Development of technological alternatives to produce renewable fuels from oleaginous microorganisms

by

Maria Isabel Espinosa Gonzalez

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

BIORESOURCE TECHNOLOGY

Department of Agricultural, Food and Nutritional Science
University of Alberta

© Maria Isabel Espinosa Gonzalez, 2014

Abstract

Microbial lipids are a promising feedstock for the production of renewable fuels. However, use of microbial lipids has been hindered due to challenges in the cultivating and processing of microbes. Thus, innovative technologies are required to enhance the feasibility of integrating oleaginous microorganisms into biofuel production strategies. Development of such technologies is the primary goal of this thesis.

Slurries of oleaginous microbial biomasses (microalgae *Chlorella protothecoides* and yeast *Cryptococcus curvatus*) were thermally hydrolyzed under subcritical conditions. Hydrolysis products including fatty acids, aqueous byproduct streams, and insolubles, were separated and characterized. Fatty acids were subsequently pyrolyzed to yield renewable fuels.

Microalgae and yeast were also cultured in aqueous byproduct streams from the hydrothermal processing of fats, oils, and oleaginous biomasses. Both model microorganisms used the glycerol byproduct from hydrolysis of fats and oils as a carbon source and displayed growth and lipid accumulation comparable to or better than cultures grown in pure glycerol. Similarly, supplementation of microbial cultures with microbial aqueous byproduct streams promoted higher biomass production compared to non-supplemented cultures.

Finally, microalgae was grown using whey permeate, a byproduct from the cheese industry. Pre-hydrolyzed whey promoted heterotrophic growth of this microalgae in both batch and fed-batch modes. Furthermore, whey permeate could be consumed by microalgae using simultaneous saccharification and fermentation.

The technologies for producing and using oleaginous biomasses proposed in this research reduce processing steps and valorize industrial waste streams. They also generate a suitable feedstock for renewable fuel production and a nutrient-rich supplement for microbial cultivation.

Preface

The research in this thesis was conducted in collaboration with members of the Biorefining Conversions and Fermentation Laboratory (2-38, AFNS) led by Professor David C. Bressler at the University of Alberta. I designed the experimental design and overall procedures referred to in chapter II (algae) with the assistance of Dr. Mussone, M.Sc. Asomaning, and Dr. Bressler. I was directly responsible for cultivation of oleaginous microorganisms (yeast and microalgae), thermal reactions, statistical analysis, and most analytical procedures referred to in all chapters. Guidance for experimental designs, overall troubleshooting, and discussion of results was provided by Dr. Archana Parashar (Chapters II (yeast), Chapter III, and Chapter IV) and Dr. Bressler (All chapters). Revision and editing was supported by Dr. Michael Chae.

Pyrolysis reactions and FTIR analysis, including interpretation and discussion (Chapter II), were conducted by M.Sc. Asomaning. Elemental and mineral analyses (Chapter II) were performed externally at the University of Alberta (Analytical and Instrumentation Laboratory of the Chemistry Department and at the Chemistry and Natural Resources Analytical Laboratory, respectively). Amino acid analysis (Chapter III) was performed with assistance from M.Sc. Lan (laboratory technician of 2-38, AFNS).

This thesis is an original work by Maria Isabel Espinosa Gonzalez.

Part of this thesis has been published:

Espinosa-Gonzalez I., J. Asomaning, P. Mussone, D.C. Bressler, Two-step thermal conversion of oleaginous microalgae into renewable hydrocarbons. Bioresource Technology, 2014.158: p.91-97.

Espinosa-Gonzalez I., A. Parashar, D.C. Bressler, Heterotrophic growth and lipid accumulation of *Chlorella protothecoides* in whey permeate, a dairy by-product stream, for biofuel production. Bioresource Technology, 2013. 155:p.170-176.

Espinosa-Gonzalez I., A. Parashar, D.C. Bressler, Hydrothermal treatment of oleaginous yeast for the recovery of free fatty acids for use in advanced biofuel production. Journal of Biotechnology, 2014. 187:p.10-15.

