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Microbial Bioconversion of Polypropylene by Yarrowia Lipolytica: Enhancing Bioconversion and Lipogenesis through Optimization of Fermentation Parameters

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MICROBIAL BIOCONVERSION OF POLYPROPYLENE BY *YARROWIA LIPOLYTICA*: ENHANCING BIOCONVERSION AND LIPOGENESIS THROUGH OPTIMIZATION OF FERMENTATION PARAMETERS

by

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Under the Direction of Eric S Gilbert, PhD

ABSTRACT

Plastic waste can serve as a feedstock for microbial bioconversion using a chemical/biological hybrid strategy. In this work, a bioconversion process for polypropylene (PP) to produce value-added oleochemicals was reported. This bioconversion process coupled thermal depolymerization with fermentation by the oleaginous yeast *Yarrowia lipolytica*. PP pellets were depolymerized by pyrolysis, generating oil that consisted of mainly branched chain fatty alcohols and alkenes. The oil was mixed with biodegradable surfactants and nutrients and mechanically homogenized. The resulting medium, termed OP4, was used for fermentation by *Y. lipolytica* strain 78-003. *Y. lipolytica* assimilated > 80% of the substrate over 312 h, including 86% of the fatty alcohols. *Y. lipolytica* produced up to 540 mg L^{-1} lipids, compared with 216 mg L^{-1} during growth in surfactant-based control medium. C 18 compounds, including oleic acid, linoleic acid, and stearic acid, were

the predominant products, followed by C 16 compounds palmitic and palmitoleic acid. Subsequently, an improved process was developed resulting in a PP-derived growth medium called OP5 that did not contain the emulsifier oleic acid and derived 81 percent of its carbon from PP. To improve on product yields during growth in OP5 medium, pH, inoculum density, C/N ratio, and osmolarity were optimized. This increased the product yield four-fold to 2.1 g L^{-1}, and led to 46 percent cellular lipid content, the highest content reported to date for plastic-to-lipid microbial bioconversion. The maximum lipid yield occurred under conditions that balanced cell growth versus lipogenesis. Using postconsumer PP, the lipid yield was significantly lower (0.74 g L^{-1}), likely due to toxic additives necessary for product stabilization. Overall, the work demonstrates the potential and the challenges associated with microbial bioconversion of plastics.

INDEX WORDS: Biotechnology, Bioconversion, Applied microbiology, Plastic, *Yarrowia lipolytica,* Oleochemicals

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DEDICATION

I would like to dedicate this work to the Mr. Mihreteab Woldegiorgis and Mrs. Saba Asihel, the strongest and most caring individuals I have ever known. Thank you for cultivating my curiosity , and never letting me succumb to challenges, but instilling in me the dogged perseverance that drives me today. I would also like to thank Yohana Mihreteab, Nahoam Mihreteab, Desalegn Ogbamichael, and Jerusalem Tekle for supporting me always, and being there whenever I needed you. This is for you.

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PP: Polypropylene

FA: Fatty Acids

1 INTRODUCTION

1.1 Plastics: versatile materials, abundant waste

1.1.1 The Waste Problem

Plastics, synthetic polymers derived from hydrocarbons are an essential part of modern global society. Due to their low density, cheap production cost, malleability, durability, and low conductivity, plastics are often preferred over traditional materials such as wood, metal, and glass (Andrady and Neal 2009; Geyer et al. 2017; Hahladakis et al. 2018; North and Halden 2013).The properties of plastics that lend to their extensive applicability also make them resistant to biodegradation. Because of their sterically hindered structure, hydrophobicity, and high molecular weight, they cannot undergo efficient microbial catabolism. Consequently, plastics have become a growing burden upon the environment.

In 2015, approximately 322 million tons of plastic products were produced worldwide, up from 180 million tons in 2000 (Geyer et al. 2017). With an average Compound Annual Growth Rate (CAGR) of 8.6% since 1950, combined with the strong correlation between global plastic waste generation and gross national income per capita (Geyer et al. 2017; Markets 2017), plastic production and waste generation is likely to continue to rise as developing nations worldwide continue to grow economically (Markets 2017). Roughly 50% of all plastics produced annually are utilized for disposable applications such as packaging and are discarded within a year of production (Geyer et al. 2017; Hahladakis et al. 2018). It has been estimated that 6.3 billion tons of plastic waste has been generated since 1950, with 12% of this plastic waste having been incinerated, and only 9% being recycled, leaving roughly 4.9 billion tons of non-biodegradable plastic accumulating in landfills and contaminating marine and soil ecosystems (Geyer et al 2017.).

1.1.2 Polypropylene

Polypropylene, the focus of this work, comprises 23.4% of the global plastics market share and is the second most abundant plastic compound produced worldwide (Markets 2017; Prof_Research_Reports 2008). In 2016, 62.3 million tons of polypropylene were produced globally, and production is estimated to reach 83.5 million tons by 2022 (Markets 2017; Prof_Research_Reports 2008). This trend of increasing plastic use is likely to continue as developing nations worldwide grow economically, and consumer products are mass produced with as well as packaged in plastic (Andrady and Neal 2009; Geyer et al. 2017; Hopewell et al. 2009; Narancic and O'Connor 2017; North and Halden 2013; Wierckx et al. 2018), resulting in increased quantities of plastic waste entering the environment and the associated toxicological consequences.

1.2 Valorizing plastic waste to reduce plastic pollution

1.2.1 Bioconversion: a renewable solution

One component of a solution to plastic pollution is to reduce the amount of postconsumer plastic entering the environment. An important element in accomplishing this goal is to give postconsumer plastic waste some economic value, i.e. to valorize it, so it is not readily discarded. In principle, standard plastic recycling techniques meet this objective, but in practice, recycled plastic products cannot easily compete with products made from virgin materials, especially when the price of petroleum is low. Approaches are needed where the input costs are low, and the value of the output is high (Hopewell et al. 2009; Mantia 2004; Narancic and O'Connor Kevin 2017; Wierckx et al. 2018; Wierckx et al. 2015). A bioconversion process can be a viable solution to the plastic waste accumulation problem. Microbial bioconversion, the conversion of natural substrates

into value-added products by microorganisms, has long been employed in industry. Several examples are presented in the following sections.

1.2.2 Therapeutics

Bioconversion has been a valuable process used in pharmaceutical production. Bioconversion, both cell-free enzymatic and whole cell bioconversion, can be used to produce antibiotics, antifungals, and anti-cancer agents. Bioconversion has been used to produce gramicidin S from immobilized *Bacillus brevis* cells (Vandamme 1981), glucoside derivatives from Tetracycline by *Bacillus lichenformis* enzymes (Pandey et al. 2018), and enzymes extracted from wastewater microbial communities to cleave and deactivate antibiotics in effluent (Zumstein and Helbling 2019). Antifungals have also been produced through bioconversion, as chitinase has been produced through both whole cell (Wang et al. 2005) and enzymatic (Asif et al. 2019) processes, and the novel antifungal Anidulafungin through expression of *Actinoplanes utahensis* deacytelase genes in *Streptococcus sp*. (Shao et al. 2013). Cancer therapeutics produced through bioconversion include arctigenin and orobol, compounds synthesized through enzymatic bioconversion (Abari and Tayebi 2019; Kim et al. 2020), and Matairesinol, a lignin-derived compounds produced through whole-cell bioconversion in recombinant *Escherichia coli* expressing reductases (Kuo et al. 2014).

1.2.3 Industrial chemicals production

Bioconversion is more often employed in industrial chemical production, for compounds used as preservatives, food flavorings, industrial and food dyes, in food and cosmetics industries, and as energy sources. Citric acid, a preservative and flavoring agent employed in a variety of roles, is produced through bioconversion of substrates such as agricultural waste and glycerol with *Aspergillus niger* (Alam et al. 2011; Jamal et al. 2007; Yang et al. 2014). Vanillin, and its

derivative vanilic acid, is a compound derived from vanilla bean that is used widely in the food, cosmetics and pharmaceutical industries as a flavor enhancer, fragrance additive, and intermediary chemical (Abdelkafi et al. 2006; Di Gioia et al. 2009). Vanillin and vanillic acid are often derived through whole-cell bioconversion of ferulic acid, and in rare occasions lignocellulosic hydrolysate, by recombinant *E. coli* or halophilic bacteria (Abdelkafi et al. 2006; Di Gioia et al. 2009; Horvat et al. 2019; Luziatelli et al. 2019; Tang and Hassan 2020). Pigments, employed as food coloring or fabric dyes, can also be derived via bioconversion. Tea polyphenols have been used to produce red, yellow and orange pigments via enzymatic bioconversion with laccase enzymes (Wang et al. 2018). Another food product that can be derived via bioconversion are sugar alcohols, sugar derivatives that are commonly used as low-calorie sweeteners. These sugar alcohols are often derived from starch hydrolysate, oil waste, and glycerol bioconversion by yeast such as *Aspergillus tereus, Y. lipolytica, Candida magnoliae, Candida tropicalis,* and *Debaryomyces guillermondii* (Arruda and Felipe 2008; da Silva et al. 2018; Li et al. 2015; Liu et al. 2017; Liu et al. 2019; Miura et al. 2015; Paidimuddala 2014; Yang et al. 2015a).

1.2.4 Biofuels

Maybe the most popular use of microbial bioconversion is the production of biofuels, energy sources derived from biomass, such as bioalcohols, biodiesel, methanol, and biohydrogen. Biofuels are often split up into first and second generation biofuels. First generation biofuels are produced from a feedstock that is unsustainable or, like sugars derived from food crops and cooking oils, negatively affects the food supply. In contrast, second generation biofuels are synthesized from sustainable and non-edible substrates such as lignocellulosic and hemicellulosic biomass, cooking waste, and algal feedstock (Bialy et al. 2011). Yeast and algae are the most employed biocatalysts for biofuel production (Canilha et al. 2012; Marella et al. 2018; Nanda et

al. 2014; Saini et al. 2020; Yan et al. 2014). Ethanol, the earliest synthesized biofuel and the most abundant substance produced through bioconversion, is often synthesized by yeast that can undergo substrate-level fermentation such as *Saccharomyces cerevisiae* (Canilha et al. 2012; Gregg and Saddler 1995; Parmar and Rupasinghe 2013; Yu et al. 2019). Glucose derived from food crop, crop waste, and crop waste hydrolysates are the usual feedstocks, and while *S. cerevisiae* is oft utilized, *Pichia sp., Aspergillus sp., Candida sp.,* and even bacteria such as *Clostridium beijerickii* can be employed to produce ethanol and other bioalcohols, such as butanol, 1,3 propanediol, and propanol (Arruda and Felipe 2008; Canilha et al. 2012; Gregg and Saddler 1995; Huang et al. 2014; Huang et al. 2017; Jutakridsada et al. 2019; Kreuger et al. 2011; Li et al. 2015; Nanda et al. 2014; Niehus et al. 2018; Paidimuddala 2014; Parmar and Rupasinghe 2013; Yu et al. 2019; Zheng et al. 2012; Zhu et al. 2012)

Biodiesel is a hydrophobic energy source derived from glucose, glycerol, and animal and vegetable oil and oil waste as feedstocks. Biodiesel is often produced by oleaginous microbes as whole cell biocatalysts to produce excess storage lipids, which are then recovered and derivatized prior to use (Abghari and Chen 2014; Chai et al. 2019; Fontanille et al. 2012; Huang et al. 2014; Huang et al. 2017; Marella et al. 2018; Tai and Stephanopoulos 2013; Yan et al. 2014). Oleaginous microbes are microbes that can accumulate a large amount of storage lipids as a physiological response to nutrient availability (Abghari and Chen 2014; Ageitos et al. 2011; Bialy et al. 2011; Kitcha and Cheirsilp 2011; Papanikolaou and Aggelis 2011). These include algae such as *Chlorella sp., Morterella sp.,* and *Mycene sp.,* and yeast such as *Lypomyces sp., Rhodosporidium sp., Rodotorula sp., Cryptococcus curvatus, and Yarrowia lipolytica* (Ageitos et al. 2011; Kitcha and Cheirsilp 2011; Papanikolaou and Aggelis 2011). Often, to improve yield, organisms are engineered to over-produce storage lipids, *allowing* some yeast and algae, such as *Y. lipolytica*, to

accumulate up to 80% of their cell weight as lipids (Abdel-Mawgoud et al. 2018; Friedlander et al. 2016; Kamisaka et al. 2007; Lazar et al. 2018; Liu et al. 2015; Marella et al. 2018; Qiao et al. 2015; Tai and Stephanopoulos 2013; Xu et al. 2016).

1.2.5 A blueprint for the bioconversion of plastics

Microbial bioconversion technology can be an viable strategy for reducing postconsumer plastic waste (Narancic and O'Connor Kevin 2017; Silva et al. 2018; Wierckx et al. 2015). Specifically, microbial growth media prepared from depolymerized plastics can be used as feedstocks for bioconversion processes (Guzik et al. 2014; Mihreteab et al. 2019). The rationale for this concept has two parts: first, several kinds of postconsumer plastics can be depolymerized into mixtures of labile molecules (Guzik et al. 2014; Mihreteab et al. 2019; Scott et al. 1990; Soják et al. 2007) and second, microbes can synthesize triacylglycerols, organic acids, enzymes and other biological products as they grow on the plastic-derived media (Fickers et al. 2005; Thevenieau et al. 2010). Depolymerized polystyrene, polyethylene, and polyethylene terephthalate (PET) have previously been employed as substrates for microbial production of biopolymers (Guzik et al. 2014; Kenny et al. 2008; Ward et al. 2006), and this valorization strategy may be employed to generate a variety of other products from other plastics, such as polypropylene.

1.3 Lipid storage and *Y. lipolytica*

1.3.1 Lipids in cell physiology and metabolism

Lipids are classified as small biomolecules which are insoluble in water, but can be solubilized in organic solvents (Mlíčková et al. 2004; Murphy 2001; Papanikolaou and Aggelis 2011).Lipids are crucial for cell structure synthesis and integrity, energy storage and production, intercellular and intracellular signaling, and apoptosis and membrane fusion mediators(Mlíčková

et al. 2004; Murphy 2001; Papanikolaou and Aggelis 2011; Saini et al. 2020). There are eight types of lipids : fatty acids, polyketides, sterols and sterol derivatives, glycerolipids, phospholipids, sphingolipids, and prenols (Klug and Daum 2014; Mlíčková et al. 2004; Murphy 2001; Papanikolaou and Aggelis 2011). The lipid types being explored as products of the bioconversion process are storage lipids. To avoid lipid toxicity and membrane disruptions due to excess polar lipids, organisms store surplus fatty acids and sterols as biologically inert storage lipids in the form of triacylglycerols (TAGs) and sterol ester (SEs). These reservoir lipids serve as reserve energy sources (TAGs) and membrane lipid constituents (SEs).

1.3.2 The storage of neutral lipids

These inert lipid molecules are stored in cellular structures known as lipid droplets, or lipid bodies. Lipid droplets are composed internal of TAGs and surrounded by multiple layers of SEs (Mlíčková et al. 2004; Murphy 2001). When the cell, due to nutrient limitations or physiological stressors, requires energy, TAGs are hydrolyzed by TAG lipases into free fatty acids, which are further oxidized and processed via catabolic pathways for ATP production, incorporated into cell membranes, used as signaling molecules, or used a building blocks for more complex lipid molecules (Mlíčková et al. 2004; Papanikolaou and Aggelis 2011; Rostron and Lawrence 2017). SEs are mobilized by SE hydrolysases into sterols and incorporates into cell and organelle membranes when needed. TAGs are of specific commercial interest due to their versatile and impactful uses in a variety of industries, including as edible vegetable oil alternatives, intermediate chemicals, lubricants, surfactants, cosmetic and pharmaceutical emulsifiers and stabilizers, and biofuels (Dulermo et al. 2015; Klug and Daum 2014; Murphy 2001; Paik et al. 2009; Papanikolaou and Aggelis 2011; Rostron and Lawrence 2017).

