two molecules of acetyl CoA.

$\text{BCA} \rightarrow 2 \text{ACA} + \text{NADH} + \text{FADH}_2$ ----(2)

Using the equations 6, 8 and 9 from the section 11.1.2,

$26 \text{ACA(c)} + 17 \text{NADPHc} + 72 \text{ATP} + 1\text{GTP} + 24 \text{NADHc} \rightarrow \text{Tripalmitin} + \text{CO}_2 + \text{FADH}_2$ ----(3) – Lipid synthesis

$\text{NADH} + \text{ATP} \rightarrow \text{NADPH}$ ---(4) – NADPH production
2 ACA(c) \rightarrow 4 \text{CO}_2 + 5 \text{NADH} + 2 \text{FADH}_2 + \text{GTP} + \text{NADPHc} \quad -(5) \quad \text{NADH production}

2 ACA(c) \rightarrow 4 \text{CO}_2 + 16.5 \text{ATP} + \text{NADPHc} \quad -(6) \quad \text{ATP production}

The above equations other than (4) are modified by using the conversion from ACA to BCA as per equation 2,

13 BCA(c) + 17 \text{NADPH} + 72 \text{ATP} + 1 \text{GTP} + 11 \text{NADH} \rightarrow \text{Tripalmitin} + \text{CO}_2 + 14 \text{FADH}_2 \quad -(7)

\text{NADH} + \text{ATP} \rightarrow \text{NADPH} \quad -(4)

\text{BCA(c)} \rightarrow 4 \text{CO}_2 + 6 \text{NADH} + 3 \text{FADH}_2 + \text{GTP} + \text{NADPHc} \quad -(8)

\text{BCA(c)} \rightarrow 4 \text{CO}_2 + 20.5 \text{ATP} + \text{NADPHc} \quad -(9)

Again the equations (4), (8) and (9), are multiplied by x, y, and z and added to (7)

\begin{align*}
(13+y+z) \text{BCA} + (17-x-y-z) \text{NADPH} + (11+x-6y) \text{NADH} + (73-21+x-5.5y-20.5z) \text{ATP} & \rightarrow \\
\text{Tripalmitin} + (4y+4z+1) \text{CO}_2
\end{align*}

Adding the ATP requirements to for butyrate to BCA, the coefficient for ATP would go up by 13+y+z

Solving for the equations, we obtain

Thus, the final equation is:

\begin{align*}
\textbf{19.6 Butyrate} & \rightarrow 27.4 \text{CO}_2 + \text{Tripalmitin}, \text{ yield being } = \frac{806}{(19.6*87)} = 0.472
\end{align*}

\textbf{A.2.6. Pentanoate}
Pentanoate, also known as valerate, also enter the fatty acid degradation pathway once it forms valeryl CoA (VCA).

Valerate + ATP \rightarrow VCA \quad -(1)

\[ \text{VCA} \rightarrow \text{ACA} + \text{PCA} + \text{NADH} + \text{FADH}_2 \quad -(2) \]

We assume each mole of ACA and PCA to be metabolized in the same way for lipid synthesis as they would be if acetate or propionate were to be used individually as separate substrates.

**Set a: Lipid synthesis reaction**

Thus, using the corresponding equations from the above sections,

\[ 26 \text{ACA} + 17 \text{NADPH} + 72 \text{ATP} + \text{GTP} + 24 \text{NADH} \rightarrow \text{Tripalmitin} + \text{CO}_2 + \text{FADH}_2 \quad -(3) \]

\[ 25 \text{PCA} + 71.5 \text{ATP} + 18 \text{NADPH} \rightarrow \text{Tripalmitin} + 24 \text{CO}_2 + 24 \text{NADH} + 24 \text{FADH}_2 \quad -(4) \]

Dividing (3) by 26 and (4) by 25 and adding them yields:

\[ \text{ACA} + \text{PCA} + 1.374 \text{NADPH} + 5.667 \text{ATP} \rightarrow 0.078 \text{Tripalmitin} + 0.998 \text{CO}_2 + 0.037 \text{NADH} + 0.998 \text{FADH}_2 \quad -(5) \]