Espinosa-Gonzalez I., A. Parashar, M. Chae, D.C. Bressler, Cultivation of oleaginous yeast using aqueous fractions derived from hydrothermal pretreatments of biomass. Bioresource Technology. 2014. DOI: 10.1016/j.biortech.2014.08.006.

Acknowledgments

I am profoundly grateful to my supervisor Dr. David Bressler, who gave me an opportunity to undertake my PhD studies. Dave, I really appreciate the patience and the extra time that you dedicated to my development. Thank you for your genuine interest in helping me to seek out and acquire different skills outside the laboratory. Your leadership and professionalism during the timespan that I have worked in your group have deeply inspired me.

I would like to show my gratitude to the members of my advisory committee, Dr. Lynn McMullen and Dr. Nat Kav, for their mentorship and guidance during my studies. Special thanks to my external examiners Dr. Mohamed Gamal El-Din and Dr. Lars Rehmann.

I want to recognize the guidance of Dr. Natisha Stashko, Dr. Archana Parashar, Dr. Paolo Mussone, and Dr. Michael Chae, who worked with me at different stages of this research. I appreciate their suggestions and feedback for the experimental part of my program and also during the communication of results in written form.

Thanks to Dr. Stephen Strelkov, Dr. Lynn McMullen, and Dr. Nat Kav who allowed me to work in their laboratories and to Kelly Dunfield, Dr. Hafizur Rahman, and Jingui Lan who trained me in different analytical techniques.

This research was possible thanks to the generous financial support provided by the Natural Sciences and Engineering Research Council of Canada and Alberta Livestock and Meat. Thank

you to Consejo Nacional de Ciencia y Tecnologia (CONACyT- Mexico) for supporting my doctoral program through the scholarship 309054. I am also thankful for the travel and professional development grants awarded by the Faculty of Graduate Studies and Research (FGSR) and the Graduate Student Association (GSA) at the University of Alberta.

One of the best experiences during my doctoral program was teaching and I would like to express my gratitude to Dr. Jonathan Curtis, Dr. Lynn McMullen, and Dr. David Bressler, who gave me the opportunity to be a teaching assistant. I also appreciate Dr. Kelvin Lien and Heather Vandertol-Vanier for their great teaching examples.

Thank you to my amazing friends Justice, Tizazu, Yiqiong, Erin, Michael, Emily, Koel, Yuko, Allie and new lab members. Thanks to all my family and friends back in Mexico and to my surrogate family in Edmonton. Thank you to my husband Edgar and my baby Isabella.

Table of Contents

I. Background

II. Two-step thermal conversion of oleaginous microorganisms into renewable hydrocarbons

III. Cultivation of oleaginous microorganisms using aqueous fractions derived from hydrothermal pretreatments of biomass

List of Tables

List of Figures

List of Abbreviations

Pentose Phosphate Pathway	PPP
Tricarboxylic Acid Cycle	TCA cycle
Embden-Meyerhof Pathway	EMP
Triacylglyceride	TAG
Polyunsaturated Fatty Acid	PUFA
Dihydroxyacetone Phosphate	DHAP
Acetyl Coenzyme A	Ac-CoA
Fatty Acid Synthase	FAS
Genetically Modified	GM
Lipid To Hydrocarbon	LTH
Yeast Extract Peptone Dextrose	YEPD
Glucose oxidase/peroxidase	GOPOD
Gas Chromatography	GC
High Performance Liquid Chromatography	HPLC
Fourier Transform Infrared Spectroscopy	FTIR
Dichloromethane	DCM
Hydroxymethylfurfural	HMF
Whey Permeate	WP
Hydrolyzed Whey Permeate	HWP

I. Background

1. Renewable fuels

Renewable fuels have emerged as promising energy alternatives to conventional oil derivatives. They can theoretically be obtained in sufficient quantities to satisfy global energy demands because they are produced from biomass, an abundant and readily available resource on earth [1-3]. The term biomass includes any living matter although it has been used mostly to refer to plants and their derivatives [4]. Biomass can be transformed into solid, liquid, or gaseous fuels such as bioethanol, biodiesel, and biocrude, which are some of the most commercialised forms [5, 6]. Beyond renewable fuels, biomass can also be converted to other commodities at biorefineries.