1.3.3 Yarrowia lipolytica

The microbe of interest for the bioconversion process being employed is *Y. lipolytica*, a well characterized, non-conventional dimorphic yeast. *Y. lipolytica* can grow on a wide variety of hydrophobic substrates and can produce a wide array of intracellular and extracellular products, including storage lipids, free fatty acids, organic acids, and extracellular enzymes and other proteins (citations from previous paper, plus new ones). *Y. lipolytica* is an oleaginous yeast and can produce high lipid yields when grown on appropriate carbon sources, leading to its application in biodiesel production. Its heavily studied genome and metabolism allows *Y. lipolytica* to be altered to increase both the volume and variety of compounds it can generate ().

Previous works have employed *Y. lipolytica* to derive microbial lipids from carbon sources varying from lignocellulosic biomass, crude glycerol, glucose, corn hydrolysate, and waste cooking oil. Wild-type and engineered *Y. lipolytica* strains have used these substrates to generate yields as high as 20 g L^{-1} and 70% cell lipid content (citations from discussion of first paper, plus new sources). Its ability to utilize varying carbon sources, its well-defined and easily engineered genome, and its oleagenicity make *Y. lipolytica* an ideal model organism for the bioconversion of polypropylene to produce high lipid titers.

1.4 Hypotheses and project objectives

The major aim of this project was to create a bioprocess that valorizes PP plastic. This is a novel undertaking for two reasons: (1) there has never been any research published on PP bioconversion or (2) plastic-to-lipid bioconversion. While there has been previous plastic bioconversion work done using polyethylene, PET and polystyrene (Guzik et al. 2014; Kenny et al. 2008; Ward et al. 2006), there has never been published works detailing a bioconversion process

utilizing PP plastic. Of the previous plastic bioconversion works, all centered using polyethylene and polystyrene polymers to produce the biodegradable polymers polyhydroxyalkanoate and polyhydroxybutyrate and did not endeavor to produce any oleochemicals. PP plastic was picked for the bioprocess because of its relative abundance in the environment, and because there have not been previous attempts to use PP plastic to produce value added chemicals

My process would employ a pretreatment procedure to depolymerize the monomers comprising PP plastic, creating a bioavailable hydrocarbon-rich feedstock. I hypothesized that successful depolymerization of PP would create a hydrocarbon-rich medium that can further be emulsified and supplemented with nutrients to make a viable microbial medium for lipid production. The project aims were to use *Y. lipolytica* as a microbial cell factory for bioconversion of this PP-derived medium. *Y. lipolytica*'s ability to utilize a wide variety of carbon sources, especially hydrocarbons, makes it an appealing microbe for the bioprocess. *Y. lipolytica's* oleagenicity also gives us a valuable end product, as storage lipids potentially have commercial value. This bioprocess would demonstrate successful valorization off PP plastic, showing it can be used as a carbon source for microbial lipid production.

After proof of concept, I then aimed to improve the process efficiency by optimizing medium and fermentation conditions to maximize lipid production. There were two strategies I considered: (1) metabolic engineering of the *Y. lipolytica* strain to increase lipid storage, and (2) process and fermentation engineering to create a medium and fermentation process optimal for lipid production for the specific *Y. lipolytica* strain. My work focused on the second strategy.

After creating a refined medium and fermentation process tailored to lipid production by our *Y lipolytica* strain, I aimed to replicate the process using postconsumer PP waste. Initial studies employed research grade virgin PP pellets, which allowed for replicable and consistent feedstock

generation; yet this feedstock did not accurately represent postconsumer PP waste, which contains added compounds and stabilizers, and has been exposed to environmental stressors. Incorporating postconsumer PP waste investigated real-world applicability. In this work, I present the findings.

2 MICROBIAL BIOCONVERSION OF THERMALLY DEPOLYMERIZED POLYPROPYLENE BY *YARROWIA LIPOLYTICA* FOR FATTY ACID PRODUCTION

2.1 Introduction

Plastic pollution is an environmental threat that continues to expand in scope. In 2015, more than 320 million tons of plastic products were produced worldwide, up from 180 million tons in 2000 (Prof_Research_Reports 2008). With an average Compound Annual Growth Rate (CAGR) of 8.6% since 1950 (Geyer et al. 2017), combined with the strong correlation between global plastic waste generation and gross national income per capita (Geyer et al. 2017), plastic production and waste generation are likely to continue to rise as developing nations worldwide continue to grow economically (Geyer et al. 2017; Markets 2017; Prof_Research_Reports 2008). Of concern, if historic trends continue, a significant fraction of plastic can be expected to reach the environment: of the estimated 6.3 billion tons of plastic waste generated since 1950, 12% was incinerated and 9% was recycled, leaving approximately 4.9 billion tons of plastic accumulating in landfills and marine and soil ecosystems (Geyer et al. 2017).

One facet of a solution to plastic pollution is to reduce the amount of postconsumer plastic entering the environment. An important element in accomplishing this goal is to give postconsumer plastic waste economic value, i.e. to valorize it, so it is not readily discarded. Standard plastic recycling techniques generally yield materials that are of lower quality than the initial product, resulting in “downcycling”, or a loss of value (Mantia 2004). In contrast, a biological approach to recycling plastic waste can potentially be economically sustainable: plastic

waste can be used as feedstocks for bioconversion processes to make biochemicals and other value-added biological products, resulting in “upcycling” . The rationale for this concept has two parts: first, several kinds of postconsumer plastics can be depolymerized into mixtures of labile molecules (4) and second, microbes can synthesize triacylglycerols, organic acids, enzymes and other biological products as they grow on plastic-derived media .This approach can be used to make products that justify the costs associated with recycling plastic waste.

Polypropylene (PP) comprises nearly 25 percent of the global plastics market share (Geyer et al. 2017), yet to date there are no reported microbial bioconversion processes for PP. A challenge for microbial metabolism of PP is its low bioavailability, resulting from its high molecular weight, hydrophobicity and a molecular structure that resists enzymatic attack (Arutchelvi et al. 2008; Jeyakumar et al. 2013; Longo et al. 2011). We hypothesized that a depolymerization pretreatment to generate PP-derived oligomers could aid in the production of a growth medium suitable for microbial bioconversion. We used pyrolysis to depolymerize and oxidize PP and then used biodegradable surfactants to disperse the resulting products in an aqueous medium suitable for microbial growth. The PP-derived growth medium was used for cultivating the oleaginous yeast *Yarrowia lipolytica*. *Y. lipolytica* grows on diverse substrates and produces a wide variety of intracellular and extracellular products, including fatty acids, organic acids, extracellular enzymes and other proteins (Abghari and Chen 2014; Ageitos et al. 2011; Aggelis 2002; Beopoulos et al. 2008; Bialy et al. 2011; Fickers et al. 2005; Rakicka et al. 2015b; Xu et al. 2016; Xue et al. 2013; Zhang et al. 2014). In this work, *Y. lipolytica* was cultivated in a PP-derived medium to produce FAs. The following report summarizes and discusses our findings.

2.2 Methods

2.2.1 Polypropylene Growth Medium Preparation

OP4 medium, a polypropylene-derived medium, was prepared by pyrolyzing virgin amorphous polypropylene (Mw=14,000) in 3 g batches at 540° C in 125 ml borosilicate flat-bottomed flasks for 190 min. The resulting pyrolysis oil (at 15 g L^{-1}) was combined with additional compounds as follows: 5.4 g L^{-1} Tween- 80®, 4.5 g L^{-1} oleic acid, 1.25 g L^{-1} $(NH_4)_2SO_4$, 2.5 g L^{-1} KH_2PO_4, and 0.830 g L^{-1} $MgSO_4 \cdot 7H_2O$. The mixture was emulsified with a hand-held food-grade homogenizer and autoclaved for 70 min at 121 °C and 15 psi.

2.2.2 Chemicals and Reagents

Virgin polypropylene pellets and oleic acid were purchased from Sigma Aldrich (Millipore Sigma, USA). Tween 80®, chloroform, methanol, cyclohexane, hexane, and all culturing compounds used were of research grade and purchased from Fisher Chemicals (Fisher Scientific, USA).

2.2.3 Cultures and Growth Conditions

Yarrowia lipolytica strain 78-003 (ATCC strain 46483) was the sole strain used in this work. Glycerol frozen stocks of *Y. lipolytica* were prepared (1 ml, OD_{600} = 20) and were stored at -80 °C. For all experiments, a frozen aliquot was thawed and added into a 250 ml Erlenmeyer flask containing 50 ml of 5% glucose medium (consisting of 50 g L^{-1} glucose and 3 g L^{-1} yeast extract). Each inoculated flask was incubated overnight at 30 °C with shaking at 200 rpm. After incubation, 1 ml samples were withdrawn and centrifuged at 10,000 rpm for 2 min, and the pellets were washed twice with 50 mM phosphate buffered saline (PBS) solution. *Y. lipolytica* was cultured using 500 ml Erlenmeyer flasks containing 50 ml OP4 medium; the flasks were inoculated with an overnight

culture of *Y. lipolytica* at an inoculation density of 0.3 (OD_{600} ml^{-1}) and incubated at 30 °C with shaking at 200 rpm.

Several versions of OP4 were prepared to evaluate the impact of medium components on lipid and biomass yields. 'Nitrogen and trace minerals only' medium consisted of: 1.25 g L^{-1} $(NH_4)_2SO_4$, 1.25 g L^{-1} yeast extract, 2.5 g L^{-1} KH_2PO_4, and 0.830 g L^{-1} $MgSO_4 \cdot 7H_2O$. 'Surfactant only' medium was comprised of 5.4 g L^{-1} Tween- 80® and 4.5 g L^{-1} oleic acid, without trace minerals or nitrogen. 'PP only' medium, which was OP4 medium without any surfactants, was comprised of 12 g L^{-1} pyrolyzed polypropylene, 1.25 g L^{-1} $(NH_4)_2SO_4$, 1.25 g L^{-1} yeast extract, 2.5 g L^{-1} KH_2PO_4, and 0.830 g L^{-1} $MgSO_4 \cdot 7H_2O$. 'Surfactant control' medium was comprised of 5.4 g L^{-1} Tween- 80®, 4.5 g L^{-1} oleic acid , 0.25 g L^{-1} $(NH_4)_2SO_4$, 1.25 g L^{-1} yeast extract, 2.5 g L^{-1} KH_2PO_4, and 0.830 g L^{-1} $MgSO_4 \cdot 7H_2O$.

The hexadecane medium used for FA profile experiments consisted of: 5% v/v hexadecane, 5 g L^{-1} yeast nitrogen broth (w/amino acids), 0.5 g L^{-1} KH_2PO_4, and 0.25 g L^{-1} MG_2SO_4._Starch medium used for FA profile experiments consisted of: 50 g L^{-1} hydrolyzed starch, 2 g L^{-1} yeast nitrogen broth (w/ amino acids), 0.5 g L^{-1} KH_2PO_4, and 0.25 g L^{-1} MG_2SO_4. OP5 medium consisted of: 5.4 g L^{-1} Tween- 80®, 1.25 g L^{-1} $(NH_4)_2SO_4$, 2.5 g L^{-1} KH_2PO_4, and 0.830 g L^{-1} $MgSO_4$. Glucose (5%) medium used for FA profile experiments consisted of: 50 g L^{-1} glucose and 3 g L^{-1} yeast extract.

2.2.4 Microscopy for Imaging Oil Droplet Production

A modified Nile Red staining method (Rostron and Lawrence 2017) was utilized to stain *Y. lipolytica* intracellular neutral lipids. Sample aliquots (1 ml) of post-fermentation culture were withdrawn and centrifuged, and pellets were washed twice with 0.9% NaCl. Pellets were suspended in 1.0 ml 10 mM PBS with 0.15M KCl and the OD_{600} was adjusted to 10. Technical

grade Nile Red powder (Millipore Sigma, USA) was dissolved in acetone to make a 10 mg mL^{-1} solution and 10 µl was added to 1.0 ml cell samples (suspended in 50 mM PBS and calibrated to an OD of ~5.0) in dark conditions, and samples were vortexed and incubated at room temperature (in dark conditions) for approximately 15 min. A Zeiss LSM 510 confocal microscope (Zeiss, USA) was used to visualize stained cells. 10 µl of sample were used for confocal analysis. Cells were excited at 488 nm, and emissions were imaged at 543 nm.

2.2.5 Intracellular Lipid Quantification

A modified Bligh and Dyer extraction (Bligh and Dyer 1959) was used to extract lipids from yeast samples. Post-fermentation OP4 culture was withdrawn and aliquoted into pre-weighed 50 ml conical tubes, and tubes were centrifuged, and pellets washed twice with 0.9% NaCl. Pellets were lyophilized overnight using a BT3.3 EL Lyophilizer Tabletop (SP Scientific, USA), weighed, and suspended in a 2:1 v/v chloroform: methanol mixture (5 ml per 50 mg cell dry weight) and sonicated using a Sonic Dismembrator (Fisher Scientific, USA) at 20 kHz, 20% amplitude with pulsing (40 s on, 20 s off for a total working time of 20 min). The chloroform layer was taken and dried under a nitrogen stream. Dried lipids were weighed and derivatized for GC/MS analysis via base-catalyzed esterification with 2.5 ml sodium methoxide (0.1 M) (Milanesio et al. 2013). The reaction was quenched using 200 µl sulfuric acid (>95%), and 2.5 ml hexane was added to each sample, which was then vortexed and centrifuged (5 min at 5000 rpm). 1.5 ml from the top hexane layer was withdrawn for GC/MS analysis.

2.2.6 GC/MS Analysis of Lipid Profile

The FA profile was characterized with an Agilent 7890A gas chromatograph (GC) attached to a 5977A mass spectrometer detector and equipped with an Agilent J&W HP-5ms UI capillary column (30 mm x 0.25 mm x 0.25 µm). 1 µl samples were injected via an Agilent 7693 Automatic

Liquid Sampler in splitless mode with an inlet temperature of 275 °C, using helium as the carrier gas at a flow rate of 1 ml min^{-1}. GC oven temperature was held at 60 °C for 1 min, and ramped to 100 °C (rate: 25 °C min^{-1}; hold 1 min). The oven temperature was then increased to 200 °C (rate: 25 °C min^{-1}; hold 1 min). The oven temperature is then increased to 220 °C (rate: 5 °C min^{-1}; hold 7 min) and then increased to an ending temperature of 300 °C (rate: 25 °C min^{-1}; hold 2 min). A C8-C24 FAME analytical standard was used during sample analyses as an external standard (Sigma Aldrich, USA).

2.2.7 Pyrolysis oil characterization via GC/MS

3 g pyrolysis oil was dissolved in 200 ml chloroform (Sigma Aldrich, USA) and 1.5 µl aliquots were analyzed via GC/MS. A GC method (Guzik et al. 2014) was used to characterize the polypropylene pyrolysis oil. 1 µl of the diluted pyrolysis oil was injected via automatic liquid sampler at an inlet temperature of 275 °C and a 2:1 split ratio. The oven method was 30 °C for 1 min, then ramping to 100 °C (rate:7.5 °C min), then ramping to 300 °C (rate 10 °C min^{-1}, hold 2 min).

2.2.8 Substrate Degradation Analysis

Substrate degradation analysis was done both gravimetrically and via GC/MS. 10 ml-aliquots of OP4 medium pre- and post-fermentation were taken for analysis. Media was centrifuged (10 min at 7000 rpm) and 5 ml of media transferred to pre-weighed conical tubes and wet weight of media documented. Samples were lyophilized and weighed, and the dry cell weight was suspended in a 1:1:1 v/v mixture of hexane, chloroform and deionized water (15 ml) and vortexed until sample was fully dissolved. Afterward, 1.5 ml of the top hexane layer was withdrawn and used for GC/MS analyses.

2.2.9 Growth Measurements

One ml of *Y. lipolytica* overnight culture was pelleted (10,000 rpm, 10 min) and pellets were washed twice with 50 mM PBS, and then resuspended in 1 ml 50 mM PBS to reach a final OD_{600} of 15. The inoculum was used to inoculate all experiments involving growing cells at the initial optical densities indicated in the text. At set timepoints, 1 ml aliquots were withdrawn and OD_{600} measurements were taken using an Eppendorf 6131 Biophotometer (Eppendorf, USA).