Adding the equation (2) to (5), we obtain the equation for lipid synthesis:

\[ \text{VCA} + 1.374 \text{NADPH} + 5.667 \text{ATP} \rightarrow 0.078 \text{Tripalmitin} + 0.998 \text{CO}_2 + 1.0369 \text{NADH} + 1.9984 \text{FADH}_2 \quad -(6) \]

**Set b: NADPH formation reaction**

\[ \text{NADH} + \text{ATP} \rightarrow \text{NADPH} \quad -(7) \]
Set c: NADH formation reaction

The equations in the above sections for acetate and propionate metabolism towards NADH synthesis are used.

\[ \text{ACA} \rightarrow 2\text{CO}_2 + 2.5 \text{NADH} + \text{FADH}_2 + 0.5 \text{GTP} + 0.5 \text{NADPH} \quad \text{(8)} \]

\[ \text{PCA} \rightarrow 3\text{CO}_2 + 4.5 \text{NADH} + 2 \text{FADH}_2 + 0.5 \text{NADPH} + 0.5 \text{GTP} \quad \text{(9)} \]

Adding the equation (2) to the above equations, we obtain

\[ \text{VCA} \rightarrow 5\text{CO}_2 + 8 \text{NADH} + 4 \text{FADH}_2 + \text{GTP} + \text{NADPH} \quad \text{(10)} \]

Set c: ATP generation reactions

Using the equations from acetate and propionate metabolism for ATP synthesis:

\[ \text{ACA} \rightarrow 2 \text{CO}_2 + 8.25 \text{ATP} + 0.5 \text{NADPH} \quad \text{(11)} \]

\[ \text{PCA} \rightarrow 3 \text{CO}_2 + 14.75 \text{ATP} + 0.5 \text{NADPH} \quad \text{(12)} \]

Adding equation (2) to the above equations,

\[ \text{VCA} \rightarrow 5 \text{CO}_2 + 27 \text{ATP} + \text{NADPH} \quad \text{(13)} \]

Set d: Final lipid formation equation from valerate

Listing the equations (6), (7), (10) and (13),

\[ \text{VCA} + 1.374 \text{NADPH} + 5.667 \text{ATP} \rightarrow 0.078 \text{Tripalmitin} + 0.998 \text{CO}_2 + 1.0369 \text{NADH} + 1.9984 \text{FADH}_2 \quad \text{(6)} \]

\[ \text{NADH} + \text{ATP} \rightarrow \text{NADPH} \quad \text{(7)} \]
VCA $\rightarrow$ 5 CO$_2$ + 8 NADH + 4 FADH$_2$ + GTP + NADPH ---(10)

VCA $\rightarrow$ 5 CO$_2$ + 27 ATP + NADPH ---(13)

Multiplying eqns. 7, 10 and 13 with x, y and z and adding (y+z+1) ATP requirement for the conversion of valerate to VCA, we obtain the equation;

$$(y+z+1) \text{VCA} + (1.378-x-y-z) \text{NADPH} + (x-8y-1.037) \text{NADH} + (5.667-2.9976+x-7y-27z+y+z+1) \text{ATP} \rightarrow 0.078 \text{Tripalmitin} + (5y+5z+0.998) \text{CO}_2$$

Solving for the balances of NADH, NADPH and ATP results in the following values: x = 1.17426, y = 0.01717, z = 0.18237.

The final overall equation for lipid synthesis from valerate is:

15.3 Valerate $\rightarrow$ Tripalmitate + 25.4 CO$_2$

The lipid yield is: 806/(15.3*101) = 0.521

A.2.7. Hexanoate

Hexanoate is metabolized into hexanoyl CoA through a synthetase and it is also degraded using the fatty acid degradation pathway into the three acetyl CoA fragments.