A biorefinery is a facility that uses a combination of technologies to generate a variety of products from different biomass feedstocks [6-8]. In a biorefinery, biomass constituents can be extracted and functionalized to produce molecules (*e.g.* chemicals and oil substitutes), materials (*e.g.* fibers and specialty lipids), and energy products (*e.g.* bioethanol and biodiesel) [9]. The most common biomasses used to generate renewable fuels include grains and lignocellulosic materials (agriculture/forestry residues, grasses, and solid animal waste); however, microorganisms can also be considered [10, 11]. The utilization of microbial biomass to produce biofuels (especially lipid-based) is an alternative to the conventional use grain feedstocks, which faces different environmental and social pressures [12-19].

The most promising high lipid content (oleaginous) microorganisms include algae, yeast, and other fungi [20]. In particular, microalgae form a heterogeneous group that possesses some attractive characteristics for the production of renewable fuels. These include high

photosynthetic efficiency, high lipid productivity, negligible competition for arable land, and the ability to grow in inexpensive media including wastewater streams [21-26].

Another group of outstanding microbes for biofuels production are oleaginous yeasts. Compared to filamentous fungi, yeasts are more suitable for lipid production because they can be cultivated more easily and faster [27]. Oleaginous yeasts can accumulate lipids in excess of 20% of their cellular dry weight, have short doubling times (< 1 h), offer easy scale-up, and are suitable for fatty acid modification [28]. Other beneficial characteristics of oleaginous yeast include their ability to reach high cell densities during fed-batch culture growth, as well as their ability to utilize alternative, low-value carbon sources for growth [29, 30].

2. Production of microbial biomass

2.1. Algae

Algae can be defined as a group of unicellular or simple multicellular photosynthetic microorganisms. Although they share some similarities with plants, algae do not achieve specialization and differentiation to the same extent [31]. The term algae encompasses an estimated 10 million species of macro and microalgae that vary in size, length, habitat, structure, and biochemical makeup [31]. Microalgae are an algal subgroup composed of colonial or single cell photosynthetic microorganisms. Microalgae can be classified into diatoms, blue-green, golden, and green algae [32]; the latter has a growing biotechnological interest [33].

2.1.1. Cultivation of algae

The various species of algae can be cultivated using photoautotrophic (autotrophic photosynthesis) methods. Some of them can also be grown under heterotrophic (respiration of organic substances) or mixotrophic (combination of photosynthesis and heterotrophy) conditions.

2.1.1.1. Autotrophy

As phototrophic organisms, algae can use light energy to fix carbon dioxide (CO_2). Commercial production of some algae can be achieved using sunlight, CO_2 from the environment, and nutrients (specifically nitrogen, phosphorus, and minerals) from simple culture media [21, 34]. Autotrophic algal production (for commercial purposes) is mostly carried out in open ponds, which offers low construction and operation costs as well as low energy inputs [34]. However, algal cultivation in open ponds has some disadvantages such as low productivity due to inefficient mass transfer and variability due to seasonal changes in sunlight, temperature, and contamination [35].

The use of closed systems (photobioreactors) for the production of microalgae overcomes some limitations associated with open pounds. For instance, light supply, contamination, and temperature can be strictly controlled [35, 36]. Disadvantages of all currently available photobioreactors are high operational costs, reduced productivity due to poor illumination and insufficient mass transfer, and challenges for scale up [36, 37].

2.1.1.2. Heterotrophy

Heterotrophy exploits the capacity for some algae to grow without light but using organic carbon sources added to the culture media. Heterotrophic cultivation of algae requires the use of bioreactors under conditions adapted from typical optimized microbial fermentations [38]. Heterotrophic algal cultivation in bioreactors provides efficient mass transfer, reduces contamination, and allows high cell density growth [39].