For gravimetric analysis of growth, 50 ml aliquots were withdrawn, centrifuged at 10,000 rpm for 10 min, the supernatant discarded, and cell pellets were washed twice with 50 mM PBS and lyophilized overnight before being weighed.

2.2.10 Cyclohexane toxicity assay

Yeast malt broth (10 gL^{-1} dextrose, 5 g L^{-1} malt extract, 3 g L^{-1} peptone, and 5 g L^{-1} yeast extract) was used to cultivate *Y. lipolytica* cells. 1 ml aliquots of overnight culture (inoculation density = 0.30 [600 nm]) were used to inoculate 50 ml of either yeast malt broth only or yeast malt broth with 0.23% w/v cyclohexane. One ml samples were withdrawn at select timepoints, and growth was measured spectrophotometrically (600 nm).

2.3 Results

2.3.1 Polypropylene thermal depolymerization

Pyrolysis of virgin amorphous polypropylene (PP) pellets generated an oil-like fluid that turned waxy when cooled rapidly. GC/MS analysis of the PP oil identified approximately 18 different compounds across the batches that were prepared. More than 80% of PP oil was branched chain compounds, with branched fatty alcohols (50.9%) and branched alkenes (25.1%) making up approximately 75% of all available carbon sources (Figure 2.1). The branched alkenes detected were all C_nH_{2n} compounds, with the most abundant compound being 2,4-dimethylhept-1-ene (~14%), followed by 2,4,6,8-tetramethyl-1-undecene (~6%) and 1,4-dimethyl-decene (~5%). The branched fatty alcohols detected were C_nH_{2n+2} compounds, with 2-hexyl-1-decanol (~41%) being the predominant compound, followed by 2-methyl-1-decanol (~10%).

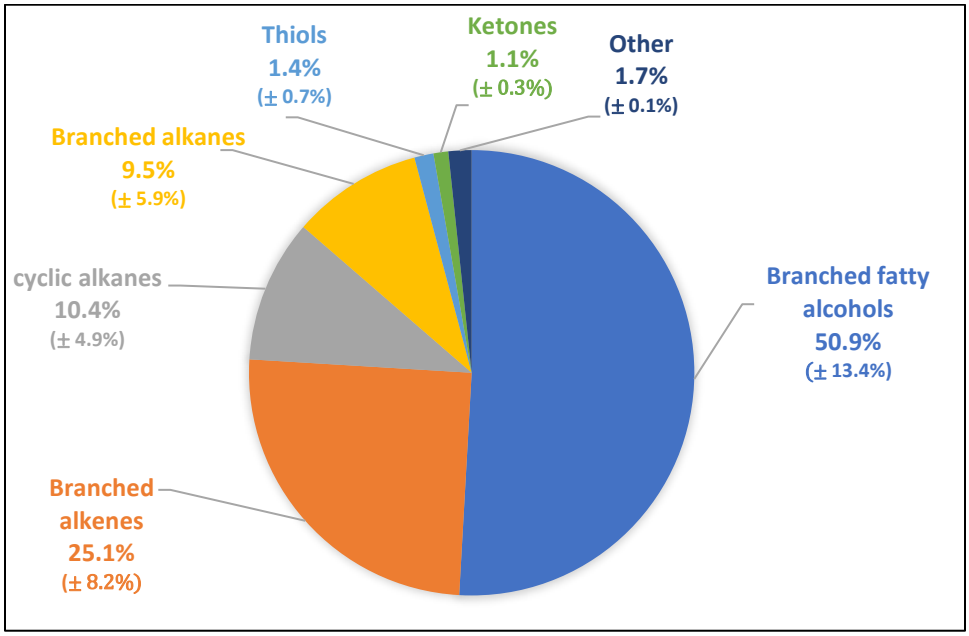


Figure 2.1 *Carbon composition of PP oil after thermal depolymerization.*

2.3.2 Analyzing Y. lipolytica growth and activity in OP4 medium: comparisons with a 'surfactant control' medium

48.9 percent of the total carbon in OP4 medium, on a moles C basis, was derived from the biodegradable surfactant. The remainder of the carbon in the medium was found in the PP oil, with trace amounts derived from the supplemented yeast extract. To determine the extent to which the PP oil in OP4 contributed to *Y. lipolytica* growth and to formation of biochemical products, a series of experiments were conducted comparing the activity of *Y. lipolytica* grown in OP4 medium versus a 'surfactant control' medium. The 'surfactant control' was identical in composition to OP4 medium except that it contained no PP oil.

2.3.3 Measuring Y. lipolytica growth

To determine the extent that PP oil contributed to *Y. lipolytica* growth, cell growth was measured gravimetrically. The analysis indicated an average of 27% less biomass when cells were grown on OP4 medium (Figure 2.2a). Biomass accumulation peaked after 72 h. The biomass remained at 2.4 g L^{-1} or greater through 192 h.

2.3.4 OP4 uptake during growth

To determine the extent of OP4 medium uptake during growth, the mass of medium remaining in solution was measured. More than 80 percent of OP4 medium was taken up after 13 days (312 h) (Figure 2.2b). OP4 medium contains 51 percent fatty alcohols which originate from PP and are produced during pyrolysis. To determine whether medium components originating from PP were taken up by *Y. lipolytica*, the change in fatty alcohol concentration in the medium was analyzed via GC/MS (Figure 2.2b). Approximately 51% of fatty alcohols in OP4 medium were taken up by *Y. lipolytica* by 120 h, with 86% of the fatty alcohols used by 312 h. The uptake of total FAs and fatty alcohols occurred in a similar fashion over the course of experiments.

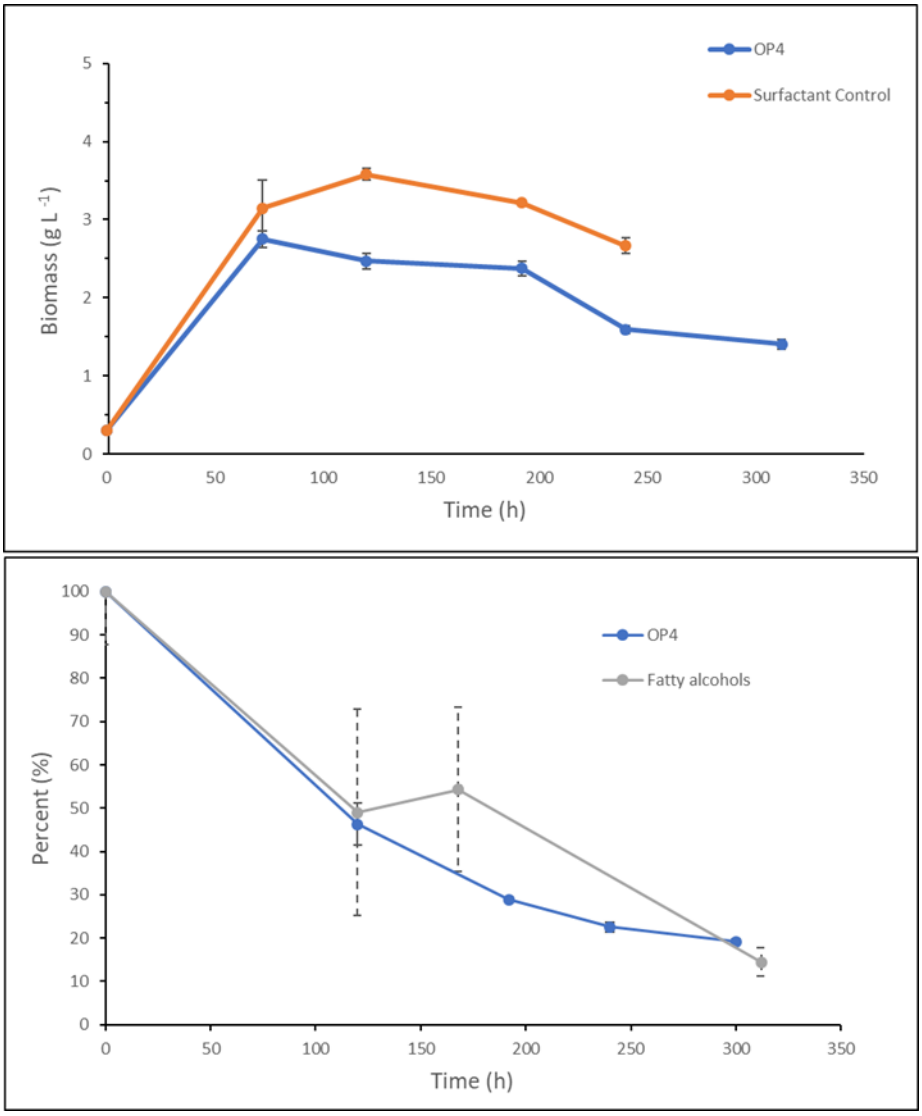


Figure 2.2 *Growth of Y. lipolytica in OP4 medium.* ***(a)*** *Growth in OP4 medium versus a 'surfactant control' medium. Growth was measured as cell dry weight. The 'surfactant control' included all emulsifiers, nitrogen, phosphorous, and trace nutrient sources found in OP4 medium but did not include PP-derived compounds.* ***(b)*** *OP4 medium use during Y. lipolytica growth. The change in OP4 concentration was measured gravimetrically. Fatty alcohol consumption was measured by GC/MS. Mean ± SD plotted for each data set.*

2.3.5 Impact of OP4 components on growth and FA production

OP4 medium contains several components that contribute to the overall growth of *Y. lipolytica*. To determine the effect of individual components of the medium on cell growth, a series of comparisons were made (Figure 2.3). Yeast extract was used as a supplemental nitrogen source

and contributed approximately 25 percent of the maximum measured biomass. A comparison of the 'surfactant only' and 'surfactant control' treatments confirmed that supplemental nitrogen and salts were necessary to increase the yield. Comparing the 'PP only' and 'OP4' treatments indicated that the presence of surfactant in the medium increased the yield more than six-fold. On the other hand, comparisons of 'OP4' and 'surfactant only' treatments or 'PP only' and 'nitrogen and trace minerals only' treatments determined that PP oil in the growth medium inhibited *Y. lipolytica* growth. Although PP oil in OP4 medium inhibited growth, it significantly increased the FA yield (Table 2.1).

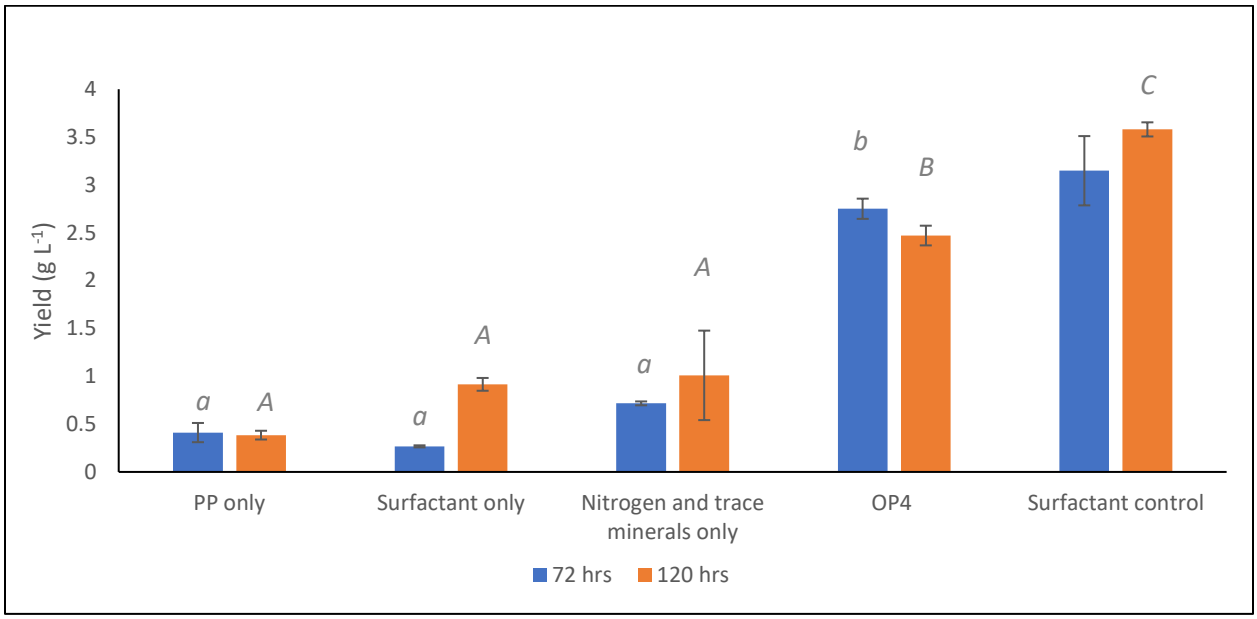


Figure 2.3 *Growth on OP4, 'surfactant control', and their various individual components. Growth was measured gravimetrically. Significance determined by ANOVA. Samples were considered significantly different if $p < 0.05$. Treatments that share the same lower case or upper case letter are not significantly different than one another.*

2.3.6 Effect of cycloalkanes on Y. lipolytica growth

Cyclic alkanes have been reported elsewhere to be poor growth substrates for *Y. lipolytica* and other industrial yeasts (Beam and Perry 1974; Das and Chandran 2011; Mauersberger et al. 1996). OP4 contains 0.15 ± 0.08 percent (w/v) cyclic alkanes. To determine whether cyclic alkanes

inhibited growth of *Y. lipolytica* at the concentration that they are found in OP4 medium, we compared growth in a rich medium with or without 0.23 percent (w/v) cyclohexane added (Figure 2.4). Cyclohexane was selected as a representative cyclic alkane. The presence of cyclohexane reduced cell growth relative to control cells by 72 hours, a difference which persisted through 120 hours. The extent of growth inhibition ranged from 6 percent to 32 percent over the course of the experiment.

Table 2.1 *Intracellular lipid yields by Y. lipolytica during growth in OP4 medium or its components*[ab]

	Yield (mg/L)	Significance
OP4	526.3 ± 12.5	*a*
Surfactant-based control	215.9 ± 20.2	*b*
Surfactant only'	117.6 ± 9.3	*c*
Nitrogen+ trace minerals'	7.3 ± 1.9	*d*
PP + Nitrogen +trace minerals'	2.8 ± 0.4	*d*

[a]Significance determined by ANOVA. Samples were considered significantly different if $p < 0.05$. [b]Treatments that share the same lower case letter are not significantly different than one another.

2.3.7 Microscopy analysis of lipid accumulation

Lipid accumulation by *Y. lipolytica* cells grown in OP4 medium or 'surfactant control' medium was analyzed by Nile Red staining and confocal microscopy followed by quantitative image analysis. Differences in the cellular lipid content were visibly noticeable in compositely stained cells (Figure 2.5a). Cells grown in OP4 medium tended to aggregate compared to those grown in surfactant-based medium. The mean fluorescence for cells grown in OP4 medium was nearly twice that of cells growing in surfactant-based medium ($p < 0.05$), indicating greater lipid accumulation when cells were grown in OP4 medium (Figure 2.5b).