Hexanoate + ATP $\rightarrow$ HCA --- (1)

HCA $\rightarrow$ 3 ACA + 2 NADH + 2 FADH$_2$ --- (2)

The same strategy as used with the butyrate metabolism was used to arrive at the following final equation:
26 ACA(c) + 17 NADPHc + 72 ATP + 1GTP + 24 NADHc \rightarrow \text{Tripalmitin} + \text{CO}_2 + \text{FADH}_2 \hspace{1cm} (3) - \text{Lipid synthesis}

NADH + ATP \rightarrow \text{NADPH} \hspace{0.5cm} (4) - \text{NADPH production}

2 ACA(c) \rightarrow 4 \text{CO}_2 + 5 \text{NADHc} + 2 \text{FADH}_2 + \text{GTP} + \text{NADPHc} \hspace{0.5cm} (5) - \text{NADH production}

2 ACA (c) \rightarrow 4 \text{CO}_2 + 16.5 \text{ATP} + \text{NADPHc} \hspace{0.5cm} (6) - \text{ATP production}

Modifying the above equations using equation (2), we get

\[ \frac{26}{3} \text{HCA} + 17 \text{NADPH} + 72 \text{ATP} + \text{GTP} + 6.67 \text{NADH} \rightarrow \text{Tripalmitin} + \text{CO}_2 + 18.33 \text{FADH}_2 \hspace{1cm} (7) \]

NADH + ATP \rightarrow \text{NADPH} \hspace{0.5cm} (8) - \text{NADPH production}

\[ \frac{2}{3} \text{HCA(c)} \rightarrow 4 \text{CO}_2 + 6.33 \text{NADHc} + 3.33 \text{FADH}_2 + \text{GTP} + \text{NADPHc} \hspace{0.5cm} (9) - \text{NADH production} \]

\[ \frac{2}{3} \text{HCA (c)} \rightarrow 4 \text{CO}_2 + 21.83 \text{ATP} + \text{NADPHc} \hspace{0.5cm} (10) - \text{ATP production} \]

Multiplying the equations (8), (9) and (10) by x, y, and z and adding we get,

\[ \left(\frac{26}{3}+\frac{2y}{3}+\frac{2z}{3}\right) \text{HCA} + (17-x-y-z) \text{NADPH} + (6.67+x-6.33y) \text{NADH} + (73-27.49+x-5.995y-21.83z) \text{ATP} \rightarrow \text{Tripalmitin} + (4y+4z+1) \text{CO}_2 \]

Adding for the ATP requirements for conversion of hexanoate to HCA, we get

\[ \left(\frac{26}{3}+\frac{2y}{3}+\frac{2z}{3}\right) \text{Hexanoate} + (17-x-y-z) \text{NADPH} + (6.67+x-6.33y) \text{NADH} + (73-27.49+x-5.995y-21.83z+\frac{26}{3}+\frac{2y}{3}+\frac{2z}{3}) \text{ATP} \rightarrow \text{Tripalmitin} + (4y+4z+1) \text{CO}_2 \]

Solving for the balances, \(x = 11.71, y = 2.9\) and \(z = 2.38\).
Thus, the final equation for lipid synthesis would be:

**12.2 Hexanoate $\rightarrow$ Tripalmitin + 22.1 CO$_2$**

Yield is $= \frac{806}{(12.2*115)} = 0.574$

**A.3. Non-lipid equation derivation**

As per Liu et al., 1999, the average chemical formula for yeast biomass is C$_{5}$H$_{8}$O$_{2.7}$N$_{0.7}$. It would correspond to a molecular weight of 121.3. The next part of the analysis follows similar to the one shown in Fei et al., 2011.

Shuler and Kargi provide an estimate for the yield of dry cell weight per electron available – 3.14 g DCW/ electron. Since acetate has 8 electrons, it would correspond to a biomass (non-lipid) yield of

Biomass yield per mol of acetate $= 3.14*8 = 25.1$ g DCW/mol acetate.

In terms of an equation, the relation would be: Acetate $\rightarrow$ 0.21 biomass + 0.95 CO$_2$ \quad (1)

Here the moles of CO$_2$ were obtained by subtracting the carbon moles in biomass from the carbon moles in acetate. (=2-0.21*5).

In addition, there would be ATP expended by the cell for biomass production. According to Verduyn, 1991, 105.5 mmol of ATP is required per gm dry biomass, which translates to the fact that for every 0.21 mol of biomass produced, 2.65 mol ATP would be required.