However, heterotrophic growth has some limitations. Heterotrophic cultivation is expensive, prone to contamination and substrate inhibition, can only be used for certain species, and leads to deficiencies in high-value, light-induced metabolites such as pigments [39]. Despite

these limitations, heterotrophy provides the highest theoretical yields of biomass (high thermodynamic efficiency) from the supplied energy compared to any other cultivation method [40]. In addition, some microalgal species have displayed higher growth rates under heterotrophic conditions compared to autotrophic conditions [41]. Furthermore, common bioreactors that are used to grow heterotrophic cultures are easy to operate and maintain compared to a photobioreactor or pond. Most importantly, they offer the possibility for high cell densities, which represents an advantage for large-scale production of microbial biomass or metabolites [41]. For example, heterotrophic cultures have been incorporated industrially for the production of several metabolites such as eicosapentaenoic and docosahexaenoic fatty acids (from oils), phytochemicals (pigments), and lipids (for biofuel applications) [42-46].

2.1.1.3. Mixotrophy

Mixotrophic growth is a mixture of metabolic conditions in which the respiratory and photosynthetic pathways work at the same time; CO_2 and organic carbon are simultaneously used [47-48]. As a consequence, growth is influenced by light and carbon source(s) added to the culture media. One of the main advantages of mixotrophic cultures is that they will still produce light-induced metabolites [39]. However, the growth rates of microalgae growing under mixotrophic conditions are generally lower than those grown under heterotrophic conditions [41].

2.1.2. Heterotrophic carbon metabolism in algae

Algae use the same respiratory pathway as higher plants. During respiration, an organic substrate is oxidised, oxygen is consumed, and CO_2 is produced. There are two main mechanisms for algal carbon uptake: active transport systems (for hexoses and amino acids) and simple diffusion across membranes (for organic acids and glycerol) [47].

Glucose, the preferred carbon source for algae, activates the active transport system within minutes of exposure [49]. After passing through the cell membrane, glucose is phosphorylated and then metabolized through one of the two glycolytic process observed in algae: Embden-Meyerhof Pathway (EMP) or Pentose Phosphate Pathway (PPP) (Figure 1) [40]. Glycolysis generates pyruvate molecules that can enter the TCA cycle [50].

Glycerol, another typical carbon source, easily diffuses into the cell where it is phosphorylated and converted into glyceraldehyde-3-phosphate, which can then enter the EMP (Figure 1) [50]. Although glycerol has been used for growth of different microalgae during production of various metabolites, knowledge of its metabolism under heterotrophic conditions is limited [41].