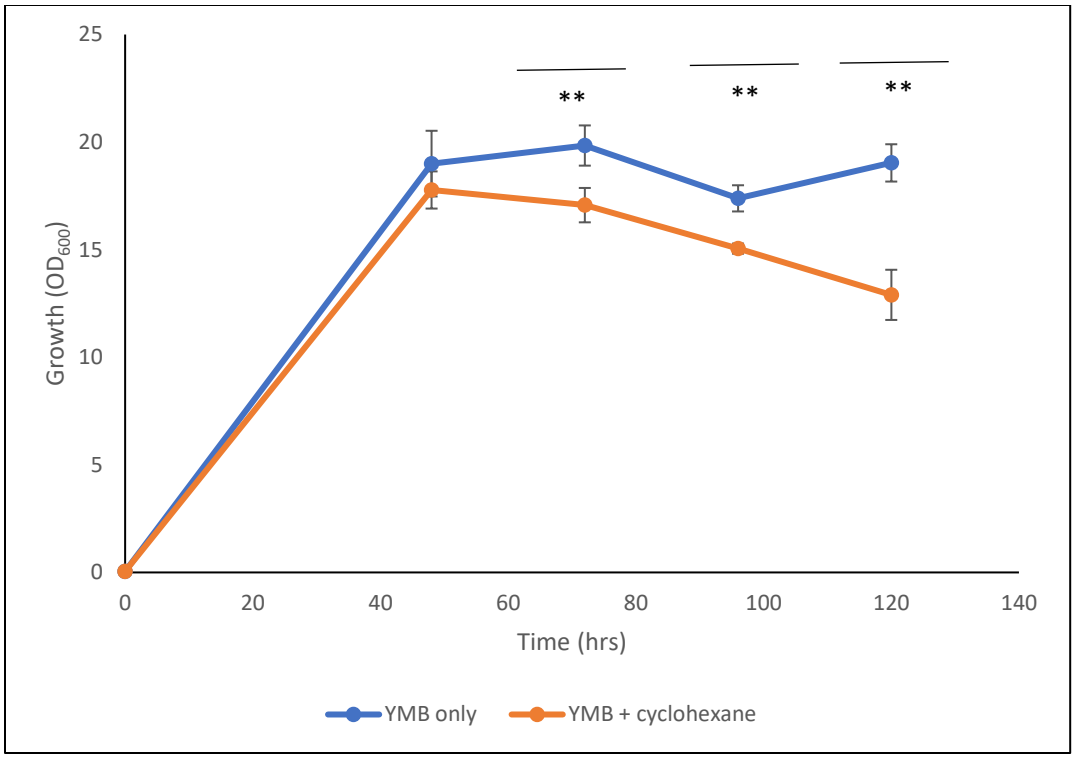


Figure 2.4 *Growth of Y. lipolytica on rich medium with and without 0.3% w/v cyclohexane. Growth was measured as OD600 absorbance via spectrophotometer at various timepoints. Significance determined by student's T-test. (**) indicates a significant difference ($p < 0.01$) between treated samples and the untreated control.*

2.3.8 Fatty acid yields during growth in OP4 medium versus surfactant control

To determine the extent that PP contributed to the production of fatty acids by *Y. lipolytica*, a comparison of FA production between cells grown in OP4 medium versus surfactant-based medium was conducted. Lipid yields were determined via GC/MS analysis. FA yields were significantly higher when *Y. lipolytica* grew on OP4 medium compared to the 'surfactant control' medium (Figure 2.6a). This was true at each of the measured timepoints over the course of experiments. *Y. lipolytica* generated most of its lipid bulk between 72 and 120 h**.** The bulk of the FAs produced were C-18, followed by C-16, with small amounts of C-20 and trace amounts of C-14 FAs at 240 h (Figure 2.6b). The bulk of the FAs produced were unsaturated; there was no significant difference in the proportion of unsaturated to saturated FAs between the 'surfactant

control' and the OP4 medium (Figure 2.6c). However, there was a significant increase in the percentage of saturated FAs produced as the experiment progressed: between 72 h and 240 hr the fraction of saturated FAs increased from 10 ± 1 percent to 35 ± 8 percent ($p < 0.05$). The majority of FAs produced were monounsaturated C18:1 FAs (Figure 2.6d).

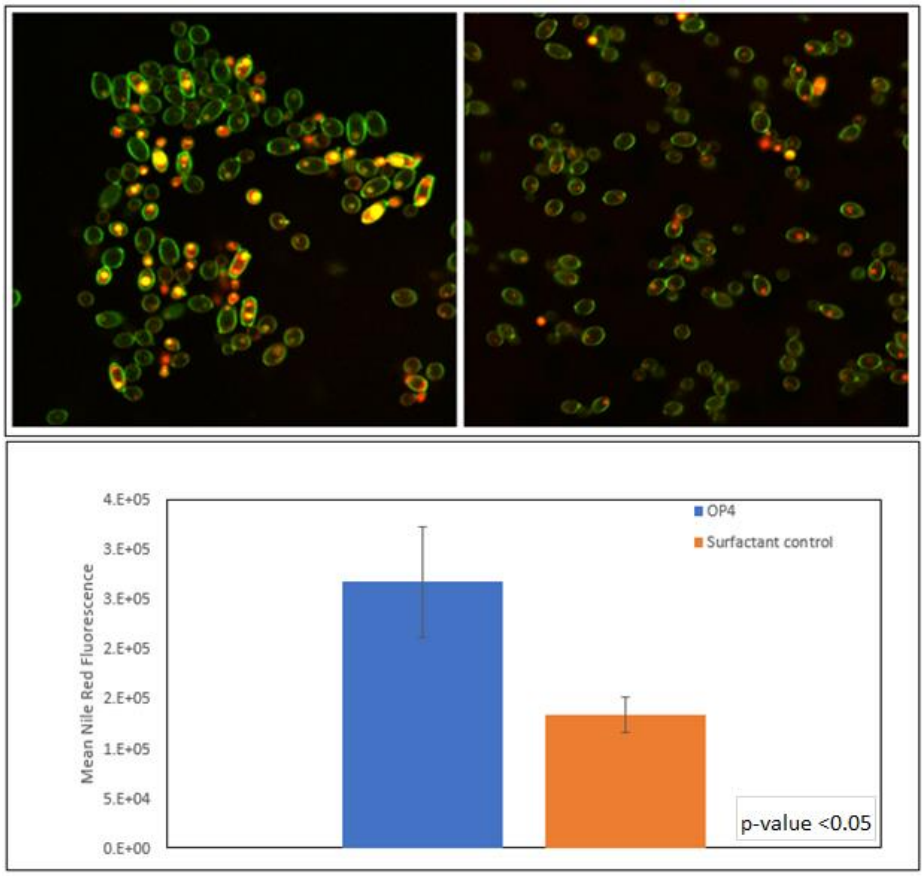


Figure 2.5 *FA production during growth in OP4 medium. Cells were imaged after 96 h. Magnification 400×. (a) left, OP4 medium; right and surfactant control. Note lipid inclusion bodies in yellow. (b) Mean Nile Red fluorescence quantified by digital image analysis.*

2.3.9 Effect of lipids or carbohydrates on FA production

Oleaginous yeast are often grown on carbohydrates and produce FAs by *de novo* biosynthesis; in contrast, OP4 medium contains hydrocarbons derived from PP depolymerization and from biodegradable surfactants, likely inducing *ex novo* FA biosynthesis. To help understand how the composition of OP4 affected FA production, *Y. lipolytica* was grown in media containing

lipids or carbohydrates as the carbon source (Figure 2.7). The substrates that were examined were: glucose (5%), starch (2%), hexadecane (5%) and OP4 medium (1.5% PP-derived compounds). Hexadecane was selected as a representative hydrophobic substrate found in OP4 medium. The C-18 FA oleic acid was the dominant FA produced by *Y. lipolytica* during growth in each medium except hexadecane. C-16 and C-18 FA distribution was consistent among cells grown on both carbohydrate-based substrates and OP4, with deviation occurring on hexadecane-grown cells. Palmitic or palmitoleic acid, C-16 FAs, were the second most abundant, followed by C-18 stearic acid. In hexadecane-containing medium, saturated palmitic acid was the main FA produced. Only 5 FAs were produced when *Y. lipolytica* was grown in OP4 or hexadecane-containing medium. In contrast, the FA profiles of *Y. lipolytica* grown on glucose and starch were more complex, with 10 different types of FAs produced, including the shorter chain FAs myristic, dodecanoic, pentadecanoic, and tridecanoic acids.

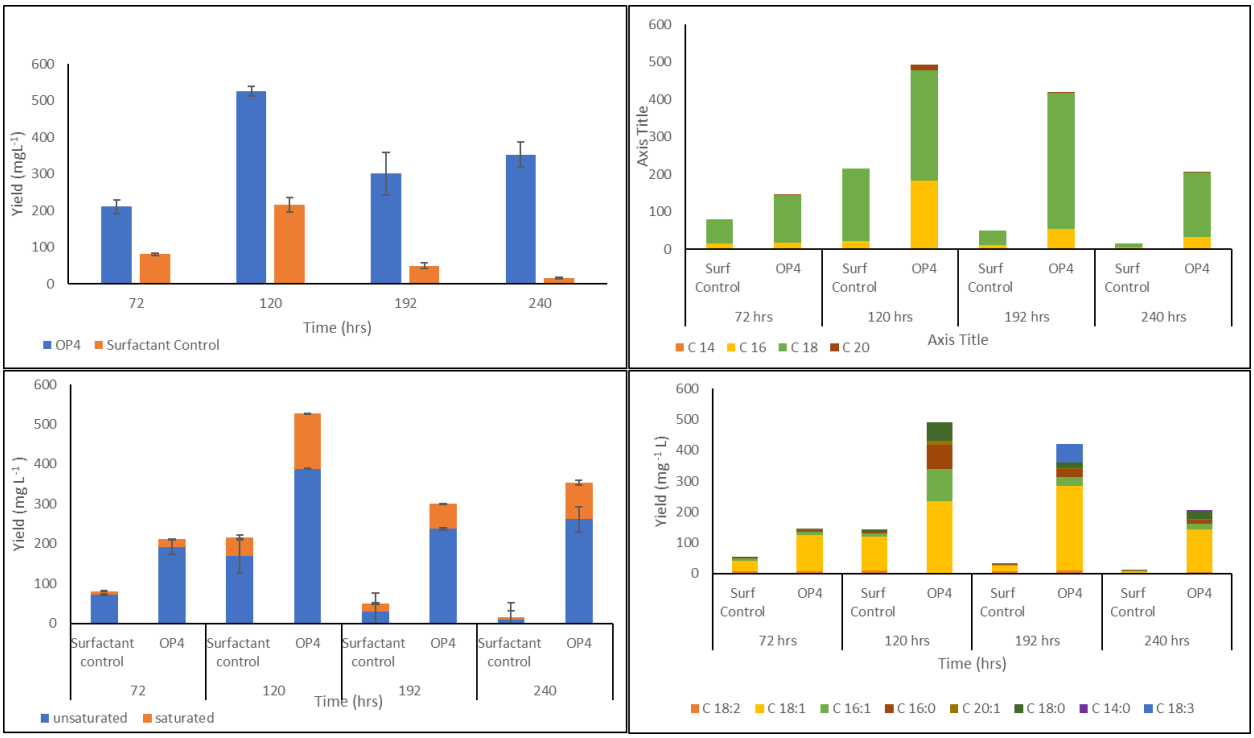


Figure 2.6 *Analysis of fatty acid production by Y. lipolytica during growth on OP4 medium vs surfactant control* ***(a)*** *total FA yield.* ***(b)*** *distribution of FAs by carbon number.* ***(c)*** *distribution of saturated and unsaturated FAs* ***(d)*** *distribution of unsaturated FAs.*

To determine whether the PP content of OP4 influenced the type of FA products produced, the FA profiles from growth in OP4 medium versus in the 'surfactant control' medium were compared (Table 2.2). Several features were evident. First, with the exception of 192 h, palmitoleic acid was a greater fraction of the FAs produced in OP4 medium than the 'surfactant control'. Conversely, at each timepoint except 120 h, the palmitic acid fraction was larger in the 'surfactant control'. Second, there was a spike in the fraction of oleic acid in the OP4 medium with respect to the 'surfactant control' at 192 h. Lastly, very long chain fatty acids (≥ C-20) were only found in cells grown in OP4 medium.

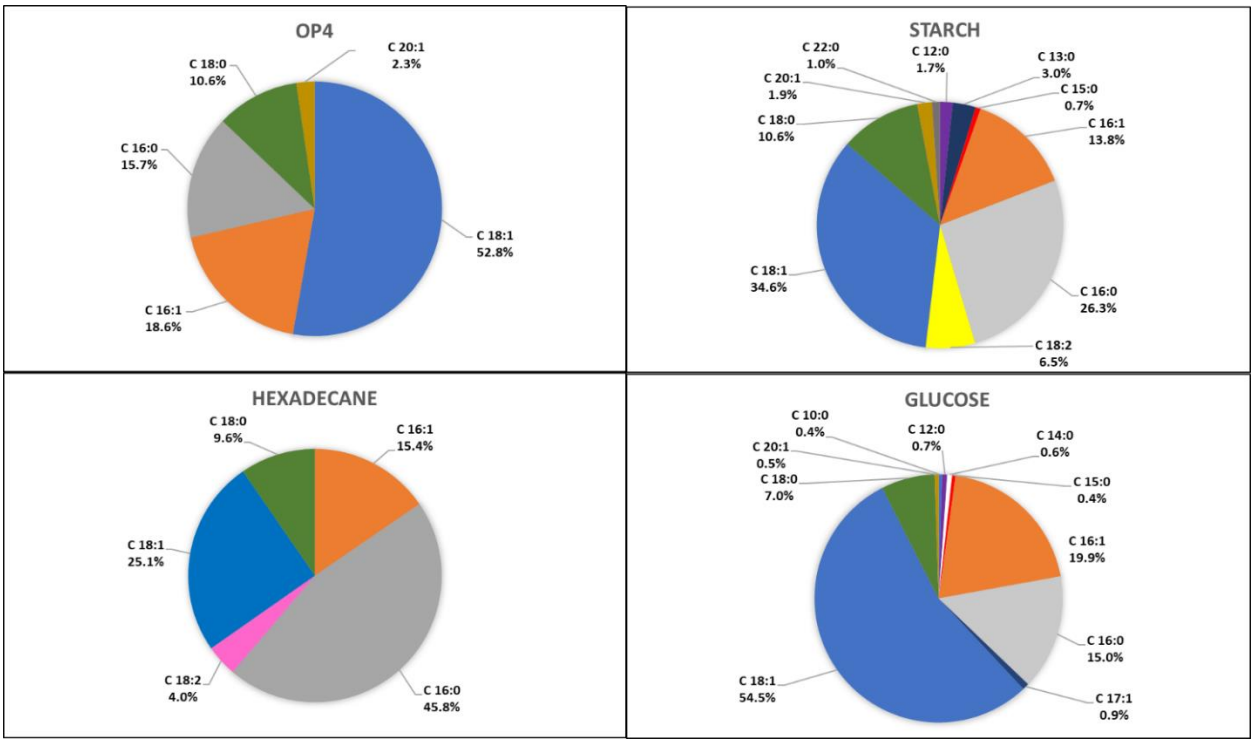


Figure 2.7 *Effect of lipid- versus carbohydrate-containing media on fatty acid production. Cells were grown for 120 h prior to analysis.*

2.4 Discussion

If plastic waste can be used in bioconversion processes, then an incentive will exist to keep it out of the environment. In this work we demonstrated that compounds generated from PP pyrolysis can be transformed by *Y. lipolytica* into fatty acids suitable for use in industry or other diverse applications. We are not aware of work by others reporting PP as a growth substrate for microbial bioconversion. The yield of FAs produced by *Y. lipolytica* during growth in OP4 medium was comparable to related bioconversion processes. There are limited examples of bioconversion processes where a plastic was used as the feedstock, but in the most related process to the work presented here, Guzik used polyethylene (PE) pyrolysis oil for polyhydroxyalkanoate (PHA; "bioplastic") production by *Pseudomonas sp.* (Guzik et al. 2014). After 48 h, 84.4% of the PE oil was metabolized, producing 0.23 g L^{-1} CDW, of which 9.8% was PHA, a biomass to

substrate yield of 0.14 g gC^{-1} and a PHA to substrate yield of 0.01 g gC^{-1} (Guzik et al. 2014). In comparison, growth of *Y. lipolytica* 78-003 in OP4 medium yielded 2.34 g L^{-1} CDW, a biomass to substrate yield of 0.13 g gC^{-1}, and a FA to substrate yield of 0.03 g gC^{-1} (0.54 g L^{-1} FAs). Similarly, when *Y. lipolytica* was grown on 5 g L^{-1} food oil waste, a FA-rich feedstock similar in hydrophobicity and carbon content to OP4 medium, the yield was 3 g L^{-1} CDW and 0.75 g L^{-1} FAs after 6 days (Bialy et al. 2011). In general, lower FA accumulation is observed during growth of oleaginous yeast on hydrophobic substrates and additional measures must be taken to increase the yield of FAs (Papanikolaou and Aggelis 2011).