Thus equation (1) would be modified as: Acetate + 2.65 ATP $\rightarrow$ 0.21 biomass + 0.95 CO$_2$ \quad (2)
Now ATP production from acetate follows the equation: (above analysis on lipid yield showed the equation) \( \text{Acetate} \rightarrow 2 \text{CO}_2 + 8.5 \text{ATP} \)

Therefore, the extra acetate to be expended for the production of 2.65 mol of ATP would be:

\[
0.32 \text{ Acetate} \rightarrow 0.64 \text{ CO}_2 + 2.65 \text{ ATP} - (3)
\]

Hence the total amount of acetate required for the production of 1 mol of biomass would be the addition of equations (2) and (3) and multiplying by the respective normalizing factor to make the coefficient of biomass 1, 

\[
6.3 \text{ Acetate} \rightarrow 7.7 \text{ CO}_2 + \text{ biomass}
\]

**A.4. MATLAB code for model**

```matlab
function flowrate_n
% Written by Sagar Chakraborty - Aug 2013
% For the purpose of determining the salt feed and permeate flow rates in the recycle reactor so as to maintain S, substrate at So and volume V, at Vo
% A particular case shown below - where we are trying to compute the flow rates for the 81-96h time period by plugging in the measurements made at 81h

vo=2.1;%target volume, unit-L
vt=2.06;%measured volume at current time point, unit-L
so=0.06;%target substrate concentration, unit-moles/L
st=0.114;%measured substrate concentration at current time point, unit-mol/L

tn_=72;% time point previous to current one, unit - hour
tn=81;% current time point, unit - hour
tnn=96;%future time point, unit - hour
df1m=(1.649-0.975)/(tn-tn_);% acid flow rate in the last time interval, tn_ to tnn, unit - L/hour
od1=158;% OD measurement (proxy for biomass) at previous time point, tn_.
od2=166;% OD measurement at current time point

mu=(log(od2)-log(od1))/(tn-tn_);% specific growth rate in the last time interval, unit - h-1
dx=mu*od2;% projected biomass increase rate for the next time interval, unit - h-1
dqm=0.833;% substrate consumption in the last time interval, unit - moles
y=(od2-od1)/dqm;% biomass yield in the last time interval, unit - moles-1
dqt=dx/y;% estimated substrate consumption rate in the next interval, unit - moles/h
dflt=df1m/dqm*dqt*(tn-tn_);% estimated acid flow rate in the next time interval, where the proportionality of acid flow rate and consumption is being used (k=dflt/dqt=df1m/(dqm*(tn-tn_)))
```

174
s1=0.98; % concentration of acid feed, unit - mol/L
s2=1; % concentration of salt feed, unit - mol/L

y = [vo, vt, so, st, dfit, dqt, tn, tnn, s1, s2];
options = optimset ('TolX', le-11, 'TolFun', le-11, 'MaxFunEvals', 2000); % setting the parameters for the non-linear solver
x = fsolve(@bioreactor_approx, [0.3 0.3], options); % the function bioreactor_approx solves a simpler system to determine good initial guesses for the actual complex system

options = optimset ('TolX', le-11, 'TolFun', le-11, 'MaxFunEvals', 2000); % setting the parameters for the non-linear solver
z = fsolve(@bioreactor_1, [x(1) x(2)], options); % the initial guesses x(1) and x(2) for the flow rates are used to solve for the more complex system of equations

z(1) % df2, salt flow rate output
z(2) % df3, permeate flow rate output

end

function k=bioreactor_approx(v)
% Written by Sagar Chakraborty - August 2013
% Purpose to solve the simpler set of linear equations to generate good initial guesses for the non-linear system of equations
% v has a randomly assigned set of initial guesses - they can be random because this system will have only a unique solution

df2=v(1); df3=v(2);
x= [df2, df3];
% The below set of variables formulates the system exactly as in the main function flowrate_n
vo=2.1; % L
vt=2.06; % L
so=0.06; % mol/L
st=0.114; % mol/L
tn_=72; % h
tn=81; % h	nnn=96; % h
df1m=(1.649-0.975)/(tn-tn_); % l/h
od1=158; % OD
od2=166;
mu=(log(od2)-log(od1))/(tn-tn_);
dx=mu*od2;
dqm=0.833;
y=(od2-od1)/dqm;
dqt=dx/y;
dflt=dfilm/dqm*dqt*(tn-tn_);