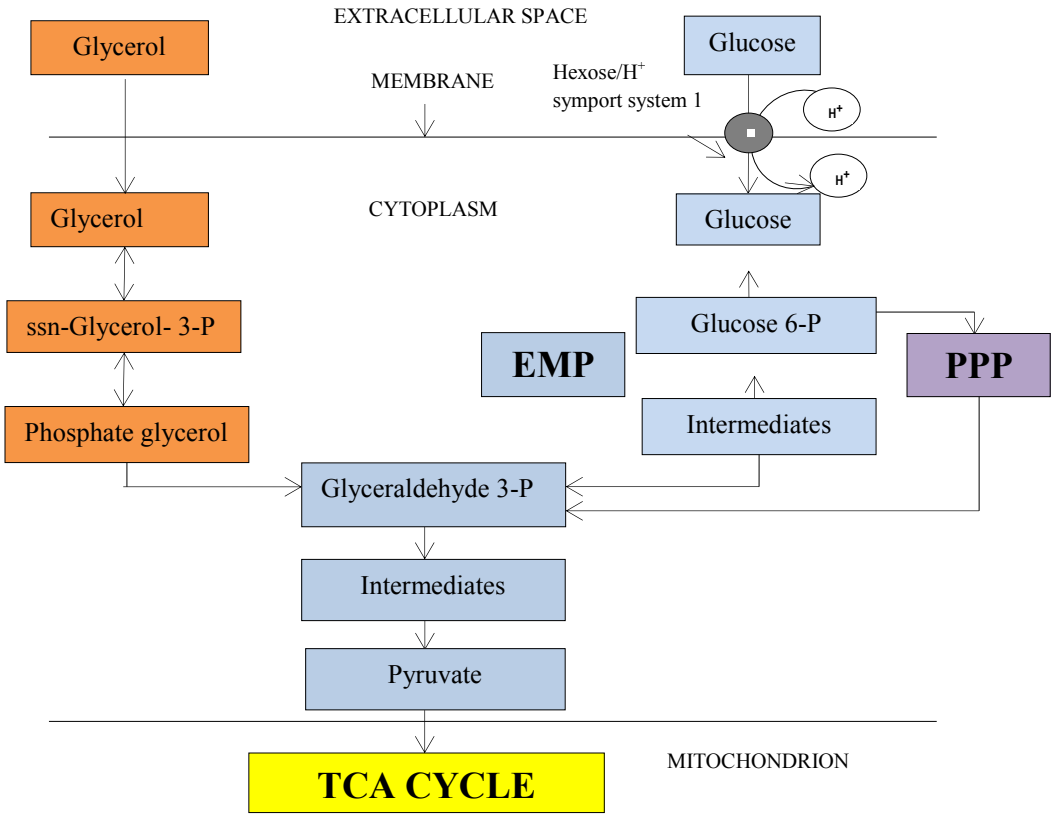


Figure 1. Main routes for heterotrophic glucose and glycerol metabolism in microalgae. EMP, Embden-Meyerhof Pathway; PPP, Pentose phosphate pathway [41].

2.1.3. Lipid metabolism in algae

Algal lipids can be divided into non-polar and polar lipids. Microalgae that accumulate non-polar lipids, predominantly triacylglycerides (TAG), as storage products are termed oleaginous [51]. Algal TAGs are generally characterized as being saturated or monounsaturated fatty acids although long chain polyunsaturated fatty acids (PUFAs) may also occur [52]. Synthesis of TAGs takes place mainly in two organelles: plastids and the endoplasmic reticulum. In the model green algae *Chlamydomonas reinhardtii*, synthesis of fatty acids occurs in plastids and is mediated by a fatty acid synthase [53, 54]. The synthesized fatty acids, mainly palmitate, stearate, and oleate, may undergo further processing such as the introduction of double bonds [55]. Finally, the TAGs are elongated and assembled in the endoplasmic reticulum, and then stored as lipid bodies [53, 56]. The accumulation of lipids in microalgal cells can be influenced by culturing conditions such as temperature, pH, nutritional content, and the age of the culture [21, 57].

2.1.4. Microalgae *Chlorella protothecoides*

2.1.4.1. Biology

The genus *Chlorella* includes some of the most biotechnologically-relevant green microalgae. *Chlorella* possess double membrane-bound chloroplasts with accessory pigments [58, 59]. Some of these structures are non-photosynthetic plastids that allow microalgae to grow heterotrophically in the dark [60]. For some *Chlorella*, the main reserve polysaccharide is starch, which is deposited inside plastids [61, 62]. *Chlorella* has a thin outer cell wall, or periplast, that covers the cytoplasm. This outer cell wall is composed of cellulose that forms randomly oriented microfibrils [63]. Outer cell walls are highly resistant to chemical and enzymatic hydrolysis due to the presence of algaenans, which are polymethylenic chains associated with amide groups and

minor amounts of N-alkyl substituted pyrroles [64]. The cytoplasm contains typical eukaryotic components including a nucleus, chloroplasts, Golgi apparatus, endoplasmic reticulum, ribosomes, mitochondria, vacuoles, contractile vacuoles, double-membrane plastids, lipid globules, flagella, and microtubules [65]. *Chlorella* reproduces through asexual division to produce identical autospores [65, 66].

Chlorella protothecoides belongs to the phylum Chlorophyta, class Trebouxiophyceae, and order Chlorellales [67]. *Chlorella protothecoides* (var. communis) cells are spherical (3-10 μm diameter), and are green when grown under autotrophic conditions or yellow/white when a carbon source is supplied [68]. These algae strictly require thiamin (vitamin B_1) for growth [68].