Table 2.2 *Fatty acid profiles of Y. lipolytica grown in OP4 medium or surfactant control medium*[ab].

	72 hrs		120 hrs	
Name	**Surfactant Control**	**OP4**	**Surfactant Control**	**OP4**
C 16:0	9.6 ± 0.9	6.4 ± 0.1	10.2 ± 0.8	**15.7 ± 0.3**
C 16:1	4.1 ± 0.3	**6.6 ± 0.4**	5.5 ± 0.6	**18.6 ± 0.2**
C 18:0	1.7 ± 0.1	2.2 ± 0.4	11.0 ± 0.4	10.6 ± 0.7
C 18:1	67.4 ± 1.3	68.2 ± 4.5	51.8 ± 4.8	52.8 ± 1.1
C 18:2	17.2 ± 1.3	15.0 ± 3.4	21.5 ± 2.7	n.d.
C 20:1	n.d.	0.74 ± 0.1	n.d.	2.3 ± 0.1
Other[d]	n.d.	0.95 ± 0.1	n.d.	n.d.
	192 hrs		**240 hrs**	
Name	**Surfactant Control**	**OP4**	**Surfactant Control**	**OP4**
C 14:0	n.d.	n.d.	n.d.	0.8 ± 0.2
C 16:0	**21.3 ± 0.4**	5.8 ± 0.5	**21.8 ± 2.1**	4.8 ± 1.0
C 16:1	3.6 ± 1.9	6.6 ± 0.7	4.8 ± 1.3	**21.6 ± 0.6**
C 18:0	**17.9 ± 0.4**	**4.1 ± 0.4**	20.3 ± 2.7	20.1 ± 0.7
C 18:1	50.4 ± 11.0	**79.1 ± 15.1**	45.0 ± 9.0	44.5 ± 6.6
C 18:2	6.7 ± 2.3	3.3 ± 2.8	**10.0 ± 2.1**	7.3 ± 0.5
C 18:3	n.d.	0.1 ± 0.01	n.d.	n.d.
C 20:1	n.d.	0.9 ± 0.1	n.d.	0.7 ± 0.1

[a] Values are percent of total FAs present at the indicated time. Mean ± SD.
[b] At each time point, Student's t test was used to evaluate whether there was a significant difference in the amount of each compound produced. Values that are significantly greater ($p < 0.05$) are in **bold** font.

[c] n.d.; not detected.
[d] compounds include heptadecanoic and tridecanoic acids

Did cultivating *Y. lipolytica* on a PP-derived growth substrate impact FA production? By comparing FA production in OP4 medium versus a 'surfactant control', a few differences in the FA profile were evident, notably an increase the palmitoleic acid fraction, the presence of C-20 compounds and a larger product yield. It remains to be seen in future experiments whether specific PP-derived compounds directly influenced the observed FA product profile. On the other hand, a comparison of the C-16 and C-18 FA profile measured in this work during growth in OP4 medium with FA profiles reported by others revealed a remarkable similarity (Table 2.3). These data suggest that the *Y. lipolytica* FA profile is influenced to a significant extent by cellular metabolism rather than substrate characteristics.

Additionally, by comparing FA profiles for cells grown on hydrophobic substrates (OP4 medium, hexadecane) and cells grown on hydrophilic substrates (glucose, starch), some differences were evident, notably the greater diversity of FAs produced during growth on the carbohydrate media. In general, the observed FA profiles were consistent with *de novo* lipid synthesis during growth on glucose and starch, where FAs are formed as secondary metabolites after nitrogen depletion warrants carbon storage and *ex novo* lipid synthesis during growth on OP4 medium and hexadecane, where FA synthesis is a growth-coupled process that assimilates hydrophobic substrates into lipids while simultaneously utilizing them for growth and maintenance (Papanikolaou and Aggelis 2011). Overall, the data support the view that FA production by *Y. lipolytica* in a PP-derived medium is similar to FA production during growth on naturally-occurring substrates.

Table 2.3 *C 16 and C 18 FA profiles of Y. lipolytica growing on various media in this and other work. Values are reported as percentages. After [36]*

	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2	Source
OP4 medium	19	16	11	54	n.d.	this work
Starch	29	15	12	38	7	this work
Glucose	16	21	7	56	n.d.[a]	this work
Hexadecane	46	15	10	25	4	this work
Glycerol	15	2	11	47	21	(Aggelis 2002)
Glycerol	12	11	9	57	11	(André et al. 2009)
Glycerol	13	17	6	55	7	(Makri et al. 2010)

[a] n.d.; not detected.

Two additional points regarding the presented data warrant further comment. First, *Y. lipolytica* did not grow as extensively in OP4 medium as it did in the 'surfactant control'. The most likely explanation is that there were growth-inhibitory compounds associated with PP that were not present in the control, mainly cyclic alkanes. Cyclic alkanes are not an adequate growth substrate for *Y. lipolytica*, as the P450 monooxygenases mainly responsible for hydrophobic substrate oxidation during assimilation are not able to oxidize cyclic alkanes (Beam and Perry 1974; Das and Chandran 2011; Mauersberger et al. 1996). Our work shows that concentrations of cyclic alkanes as minimal as 0.3% w/v impeded growth. Notably, in spite of the lower biomass yield during growth on OP4 medium, the FA yield was significantly higher. Cellular stress, including nutrient limitations (Aggelis 2002; André et al. 2009; Beopoulos et al. 2008; Kitcha and Cheirsilp 2011; Klug and Daum 2014; Kuttiraja et al. 2016) increases FA storage by *Y. lipolytica*;

chemical toxicity may have caused a similar stress response. Second, the PP component of OP4 medium contributed to *Y. lipolytica* growth and FA production. This result was evident in three ways. 1) gravimetric analysis of changes in the growth medium mass during experiments determined that 81% of the medium was taken up by *Y. lipolytica*. Even if all the non-PP components of OP4 medium were consumed first, these were less than half of the medium, meaning that at least 30% of the substrate taken up by *Y. lipolytica* was PP-derived. 2) The concentration of branched fatty alcohols in OP4 medium declined by 86% over the course of experiments. These compounds were the most abundant constituent of PP oil and were not part of the biodegradable surfactant in OP4 medium; the reduction in concentration indicates that *Y. lipolytica* assimilated at a minimum one major component of PP oil. 3) *Y. lipolytica* produced considerably more FAs when grown on OP4 media than when grown on the 'surfactant control', indicating that PP-derived compounds contributed to FA production.

The production of FAs by *Y. lipolytica* can be optimized by altering fermentation conditions and by metabolic engineering. For example, supplementing a food oil waste-derived growth medium with 10 g L^{-1} glucose increased the biomass yield from 3 g L^{-1} to 13 g L^{-1} with a concomitant increase in the FA yield from 0.75 g L^{-1} to 7.3 g L^{-1}. Additionally, the bioavailability of the growth substrate can be increased; for example, bioconversion of waste cooking oil was enhanced by ultrasonication of the growth medium, leading to an increase in FA production (Patel and Matsakas 2018). When the FA degradation and remobilization genes, *Pox1-6* and *TGL4*, were inhibited, *Y. lipolytica* grown on 250 g L^{-1} glycerol was able to obtain a lipid yield of 15.5 g L^{-1}, with lipid content constituting 31% CDW (Rakicka et al. 2015b). Qiao (2015) determined that simultaneous overexpression of *Y. lipolytica* stearyl coA desaturase, acetyl-CoA carboxylase, and diacylglycerideacyl-transferase genes yielded a strain with fast cell growth and a high lipid titer

(55 g L^{-1}) (Qiao et al. 2015). Based on these results, it is likely that a strategy can be found to increase the yield of FAs for cells grown in OP4 medium. More specifically, we hypothesize that adding carbon sources such as glucose or glycerol will favor biomass accumulation during the growth of the inoculum, and the larger microbial population should lead to quicker uptake of PP-derived compounds and greater FA accumulation. Alternatively, we hypothesize that overexpressing enzymes responsible for *ex novo* FA biosynthesis including fatty alcohol dehydrogenase, fatty alcohol oxidase, and fatty aldehyde dehydrogenase will favor FA accumulation over catabolism by peripheral pathways, leading to increased yields. Overall, we think that bioconversion can be part of a terminal recycling solution for plastic waste.

3 ENHANCING POLYPROPYLENE BIOCONVERSION AND LIPOGENESIS BY *YARROWIA LIPOLYTICA* THROUGH OPTIMIZATION OF FERMENTATION PARAMETERS

3.1 Introduction

Plastic pollution in the environment is a challenging problem that requires innovative strategies to solve. Microbial bioconversion of plastics for producing value-added products is potentially part of the solution (Cacciari et al. 1993; Guzik et al. 2014; Kenny et al. 2008; Mihreteab et al. 2019; Narancic and O'Connor 2017; Silva et al. 2018; Skariyachan et al. 2017; Syranidou et al. 2019; Ward et al. 2006; Wierckx et al. 2018; Wierckx et al. 2015). Microbial bioconversion and biodegradation processes have previously been reported for polyesters, polyethylene, polystyrene, and nylon (Brandon et al. 2018; Chertkov O Fau - Sikorski et al. 2011; Deguchi et al. 1997; Kawai et al. 2014; Ribitsch et al. 2012; Skariyachan et al. 2017; Syranidou et al. 2019; Tanasupawat et al. 2016; Wei et al. 2014; Yang et al. 2018b; Yang et al. 2015b; Yang et al. 2015c; Yoshida et al. 2016). In recent work, we developed a process for microbial bioconversion of polypropylene (PP) that used the oleaginous yeast *Yarrowia lipolytica* to produce fatty acids (Mihreteab et al. 2019). This process employed pyrolysis to depolymerize PP and create a hydrocarbon- and fatty alcohol- rich oil. The oil was mixed into a nutrient-supplemented aqueous solution using biodegradable emulsifiers, including oleic acid, resulting in a PP-derived growth medium named OP4. *Y. lipolytica* was able to assimilate more than 80% of OP4, producing a yield of up to 530 mg L^{-1} lipids, of which 59 percent was derived from PP (Mihreteab et al. 2019). After demonstrating the potential for PP bioconversion using OP4 medium, we endeavored to make a PP-derived growth medium that required less adjuvants and a process that could yield more product. In this work, we report on OP5 medium, a PP-

derived growth medium that is free of added oleic acid and derives 77 percent of its carbon from polypropylene. We evaluated several approaches to increase the product yield during the growth of *Y. lipolytica* in OP5 medium and then examined the impact of using postconsumer PP waste in a bioconversion process.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Virgin amorphous polypropylene pellets (Mw= 14,000) were purchased from Sigma Aldrich (Millipore Sigma, USA). Tween 80®, chloroform, methanol, cyclohexane, and hexane and all culturing compounds were of research grade and purchased from Fisher Chemicals (Fisher Scientific, USA).

3.2.2 Polypropylene Growth Medium Preparation

OP5 medium was prepared as follows. 125-ml flat-bottomed borosilicate flasks were used to pyrolyze either virgin PP pellets or sheared postconsumer PP in 3 g batches at 540 °C for 195 minutes. The pyrolysis oil (at 15 g L^{-1}) was combined with: 5.4 g L^{-1} Tween- 80®, 5 g L^{-1} yeast nitrogen base, 2.5 g L^{-1} KH_2PO_4 , 2.5 g L^{-1} yeast extract and 1.25 g L^{-1} $MgSO_4 \cdot 7H_2O$. The mixture was emulsified with a hand-held food-grade homogenizer. To compensate for the lack of oleic acid as an emulsifier as was used in OP4 medium, we increased the homogenization time of OP5 medium from 90 seconds (mixing time for OP4 medium) to 3 minutes. The resulting homogenized medium was autoclaved for 30 min at 121 °C and 15 psi.

3.2.3 Cultures and Fermentation Conditions

Yarrowia lipolytica ATCC strain 46482 was the sole strain used in this work. Overnight cultures were prepared from 1 ml 60 % glycerol stocks of *Y. lipolytica* stored at -80 °C. For all experiments, frozen cells were thawed and used to inoculate 250 ml Erlenmeyer flasks containing 50 or 100 ml of Yeast-Extract-Peptone-Dextrose medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, and 20 g L^{-1} dextrose). Inoculated flasks were incubated overnight at 30 °C with shaking at 200 rpm overnight. All shake flask fermentations were conducted using 500 ml Erlenmeyer flasks containing 50 ml OP5 medium; triplicate flasks were inoculated with an overnight culture of *Y. lipolytica* at varying inoculum densities as indicated and incubated at 30 °C with shaking at 200 rpm.

3.2.4 Growth Measurements

Y. lipolytica growth measurements were taken spectrophotometrically and gravimetrically. Aliquots of 1 ml were withdrawn, and growth was measured by optical density at 600 nm using an Eppendorf 6131 Biophotometer (Eppendorf, USA). For growth measurements using gravimetric methods, 50 ml volumes of culture were withdrawn after fermentation and centrifuged at 7,000 rpm. Supernatant was decanted and stored, and cell pellets were washed twice with 50 mM Phosphate Buffered Saline (PBS) solution. Cells were then flash frozen in liquid nitrogen and lyophilized overnight using a BT3.3 EL Lyophilizer (VirTis/SP Scientific, USA) and weighed on an analytical balance.

3.2.5 Microscopy

Brightfield microscopy images were taken using an AmScope BL120c microscope (United Scope, California) equipped with an Amscope MD35 camera attachment at 1000x magnification. Slides

were prepared by aseptically pipetting 10 □l of OP5 medium containing *Y. lipolytica* after 120 h growth. No stains were used.

3.2.6 Fermentation Optimization

For pH experiments, the pH of OP5 medium was adjusted to 4.0, 5.0, or 6.0 using 2.0 M H_2SO_4 or 4.0 M NaOH. A Corning 320 pH meter (Corning, USA) was used to measure pH. For inoculum density experiments, OP5 medium was inoculated at an inoculum density of either 1.0, 3.0, or 6.0. To prepare the cultures, the optical density of the overnight culture was measured (600 nm). Based on the measurement, the required volume of cells was collected and centrifuged at 4,000 rpm. Pelleted cells were washed twice with 50 mM PBS solution and used to inoculate OP5 medium. For carbon-nitrogen ratio experiments, the nitrogen content of OP5 medium was adjusted by modifying the amount of yeast nitrogen base (contains 15% nitrogen w/v) and yeast extract (contains 10% nitrogen w/v) added to the OP5 medium prior to autoclaving, and a medium pH of 6.0 and inoculum density of 3 were used carbon-nitrogen ratios of 20, 40, 80, and 100 were tested. For osmolarity experiments, NaCl was used to adjust the medium's osmolarity. NaCl was added to OP5 medium at concentrations of 0.75, 1.5 and 3 g L^{-1} prior to autoclaving, and a C/N ratio of 80, inoculum density of 3, and pH of 6.0 were used. For all experiments, cells were grown for 120 hours.

3.2.7 Substrate Assimilation Analysis

The uptake of growth substrate in OP5 medium was measured gravimetrically. 50 ml of either pre-fermentation or spent OP5 medium were added to 50 ml conical tubes and lyophilized. After all moisture was removed, the remaining mass of medium was weighed, and substrate uptake was determined as the different in the mass of medium solids before and after fermentation.