s1=0.98;%mol/L
s2=1;%mol/L

k=[0 0];% initializing the k vector
y = [vo vt so st dflt dqt tn tnn sl s2];% storing all the key variables into a vector y
k(1) = y(1)-y(2)-(y(5)+x(1)-x(2))*(y(8)-y(7)); % the solution of a linear system of equations
k(2) = y(5)*y(9)+x(1)*y(10)-y(6)-x(2)*y(3)-((y(3)*y(l)-y(4)*y(2))/(y(8)-y(7)));
% the df2 and df3 estimates obtained this way are sent back to the main function and stored there in the vector x
end

function z=bioreactor_1(v)
% Written by Sagar Chakraborty - August 2013
% Purpose to solve the non-linear system of equations using the good initial guesses generated from the linear solution
% v has the good initial guesses passed from the main function flowrate_n

z=[0 0];% initializing z, the final solution vector
df2=v(1); df3=v(2);
x=[df2, df3];
% The below set of variables formulates the system exactly as in the main function flowrate_n

vo=2.1;%L
vt=2.06;%L
so=0.06;%mol/L
st=0.114;%mol/L

tn_=72;%h
tn=81;%h	nn=96;%h
dfilm=(1.649-0.975)/(tn-tn_);%l/h
od1=158;%OD
od2=166;

mu=(log(od2)-log(od1))/(tn-tn_);
dx=mu*od2;
dqm=0.833;
y=(od2-od1)/dqm;
dqt=dx/y;
dflt=dfilm/dqm*dqt*(tn-tn_);
s1=0.98;%mol/L
s2=1;%mol/L

y = [vo, vt, so, st, df1t, dqt, tn, tnn, s1, s2];

% the below variables k,p,q,r,etc. store the expression values into one variable
k = y(5)*y(9)+x(1)*y(10)-y(6);
p = k-y(3)*(y(5)+x(1));
q = k-y(4)*(y(5)+x(1));
r = y(2)+(y(5)+x(1)-x(2))*(y(8)-y(7));
c = y(2);
l = -1/(y(5)+x(1));
u = 1/(y(5)+x(1)-x(2));
z(1)= 1*log(p/q)-u*log(r/c); %defining the flow rate df2
z(2)= y(1)-y(2)-(y(5)+x(1)-x(2))*(y(8)-y(7)); % defining df3
% df2 and df3 are sent back in the vector z
end

A.5. Other studies – Lipase activity measurements

A.5.1. Reasons for lipase activity measurements

We had been observing a plateauing of the lipid content curves at 60% as mentioned in the bioreactor studies both on acetate and VFAs. Also, in the batch study conducted on glucose by Mitchell Tai (Tai, 2012), a maximum lipid content of 62% was obtained. Thus, it is quite likely that an internal factor is preventing the cells from storing lipids to greater than 62% lipid content. One of the possible explanations is the lipase activity. Studies in literature point to the presence of lipase genes in Y. lipolytica (Corzo & Revah 1999; Fickers et al. 2003). Thus, it was worth investigating if the microbe was indeed producing detectable amounts of lipase in our bioreactor runs.

A.5.1. Extracellular lipase detection assay

The following materials are required: NaCl - Salt from Macron Chemicals, NaH2PO4.2H2O – Salt from sigma, Para-nitro phenyl butyrate (pPNB) – Liquid from Sigma Aldrich, Lipase from Candida rugosa – Lyophilized powder from Sigma Aldrich, Multiwell plate – Costar 3595 and Eppendorf tubes

a. Method to prepare the reaction buffer

1. Prepare 30 ml of 100 mM NaH2PO4.2H2O in milliQ water. (468 mg of the salt in 30 ml H2O)

2. Add NaCl to the solution to a concentration of 100 mM. (add 175.5 mg NaCl)

3. Use 10M NaOH to set the pH to 7.2.

4. Add pPNB to absolute ethanol to a stock concentration greater than 100 mM (e.g. 85.4 mg added to 1ml EtOH – concentration of 0.38 M)

5. Aliquot stock pPNB solution into the sodium phosphate buffer so as to obtain a final concentration of 1 mM of pPNB in the buffer (for the above example, 78.9 ul of 0.38 M solution added to the 30 ml buffer)