2.1.4.2. Biotechnological applications of *C. protothecoides*

C. protothecoides has been used for the production of carotenoids (lutein) and ascorbic acid, and has also been utilized as a food ingredient [69-72]. However, since lipids from *C. protothecoides* can be used as a feedstock for the production of biofuels, characterization of these algal species (mainly culturing and post-processing methods) has become an increasingly important field of research. For instance, one of the most critical aspects for the heterotrophic growth of *C. protothecoides* is the carbon source required for cultivation. Previous research was directed towards finding suitable substrates for the growth of and lipid production in *C. protothecoides*, and mainly focused on cultivation using low-value carbon sources (Table 1).

Table 1. Alternative substrates used for the growth and lipid accumulation of *C. protothecoides.*

Substrate	Strategy	Biomass (g/L)	Lipid (%)	Ref.
Hydrolyzed sugar cane	FB	121	45	[73]
Hydrolyzed cassava starch	B	4	50	[74]
Hydrolyzed corn powder	B	5	40	[75]
Hydrolyzed cassava starch	FB	54	53	[75]
Cassava starch	SSF	49	55	[75]
Hydrolyzed molasses	FB	97	57	[76]
Hydrolyzed molasses	FB	71	58	[76]
Hydrolyzed *Jerusalem*	B	16	44	[77]
Hydrolyzed sweet	B	3	53	[78]
Sweet sorghum juice	B	5	53	[78]

FB, Fed-batch; B, Batch; SSF, Simultaneous Saccharification and Fermentation

2.2. Yeast

Yeasts are fungi that divide though budding or fission and do not form a fruiting body [79]. Yeasts have been widely used for various applications in food (beers, ciders, wines, sakes, distilled spirits, bakery products, cheeses, and sausages), medicine (heterologous production of pharmaceutical enzymes and proteins), agriculture (biocontrol, bioremediation, and as an environmental indicator) as well as other industries (production of fuel ethanol, single cell proteins, feed and fodder, enzymes, and small molecular weight metabolites) [79, 80].

Saccharomyces cerevisiae is the model yeast used for most biotechnological applications. However, non-*Saccharomyces* (non-conventional) yeasts include some Ascomycetes and Basidiomycetes, which also have specific properties and potential for biotechnology [81]. Ascomycetes have been used for heterologous expression of proteins and synthesis of fine

chemicals and compounds with medical and nutritional applications [82]. Basidiomycetes also have biotechnological applications in the synthesis of enzymes or production of unique secondary metabolites stemming from their ability to use complex carbon sources [80]. Some Basidiomycetes have also been used for the production of lipids with value added applications (cocoa butter equivalent) [83, 84] or biofuel feedstock [30].

Yeast cultures require the use of stirred and aerated fermentation vessels (bioreactors). Industrial yeast cultivation requires: 1) automatic controls to monitor all parameters and to ensure no contamination, 2) carbon sources and other nutrients such as nitrogen and minerals, and 3) inoculation from smaller bioreactors [27].

2.2.1. Carbon metabolism in yeast

Carbon metabolism in conventional and non-conventional yeasts generally follows the same pathway with minor variations at specific biochemical steps or mechanisms [81]. Simple sugars, primarily pentoses and hexoses, are transported by facilitated diffusion through the action of permeases [81]. Once inside the cell, the sugars enter the glycolytic pathway to produce pyruvate (as described for algae in Figure 1). Non-conventional yeasts grown under aerobic conditions, oxidize pyruvate to CO_2 through the TCA cycle [81].

Some yeasts can use glycerol as a carbon source during aerobic cultivation. Glycerol uptake occurs mainly by passive diffusion, although active transport may be possible in some species [81, 85, 86]. Glycerol metabolism is achieved through two mechanisms: 1) phosphorylation followed by oxidation, and 2) dehydrogenation and subsequent phosphorylation [87, 88] (Figure 2). These distinct pathways, which are observed in *S. cerevisiae* and *S. pombe,* respectively, both result in the production of dihydroxyacetone phosphate (DHAP).