3.2.8 GC/MS analysis of OP5 hydrophobic fragments

We quantified the consumption of organic constituents of OP5 medium during fermentation by GC/MS. Liquid-liquid extractions were used to collect the organic constituents before and after the growth of *Y. lipolytica* in OP5 medium. Fifty ml hexane was added to 50 ml pre-fermentation or spent OP5 media, and the mixture was transferred to 250 ml screw cap Nalgene tubes, sealed with Teflon tape, and shaken at 30 ºC and 150 rpm for 24 hours. After incubation, samples were centrifuged at 5000 rpm for 10 minutes, and 1.5 ml of the hexane (top) layer was withdrawn and used for GC/MS analysis. The GC method of Guzik et al. (2014) was used to characterize and quantify the constituents of the hexane layer (Guzik et al. 2014). Details about the GC/MS instrumentation and protocol are described in a section below. One µl was injected via automatic liquid sampler at an inlet temperature of 275 °C. The oven method was: 30 °C for 1 min, then ramping to 100 °C (rate:7.5 °C min^{-1}), then ramping to 300 °C (rate 10 °C min^{-1}, hold 2 min). Separated peaks were quantified using MassHunter® Qualitative Analysis software (Agilent, USA).

3.2.9 OP5 medium components: impact on product yield

Media were made using the various components of OP5 medium. Each component medium was made using deionized water and buffered to a pH of 6.0 using 0.1 M HCL or 0.1 M NaOH . Tween ® 80 only medium contained 5.4 g L^{-1} Tween ® 80 surfactant only. PP-only medium was composed solely of 15 g L^{-1} PP oil . Tween ® 80 + nutrients medium was composed of 5.4 g L^{-1} Tween ® 80 surfactant, 5 g L^{-1} yeast nitrogen base, 2.5 g L^{-1} KH_2PO_4 , 2.5 g L^{-1} yeast extract and 1.25 g L^{-1} $MgSO_4 \cdot 7H_2O$.

Component media experiments were carried out in 500 ml Erlenmeyer flasks containing 50 ml medium, and *Y. lipolytica* was inoculated at an inoculum density of 1.0. Samples were placed

in a shaking incubator for 120 h at 30 °C. After fermentation, samples were transferred to 50 ml conical tubes, and centrifuged at 4,000 rpm for 10 minutes. The supernatant was decanted and cells washed twice with 50 mM PBS before being lyophilized, weighed as described above, and lipids extracted and quantified as described in the following section.

3.2.10 Intracellular Lipid Quantification

A previously used modified Bligh and Dyer extraction (Bligh and Dyer 1959; Mihreteab et al. 2019) was used to extract lipids from yeast samples. Post-fermentation OP5 culture was withdrawn and dispensed into pre-weighed 50 ml conical tubes prior to centrifugation at 4,000 rpm, and pellets were washed twice with 50 mM PBS solution. Pellets were lyophilized, weighed, suspended in a 2:1 v/v chloroform: methanol mixture (5 ml per 50 mg cell dry weight) and sonicated using a Sonic Dismembrator (Fisher Scientific, USA) at 20 kHz and 20 % amplitude with pulsing (40 s on, 20 s off for a total working time of 20 min). The chloroform layer was withdrawn and aliquoted into pre-weighed conical tubes, then dried under a nitrogen stream. Dried lipids were weighed to determined lipid yield.

Dried lipids were weighed and methylated for GC/MS analysis via base-catalyzed esterification with 2.5 ml sodium methoxide (0.1 M) (Milanesio et al. 2013). The reaction was quenched using 200 µl sulfuric acid (>95%), and 2.5 ml hexane was added to each sample, which was then vortexed and centrifuged for 10 min at 10,000 rpm. 1.5 ml from the top hexane layer was withdrawn for GC/MS analysis.

3.2.11 GC/MS Analysis of Lipid Profile

Fatty acid profiles were characterized with an Agilent 7890A gas chromatograph attached to a 5977A mass spectrometer detector and equipped with an Agilent J&W HP-5ms UI capillary column (30 mm x 0.25 mm x 0.25 µm). GC parameters used for fatty acid profile analysis were

previously used by Mihreteab et al (Mihreteab et al. 2019). One µl samples were injected via an Agilent 7693 Automatic Liquid Sampler in splitless mode with an inlet temperature of 275 °C, using helium as the carrier gas at a flow rate of 1 ml min^{-1}. GC oven temperature was held at 60 °C for 1 min, and ramped to 100 °C (rate: 25 °C min^{-1}; hold 1 min). The oven temperature was then increased to 200 °C (rate: 25 °C min^{-1}; hold 1 min). The oven temperature is then increased to 220 °C (rate: 5 °C min^{-1}; hold 7 min) and then increased to an ending temperature of 300 °C (rate: 25 °C min^{-1}; hold 2 min). A C8-C24 FAME analytical standard was used during sample analyses as an external standard (Sigma Aldrich, USA).

3.2.12 Postconsumer PP oil characterization via GC/MS

Three grams postconsumer PP oil was dissolved in 250 ml chloroform (Sigma Aldrich, USA) and 1.5 ml aliquots were analyzed via GC/MS. The GC method used for hydrophobic fraction analysis (Guzik et al. 2014) was used to characterize the polypropylene pyrolysis oil. One µl of the diluted pyrolysis oil was injected via automatic liquid sampler at an inlet temperature of 275 °C. The oven method was 30 °C for 1 min, then ramping to 100 °C (rate:7.5 °C min^{-1}), then ramping to 300 °C (rate 10 °C min^{-1}, hold 2 min).

3.3 Results

3.3.1 OP5 medium composition

The PP oil in OP5 medium was comprised primarily of branched fatty alcohols including hexyldecanol and methyldecanol (59 %) and the branched alkenes 2,4 dimethyl-heptene and 2,6 dimethyl-octene (16 %) (Figure 3.1c). This composition was similar to the PP oil used in OP4 medium (Mihreteab et al. 2019) which contained 51 % branched fatty alcohols and 25 % branched

alkenes. After homogenization, the medium had a milky, evenly-mixed appearance. Unlike OP4, which contained oleic acid as an added emulsifier and remained homogenized at room temperature for a period of up to 3 days, OP5 medium separated within 2 hours without constant mixing. To compensate for this, the medium was used within an hour of homogenization and sterilization to prevent separation prior to fermentation.

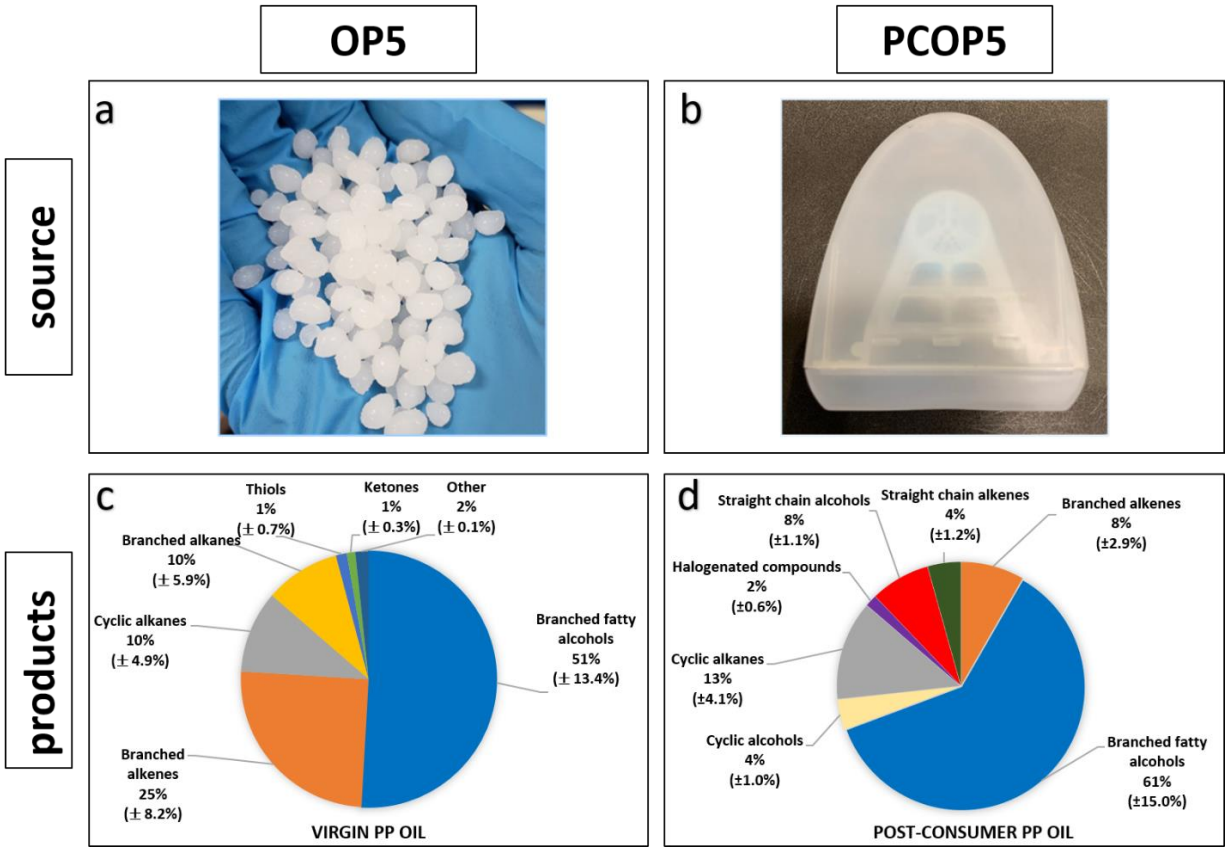


Figure 3.1 *Media were prepared from two sources of polypropylene. (a) OP5 medium was prepared from virgin amorphous PP pellets. (b) PCOP5 medium was prepared from postconsumer dental floss packaging made of PP. (c,d) The majority of the corresponding PP oils was branched fatty alcohols although differences were evident between the oils derived from the two sources of plastic.*

Over the course of experiments, OP5 went from a homogenized medium to a colloidal suspension where PP droplets aggregated into small (<50 □m diameter) droplets dispersed throughout the medium. By microscope, these droplets were shown to associate with *Y. lipolytica*, which formed biofilm-like aggregates around the droplets (Figure 3.2).

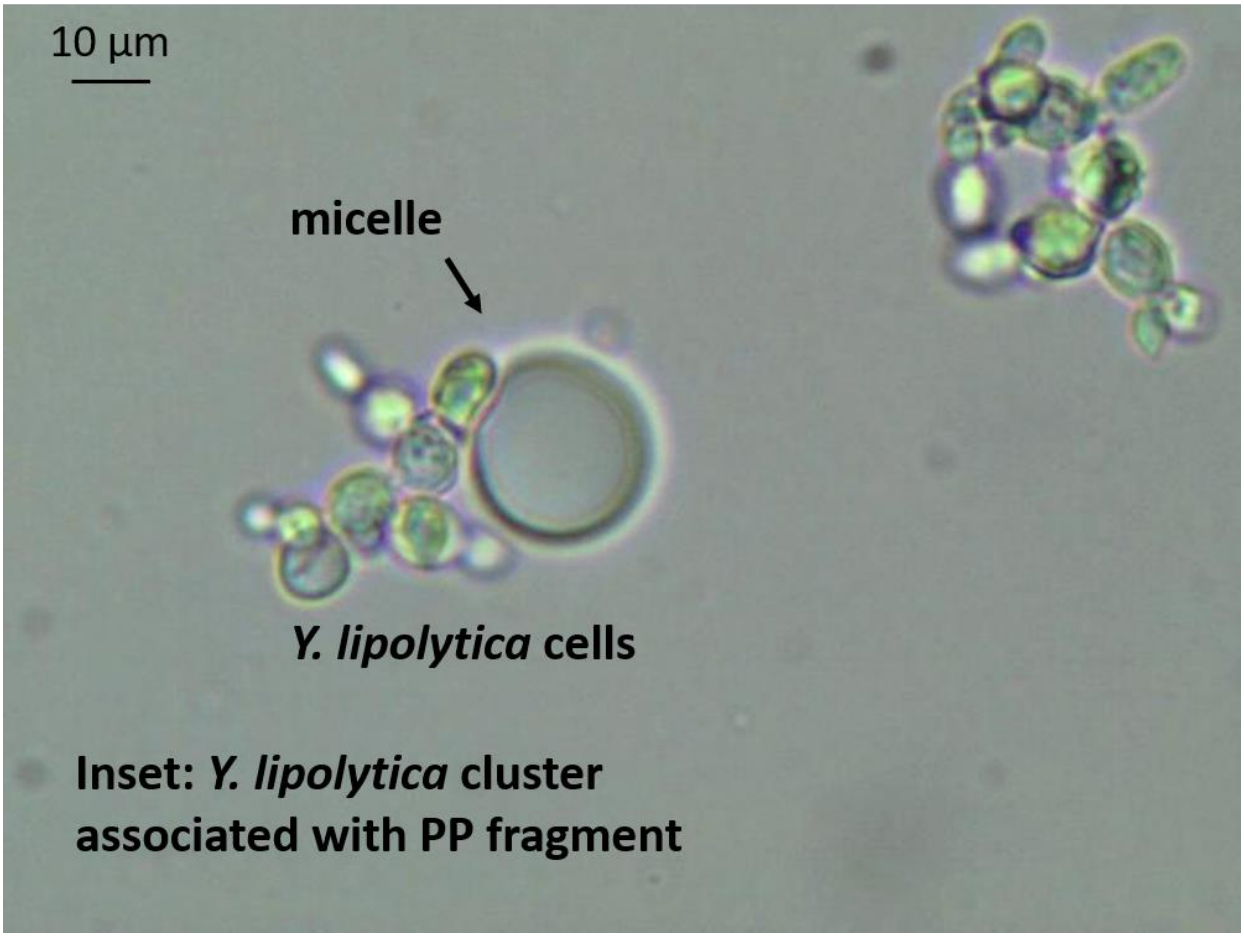


Figure 3.2 *Y. lipolytica cells in association with PP micelles from OP5 medium during fermentation. Cell aggregates form in the medium around the micelles. Image taken at 1000x magnification.*

3.3.2 Growth and lipogenesis on OP5 versus OP4 medium

We compared the growth of *Y. lipolytica* in OP5 versus OP4 medium to determine if the medium composition would affect cell yield or product yield (Figure 3.3). Cells grown in OP5 medium reached intracellular lipid yields of 0.53 g L^{-1} (Figure 3.3a), similar to lipid yields of cells grown on OP4 medium (Figure 3.3a). Biomass yields were lower when cells grew in OP5 medium, as the maximum biomass generated was 1.1 g L^{-1}, less than half that of *Y. lipolytica* grown on OP4 medium (Figure 3.3b). Cellular lipid content was higher during growth in OP5 medium compared to OP4 medium at each timepoint. Cells grown on OP5 achieved a maximum cell lipid content of 45 percent, compared to only 22 percent for cells grown on OP4 (Figure 3.3c).