6. Store the solution in a refrigerator and wrap it with aluminum foil to avoid exposure to light

Note: With time, the solution gets more yellow (pPNB breaks down) and hence, the base OD obtained from the spectrophotometer would go up. It is best to avoid taking out the stock bottle
from the refrigerator often. Thus, the best approach is to aliquot 1 ml volume of the reaction buffer into many eppendorfs (~20) which can be used on an as needed basis.

b. Method to prepare the positive control lipase solution

1. Prepare a 25 ml solution of lyophilized lipase from *Candida rugosa* in milli-Q water at a concentration of 0.1 g/L and store it in refrigerator wrapped in aluminum foil.

2. Aliquoting the lipase solution into eppendorfs is advised.

c. Procedure

1. 196 ul of reaction buffer is required for each assay. Typically, the assay is repeated in duplicate for each sample. Also, the control is run each time an experimental sample is run. Accordingly, one might need to take out required number of eppendorf tubes out of the freezer. The tubes should be allowed to equilibrate to room temperature for some time.

2. The cell broth sample to be analyzed for lipase is centrifuged and the supernatant collected.

3. At this point, one can go to the plate reader room, activate the M2e device and set the reaction temperature to 37°C. The instrument will take a few minutes to reach 37°C.

4. The multi well plate, eppendorf with reaction buffer, eppendorf with lipase (positive control) and the cell supernatant are then taken into the plate reader room.

5. Into a chosen well, 196 uL of reaction buffer is added and 4 uL of control or supernatant is added depending on the sample. As mentioned before, both these samples are analyzed in duplicate.

6. Choose the option of ‘Automix – before first read for 5 s’ in the settings so as to ensure proper mixing of the lipase enzyme into the buffer. Choose 4 mins as the time for measurement. Absorbance is measured at 405 nm.
d. Analysis

The absorbance measured is correlated to the para nitrophenol produced according to Beer Lambert's law. Here the molar extinction coefficient of para nitrophenol is 14,775 M⁻¹cm⁻¹. The path length for a 200 μl volume is 0.596 cm.

The data collected is analyzed to obtain a plot of para nitrophenol concentrations with time. The initial time points fit into a linear trend and the slope of this trend provides the lipase activity. 1 unit of lipase activity is equivalent to 1 μmol of para nitrophenol produced per min. Thus the lipase activity would be obtained as Units/ml in the well which should be multiplied by 50 (4 μL diluted to 200 μ) to get the lipase activity in the cell broth.

A.5.2. Results

- Lipase activity obtained in the extracellular broth during the bioreactor run is plotted and shown in Fig A.1

- Maximum activity similar to that ~ 0.1 g/L of lipase from Candida rugosa

![Lipase activity in bioreactor](image)

Figure A.1. Lipase activity trend in bioreactor study
A.5.3. Interpretation of results – Estimate the effect of the lipase

Assuming activity towards lipid in the assay (para-nitrophenyl butyrate) is same as that towards the triacylglycerides stored by *Y. lipolytica*

- 1 unit of activity corresponds to a degradation rate of 1 uM of TAG (lipid)/min
- On day 4, assuming 50 g/L of lipid in reactor (as per our runs)
- And with a molecular weight of TAG = 806 (assuming tripalmitin)
- Moles of TAGs on day 4 of the run= 50/806 = 0.062 M
- So 150 units of lipase activity = 150 uM/min=0.00015 M /min
- Time needed to degrade all of the lipids available in the bioreactor = 0.062/0.00015 = 413 mins ~ 7 h

Perhaps not all of the measured extracellular lipase is therefore active against the lipids stored by the cells. It thus means that even with a fraction of that activity, the lipase would be effective in depleting the lipids in the vacuoles such that rate of lipid accumulation = rate of degradation. Hence, plateauing of the lipid content trend is being observed after 60%.

A.5.4. Conclusion of lipase study

The above study hints that lipase might indeed be playing a role in the plateauing of the lipid content. First, the study needs to be repeated on other bioreactor runs to make sure that lipase is detected each time and also to determine if the levels of lipase production vary between runs. The next steps involve identifying and knocking out the genes associated with lipase production to determine their involvement in the plateauing effect.