Triosephosphate isomerase can convert DHAP to D-glyceraldehyde 3-phosphate (GA3P), which is an intermediate in glycolysis.

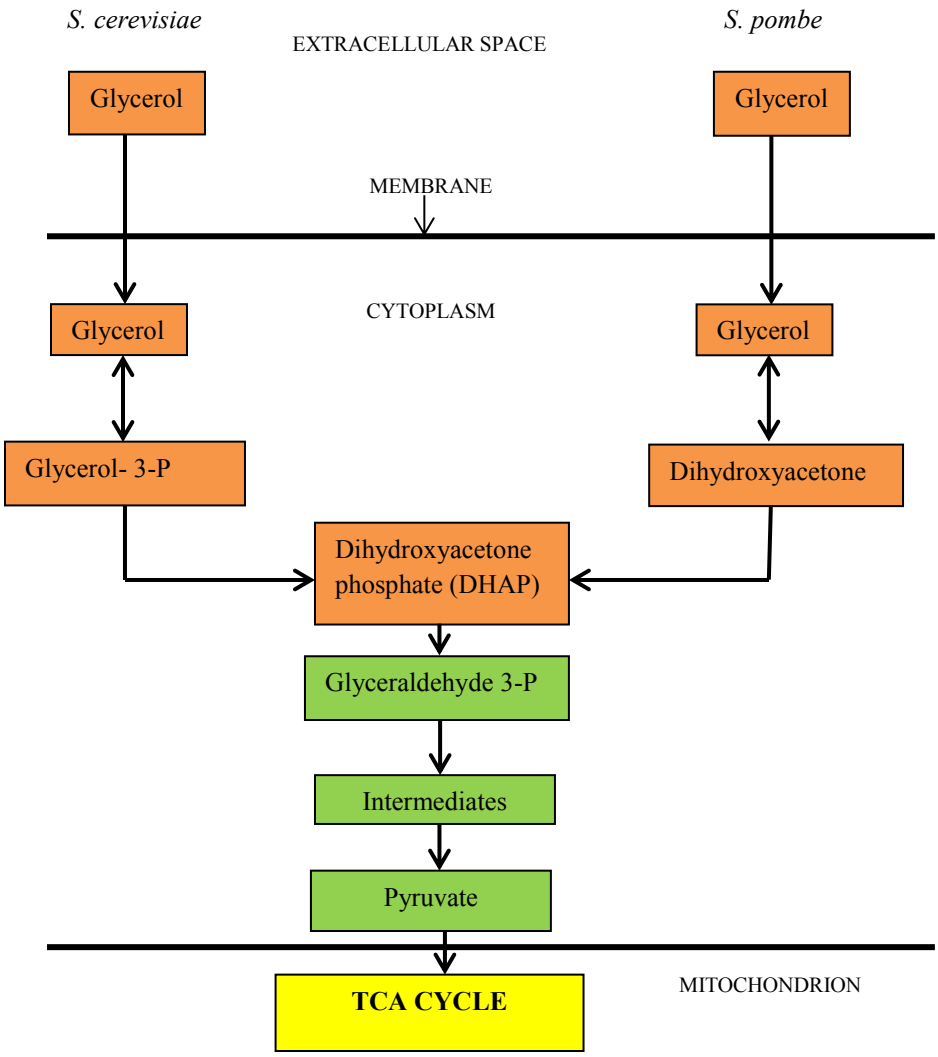


Figure 2. Glycerol uptake and metabolism in the two model yeasts *S. cerevisiae* and *S. pombe* [81].