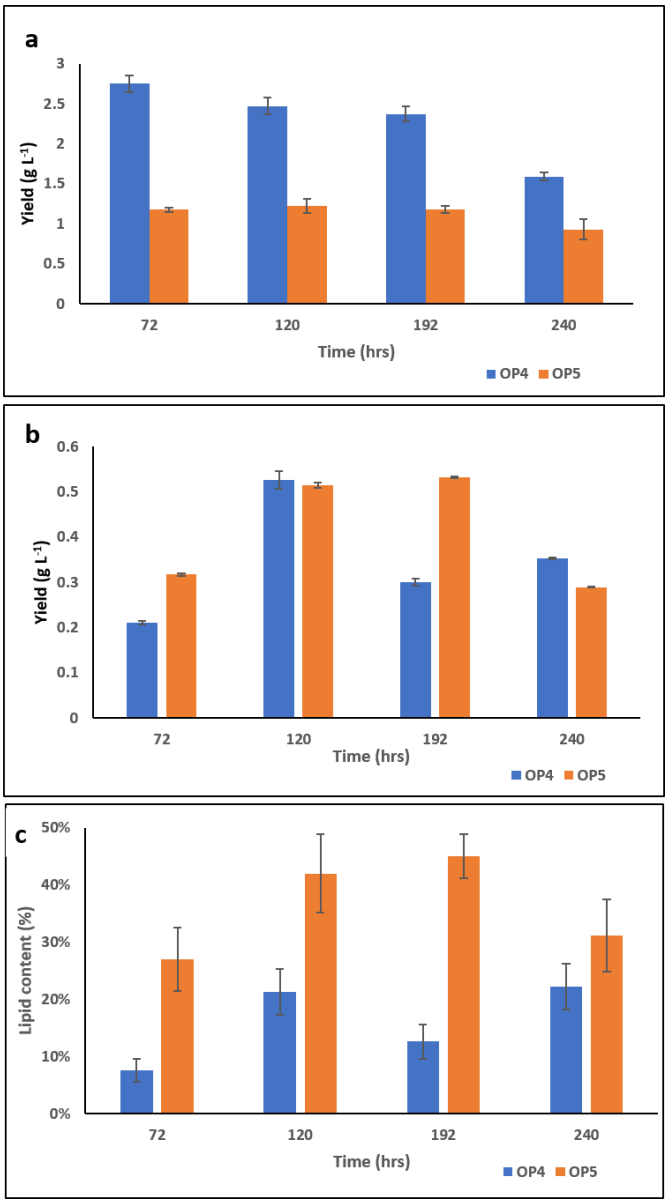


Figure 3.3 *Comparison of Y. lipolytica growth and lipid production during growth in OP4 medium versus OP5 medium. (a) biomass; (b) lipid yield; (c) lipid content per cell.*

3.3.3 OP5 substrate uptake during fermentation

Experiments were conducted to determine the rate of OP5 medium uptake by *Y. lipolytica* and to profile the constituents that were assimilated. Bulk substrate assimilation was measured gravimetrically over a 192 h period. *Y. lipolytica* used 39 percent of the bulk OP5 substrate by 120 hours, a lower amount than when grown on OP4 medium, when 53 percent was used by the same

timepoint (Figure 3.4a). By 192 hours, *Y. lipolytica* assimilated 62 percent of the bulk OP5 substrate, compared to 71 percent when grown on OP4 medium. The overall trend of substrate assimilation for the two media was similar.

An analysis of the composition of pre-fermentation OP5 medium in comparison to spent OP5 medium was carried out to determine which PP oil constituents were taken up by *Y. lipolytica* during the fermentation process (Figure 3.4b). OP5 medium was extracted into hexane at t = 0 h and t = 120 h, and the recovered analytes were quantified by GC/MS. The analysis showed 89 - 95 percent reduction in the peak area of all detected PP oil constituents (Figure 3.4b).

3.3.4 Starting medium pH and inoculum density significantly effect growth and lipogenesis

A grid design was used to assess interactions between pH and inoculum density. We examined several different parameters that have been reported to affect *Y. lipolytica* metabolite production by others, including pH, inoculum density, C:N ratio and osmolarity (Bouchedja et al. 2018; da Silva et al. 2018; Kuttiraja et al. 2018; Kuttiraja et al. 2016; Yang et al. 2015a; Zhang et al. 2019). We first investigated the impact of pH and inoculum density on growth, product yield and cell lipid content (Table 3.1). This was done by inoculating OP5 medium at varying initial pH (4.0 ,5.0 and 6.0) at various inoculum densities (1.0, 3.0 and 6.0). We found that growth was the lowest at an inoculum density of 1.0 and pH = 4.0 (1.3 g L^{-1}) and highest at an inoculum density of 6.0 and pH = 5.0 (11.9 g L^{-1}) (Table 1). Conversely, the lipid content per cell was highest at an inoculum density of 1.0 and pH = 4.0 (1.4 g L^{-1}) and lowest at an inoculum density of 1.0, pH = 5.0 (0.5 g L^{-1}) (Table 3.1). The highest overall lipid yield was with at an inoculum density of 3.0, pH = 6.0. Hereafter, an inoculum density of 3.0 and pH = 6.0 was used for all further experiments.

Table 3.1 *Effect of pH and inoculum density (ID) on Y. lipolytica growth and lipid production in OP5 medium.*

Biomass[a]			
	ID[b] = 1.0	ID = 3.0	ID = 6.0
pH 4.0	1.31 ± 0.08	2.84 ± 0.57	4.81 ± 0.08
pH 5.0	1.38 ± 0.10	1.81 ± 0.09	11.89 ± 0.01
pH 6.0	1.70 ± 0.05	5.83 ± 0.16	4.25 ± 0.11
Lipid Yield[a]			
	ID=1.0	ID=3.0	ID=6.0
pH 4.0	0.78 ± 0.10	0.69 ± 0.08	0.65 ± 0.02
pH 5.0	0.42 ± 0.15	0.70 ± 0.16	1.01 ± 0.10
pH 6.0	0.85 ± 0.12	1.42 ± 0.62	1.19 ± 0.20
Lipid Content[c]			
	ID=1.0	ID=3.0	ID=6.0
pH 4.0	0.59 ± 0.03	0.24 ± 0.03	0.14 ± 0.01
pH 5.0	0.30 ± 0.10	0.39 ± 0.09	0.09 ± 0.01

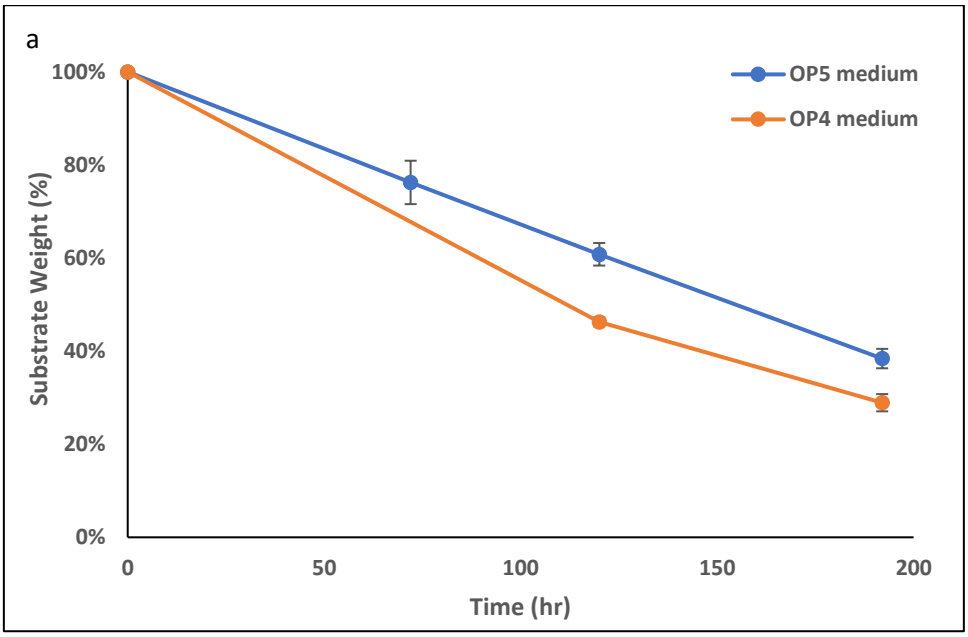
pH 6.0	0.50 ± 0.11	0.25± 0.10	0.28 ± 0.04

[a] Biomass and lipid yield figures are presented as g L^{-1}

[b]ID, inoculum density (optical density, 600 nm). See Methods for details

[c] Lipid content is the ratio of lipid mass to biomass

Highlighting: yellow, condition with highest lipid content; blue, condition with highest biomass; green, condition with highest overall lipid production



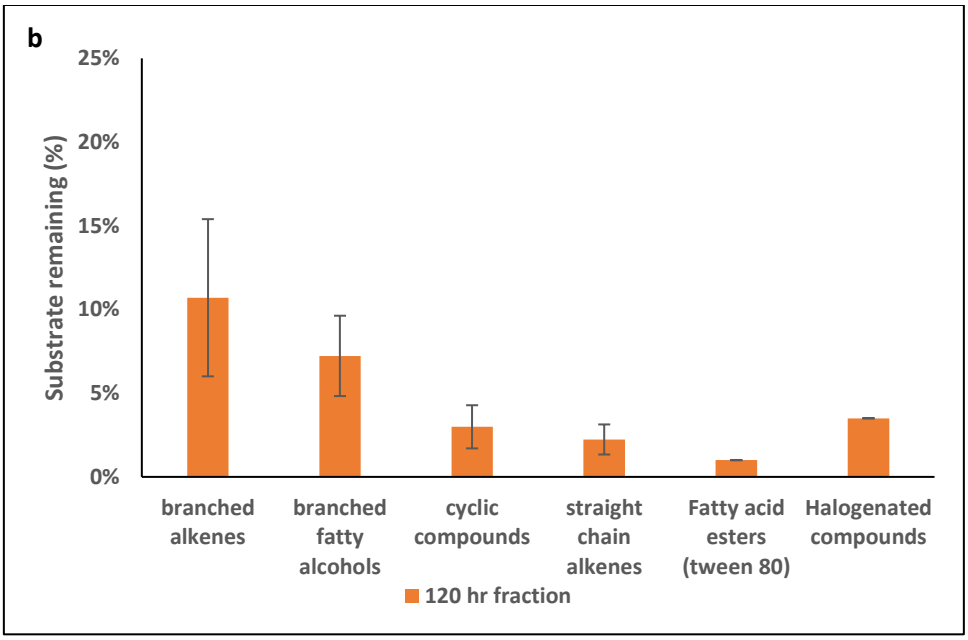


Figure 3.4 *(a) Growth medium assimilation was measure by gravimetric analysis. OP4 medium data after Mihreteab et al, 2019 (b) Metabolism of carbon compounds detected in OP5 spent media after 120 h of growth. Analysis by GC/MS.*

3.3.5 Carbon-to-nitrogen ratio optimization improves lipid yield

Because of the laborious nature of the experiments, a grid design could not be used for all the variables; rather, we switched to a sequential approach, beginning with the C/N ratio. Biomass yields were highest at the initial C/N ratio of 40 (Figure 3.5). Lipid yields were best at a C/N ratio of 80, yielding 2.1 g L^{-1}. Lipid content was highest at a C/N ratio of 80 (46 percent), and lower at C/N ratios of 20 and 40 (Figure 3.5). As a C/N ratio of 80 was best for lipogenesis, this parameter was adopted for all further experiments.

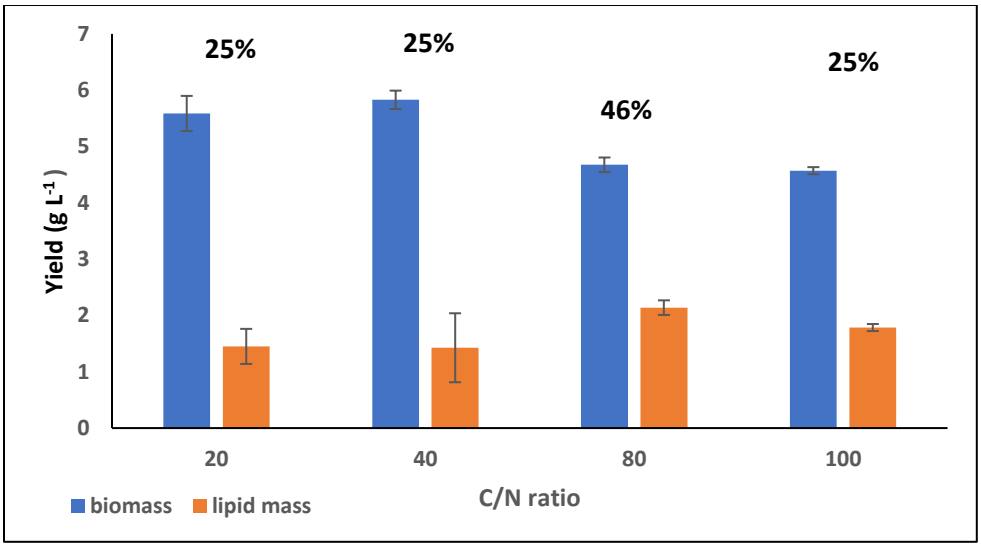


Figure 3.5 *Effect of C/N ratio on Y. lipolytica growth and lipid production. The corresponding cell lipid content is denoted above each graphic.*

3.3.6 Increased osmolarity negatively affects lipogenesis

After an optimal C/N ratio was identified, osmolarity was examined to see how it affected *Y. lipolytica* lipogenesis. Osmolarity has been demonstrated elsewhere to be a variable that can influence cellular physiology and metabolism in *Y. lipolytica* (da Silva et al. 2018; Liu et al. 2017; Yang et al. 2015a). NaCl was added to optimized OP5 medium at concentrations of 0.75, 1.5, and 3 g L^{-1}. Increasing osmolarity through NaCl addition had an adverse effect on lipogenesis, and led to marked decreases in biomass yield, lipid yields, and cell lipid content (Figure 3.6).

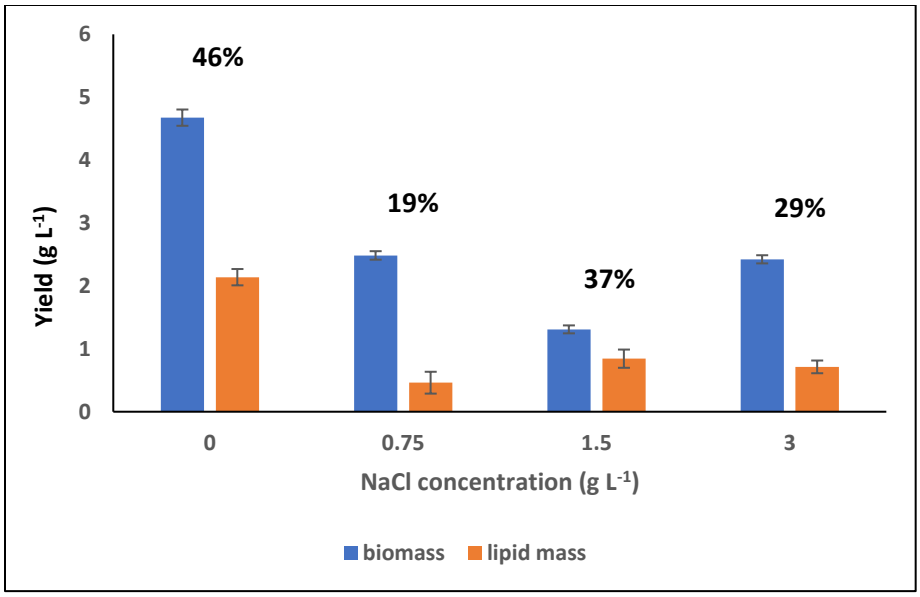


Figure 3.6 *Effect of osmolarity on Y. lipolytica growth and lipid production. The corresponding cell lipid content is denoted above each graphic.*

3.3.7 Y. lipolytica used PP from OP5 for lipid, biomass generation

OP5 medium has several components, including the surfactant Tween® 80, that can affect growth and lipogenesis. To determine the contribution of individual medium components to *Y. lipolytica* lipogenesis during growth in OP5 medium, cells were grown on media composed strictly of PP ("PP only"), Tween® 80 ("Tween® 80 only"), and OP5 medium without PP oil ("Tween® 80 +nutrients"), and biomass and lipid yields were compared with those from growth in optimized OP5 medium (Figure 6). Cells grown on optimized OP5 medium had 2.6 times higher biomass yield and 5.3 times the lipid yield when compared to cells grown on the "Tween® 80 + nutrients" (Figure 3.7). By comparing the lipid yield from the optimized OP5 medium with that of "Tween 80 + nutrients", it was determined that 81 percent (1.7 g L^{-1}) of the lipids produced were attributable to carbon derived from PP, with the remainder (0.4 g L^{-1}) attributed to carbon from Tween® 80. This analysis excluded the possibility that the majority of fatty acids measured in the product were derived from Tween® 80 rather than from bioconversion of PP.

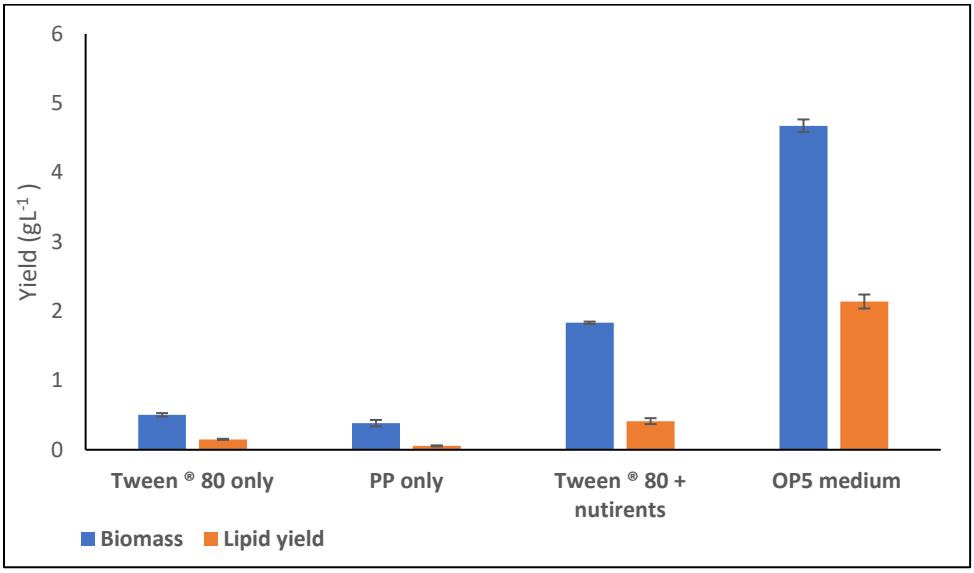


Figure 3.7 *Comparison of biomass and lipid yields after growth on media prepared from each of the OP5 medium components.*

3.3.8 Postconsumer PP oil carbon composition similar to virgin PP oil

The OP5 medium used in this work was prepared from virgin PP pellets. To determine if growth and product yield would differ if the source of PP were a postconsumer plastic, we prepared a postconsumer PP-derived OP5 medium termed PCOP5 (Figure 3.1). The carbon profiles of virgin and postconsumer PP pyrolysis oils were similar in that both PP oil sources had majority branched fatty alcohols (Figure 3.1c, 3.1d). Virgin PP oil contained 20 different compounds, with most of the carbon compounds (51%) being branched fatty alcohols, followed by branched alkenes (16%), cyclic compounds (12%), and straight chain alkenes (6%) (Figure 3.1c). In comparison, 25 different carbon compounds were found in postconsumer PP oil, with 61 percent of those carbon compounds being branched fatty alcohols, followed by cyclical compounds (15%), branched alkenes (8%) and straight chain alcohols (8%) (Figure 3.1d).

3.3.9 Postconsumer polypropylene impacts Y. lipolytica lipogenesis

Biomass yields were comparable to those measured during *Y. lipolytica* growth in OP5 medium (Figure 3.8a), but lipid yields during growth in OP5 medium were 300 percent higher than the lipid yields of cells grown in PCOP5 medium (Figure 3.8a). Average cell lipid content was also markedly lower in PCOP5 medium, with OP5-grown cells having an average lipid content of 45%, as compared to 17% for cells grown in PCOP5 medium (Figure 3.8a).

3.3.10 Fatty acid profiles for Y. lipolytica cells grown in OP5 or PCOP5 media

Intracellular FA profiles for *Y. lipolytica* cells grown on OP5 and PCOP5 for 120 hours were analyzed (Figure 3.8b, 3.8c). FA profiles were not complex for cells grown on either media, with only 4 FAs detected in each FA profile. Palmitic acid was the dominant FA on both OP5 and PCOP5, at 72 percent of FAs detected on OP5 grown cells and 81 percent for PCOP5 grown cells (Figure 3.8b, 3.8c). Pentadecanoic acid was present in OP5 grown cells, with 8.1 percent of the FA profile detected (Figure 3.8b), but it was not present in PCOP5 grown cells. Overall, FA profiles did not differ significantly when cells were grown on OP5 versus PCOP5 medium.

3.4 Discussion

Microbial bioconversion has potential for upcycling plastic waste into value-added biochemicals. In previous work, we demonstrated that thermal depolymerization of PP could be used to make a microbial growth medium suitable for *Y. lipolytica* to grow and produce fatty acids (Mihreteab et al., 2019). In this work, we report an improved PP-derived medium that did not require oleic acid as an emulsifier. By removing oleic acid, the amount of carbon derived from PP increased from 51 percent in OP4 medium to 77 percent in OP5 medium. Additionally, by optimizing the fermentation conditions, the lipid yield was more than 4 times greater compared to

the yield when *Y. lipolytica* grew in OP4 medium. These improvements are significant advances in developing a biological process for PP upcycling.

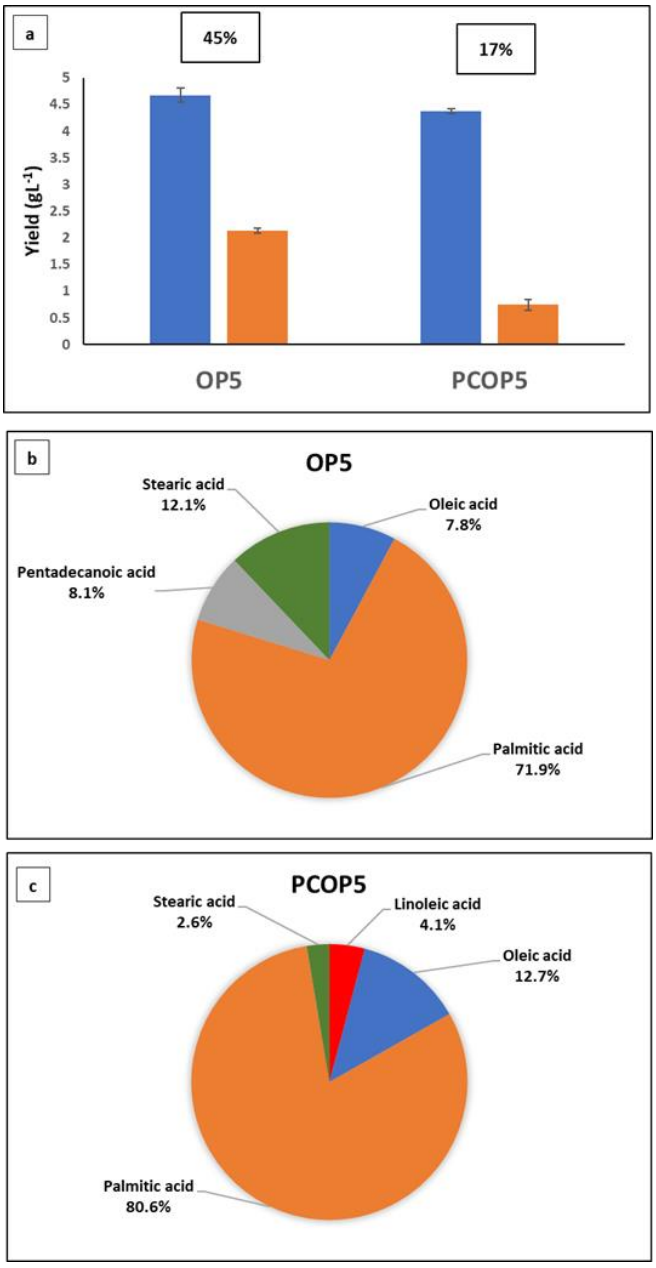


Figure 3.8 *(a) Growth, lipid production and lipid content for cells grown on either OP5 or PCOP5 media; The corresponding cell lipid content is denoted above each graphic. Fatty acid profiles of cells grown on (b) OP5 medium or (c) PCOP5 medium.*

Lipid storage by oleaginous yeast is a physiological adaptation to stress (Back et al. 2016; Fickers et al. 2005; Gonçalves et al. 2014; Kuttiraja et al. 2016; Pomraning et al. 2016; Rakicka et al. 2015b; Xu et al. ; Zhang et al. 2019). This creates a challenge for maximizing the lipid yield because the per-cell lipid production is greatest when conditions for cell growth are poor, resulting in low biomass. Conversely, when conditions for cell growth are permissive, lipid storage is low. These behaviors were evident in the presented data in two places. First, a comparison between *Y. lipolytica* growth in OP4 medium versus OP5 medium demonstrated higher biomass accumulation during growth in OP4 medium but significantly greater cellular lipid content during growth in OP5 medium. These results correlate with the presence of easily assimilated oleic acid in OP4 medium, which was absent in OP5 medium. Second, at pH = 4.0 and low inoculum density, a more stressful condition, growth was the least and lipid content per cell was the greatest; conversely at pH = 6.0 and high inoculum density, a more permissive condition, growth was the greatest but lipid content per cell was the lowest. The highest overall lipid yield occurred at pH = 6.0 and inoculum density of 3.0, a set of conditions which were intermediate relative to the others; it is notable that under these conditions, neither the growth nor the per-cell lipid content was the highest. These findings were comparable to previous studies, which showed that *Y. lipolytica* inoculum size affected both growth and product formation during fermentation, with lower inoculum density favoring higher cellular lipid content and higher inoculum density favoring biomass growth (Kucharczyk and Tuszyński 2015; Price et al. 2014; Rakicka et al. 2015b).

We decided to pursue a higher lipid yield by using as a starting point the inoculum density and pH that were most effective and then to optimize additional variables. Several environmental factors have been demonstrated to affect product formation in *Y. lipolytica*, including the C/N ratio and the osmolarity of the medium (Bellou et al. 2016; Kitcha and Cheirsilp 2011; Kuttiraja et al.

2016; Pomraning et al. 2016; Rakicka et al. 2015a; Zhang et al. 2019). A C/N ratio greater or equal to 80 resulted in an additional 50% increase in lipid yield; higher C/N ratios signal nitrogen scarcity, often resulting in lipid storage (Back et al. 2016; Kitcha and Cheirsilp 2011). In contrast, increasing osmolarity, which significantly influences erythritol formation in *Y. lipolytica,* did not have a positive effect of lipid formation (da Silva et al. 2018; Liu et al. 2019; Yang et al. 2015a) . Overall, parameter optimization resulted in a greater than 4-fold increase in lipid yield compared to the original cultivation conditions.

To simulate the real-world applicability of our process we investigated postconsumer PP which, unlike amorphous virgin PP, contains additives to promote stability and longevity. Some of the most common additives used in PP packaging include antioxidants, slip agents and heat stabilizers (Chen et al. 2008; El Mansouri et al. 1998; Hahladakis et al. 2018) . We determined that growth was not adversely affected in PCOP5 medium, but lipid yields were significantly reduced compared to growth in OP5 medium. We hypothesize that heavy metal-based slip agents and organophosphate antioxidants, compounds used to delay oxidative stress caused by UV radiation, may be impeding lipogenesis in *Y. lipolytica* (Chen et al. 2008; El Mansouri et al. 1998; Hahladakis et al. 2018). It has been shown that antioxidants can impede lipogenesis in hepatocytes by preventing reactive oxygen species from inducing lipid accumulation (Yang et al. 2018a). Heavy metals such as cadmium, tin, and lead have also been shown to increase ER stress and lipid peroxidation in eukaryotic cells (Shinkai et al. 2010; Vlahogianni and Valavanidis 2007), and might be negatively influencing *Y. lipolytica* lipogenesis (Bankar et al. 2018a).

What approaches can be pursued to upcycle postconsumer PP and to further increase the product yield? In addition to optimizing growth conditions, altering the *Y. lipolytica* genome may be beneficial. Introducing heterologous organophosphate hydrolase genes may assist *Y. lipolytica*

in degrading organophosphate additives, possibly increasing lipid accumulation (Kang et al. 2006; Zinjarde et al. 2014). Engineered metabolite detoxification has been used elsewhere; for example, introduction of an exogenous gene to decrease inhibitory xylose concentrations improved lignocellulose bioconversion (Niehus et al. 2018). A similar strategy may benefit PP bioconversion. The impact of heavy metals on *Y. lipolytica* activity is not well understood, outside of their effects on *Y. lipolytica* dimorphism and biofilm formation (Bankar et al. 2018a; Bankar et al. 2018b). Steps to mitigate their impact on fatty acid production could include incorporating the DMT1 gene, a divalent metal ion transporter gene that mitigates cadmium uptake in rodent intestines, into the *Y. lipolytica* genome (Tallkvist et al. 2001). Additionally, several metabolic engineering strategies have been investigated to improve cell lipid content. These include introduction of loss of function mutations to lipid catabolism genes (Liu et al, 2015), and overexpression of native lipogenesis genes. In general, there are several approaches that can be employed to improve the lipid yield and the potential for a biological process for plastic upcycling warrants additional investigation.

4 CONCLUSIONS

Plastic waste continues to accumulate in the environment, and as conventional recycling becomes an ineffective solution to the plastic waste problem, biotechnology has become an increasingly favorable alternate (Cacciari et al. 1993; Guzik et al. 2014; Kenny et al. 2008; Mihreteab et al. 2019; Narancic and O'Connor 2017; Silva et al. 2018; Skariyachan et al. 2017; Syranidou et al. 2019; Ward et al. 2006; Wierckx et al. 2018; Wierckx et al. 2015). Biotechnological solutions to the plastic waste problem are a sustainable approach that can have a positive impact on the global plastic waste load. To be considered a feasible alternative, a bioprocess for plastic waste processing must be economically and industrially viable. These biotechnological strategies include hydrolysis of Polyethylene Terephthalate (PET) by esterase producing bacteria (Chertkov O Fau - Sikorski et al. 2011; Kawai et al. 2014; Ribitsch et al. 2012; Tanasupawat et al. 2016; Yoshida et al. 2016), polystyrene and polyethylene degradation by mealworms (Brandon et al. 2018; Yang et al. 2018b; Yang et al. 2015b; Yang et al. 2015c), and incubation of mixed plastics with tailored microbial consortia (Skariyachan et al. 2017; Syranidou et al. 2019). These and other strategies have yielded promising results and offer sustainable alternatives to traditional recycling.

Our first work detailed a novel biotechnological process that employs *Yarrowia lipolytica,* an industrial workhorse than can utilize an extensive range of carbon sources (Abghari and Chen 2014; Ageitos et al. 2011; Aggelis 2002; André et al. 2009; Bialy et al. 2011; Chai et al. 2019; Dobrowolski et al. 2019; Dourou et al. ; Fickers et al. 2005; Liu et al. 2019; Niehus et al. 2018; Spagnuolo et al. 2018), as an oleaginous cell factory to produce fatty acids from a polypropylene-derived medium (Mihreteab et al. 2019). Our research showed that the process can be viable, but for it to be economical, our lipid output must be considerably increased. In our

second work, we showed that improving our medium and augmenting fermentation parameters significantly increased biomass and lipid yield without sacrificing cell lipid content. By removing oleic acid as a surfactant from our media, we increased the amount of bio-available carbon available from PP oil from 51 percent to 87 percent.

This reduction in easily assimilated carbon via oleic acid initially led to lower biomass and lipid yields. To improve on this, we implemented a stepwise factor optimization model that centered on improving lipid yields by experimenting with four (4) parameters: (1) the starting pH of the OP5 medium, (2) the initial inoculum density with which the medium was inoculated, (3) the C/N ratio and (4) the osmolarity of the medium. Through adoption of the parameters that are most favorable for *Y. lipolytica* lipogenesis, we were able to increase lipid yields by 400 percent while maintaining a cell lipid content of 45 percent.

There was significant progress done in increasing the industrial viability of our process, but there are several factors to consider before scaling up this bioprocess. The main factor to consider is augmenting our process and strain to better manage postconsumer PP. While fermentation augmentation led to yield gains as high as $2.1 gL^{-1}$, this was done using virgin PP pellets purchased for research purposes. Postconsumer PP products have added plasticizers, flame retardants, and stabilizers that: these added compounds, often in the form of heavy metals and halogenated compounds, impede lipogenesis of our *Y. lipolytica* strain, as seen in the significantly lower yields ($0.7\ gL^{-1}$) than achieved using virgin PP. We hypothesize improving our *Y. lipolytica* strain to better handle the stresses of metabolizing these additives should improve overall lipid yields. We also believe improving our pyrolysis process by instituting elements that would remove heavy metals and inorganic compounds from the PP oil would also improve yields by providing a better carbon source. Overall, our body of work has created an

industrially significant process to tackle the plastic waste crisis, and elucidated some obstacles to making this process scalable and economically viable.

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