2.2.2. Lipid metabolism in oleaginous yeast

Lipid metabolism in non-oleaginous and oleaginous yeasts differs only by minor biochemical changes. Lipid accumulation in oleaginous yeast is triggered by a nutrient imbalance, specifically a nitrogen deficiency and high amounts of a carbon source [51]. Under

these conditions, oleaginous yeasts induce a series of enzymatically-regulated reactions in the mitochondria that produce an excess of citrate [51]. Citrate is diverted from the TCA cycle to the cytosol where it enters a fatty acid synthesis pathway [51]. The synthesised fatty acids, primarily stearic and palmitic acids, can undergo further modifications, such as desaturation and elongation, in the membranes of the endoplasmic reticulum [89] (Figure 3). TAGs in yeast are stored as oil droplets within the cells [89].

The maximum theoretical efficiency for the conversion of glucose to TAGs is 33% (w/w) although in practice, the maximum yield is roughly 22% (w/w) [27]. This is likely because the theoretical efficiency does not take into account that some of the glucose is used by yeast to promote growth.

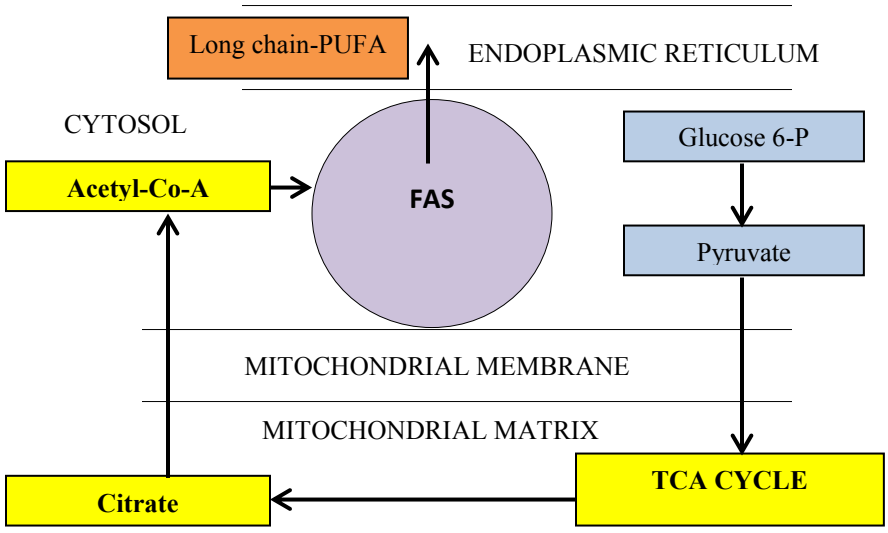


Figure 3. Hypothetic organization of lipid metabolism in yeast. Glycolysis in the cytosol produces pyruvate, which migrates into the mitochondria and enters the TCA cycle. Citrate is an intermediate of the TCA cycle. Citrate yields Acetyl-CoA, the precursor for fatty acid synthesis. Production of fatty acids is mediated by the enzymatic complex fatty acid synthase (FAS). Fatty acids can be elongated and modified in the membranes of the endoplasmic reticulum to produce long chain polyunsaturated fatty acid (PUFA) [89].

2.2.3. Yeast *Cryptococcus curvatus*

2.2.3.1. Biology

The yeast *C. curvatus* belongs to the phylum Basidiomycetes, subphylum Agaricomycotina, class Tremellomycetes, and order Trichosporonales [90]. *C. curvatus* is a unicellular, anamorphic yeast with an ovoid shape (2.7 to 4.7 × 3.3 to 9.4 μM) and has a polysaccharide capsule and multilamelate cell wall containing xylose, mannose, and mannitol [91]. These yeasts form soft mucoid colonies that can become yellow and wrinkled after one month [91]. *C. curvatus* requires thiamine (vitamin B_1) for growth and reproduces through enteroblastic budding, which occurs through evagination and growing of the innermost layer of the cell wall following its rupture [91]. *C. curvatus* is also capable of *de novo* lipid synthesis and is able to alter lipid substrates (fatty acids) and incorporate them into their triacylglyceride reserves [92, 93].

2.2.3.2. Biotechnological applications of *C. curvatus*

C. curvatus is an oleaginous yeast of great biotechnological interest. *C. curvatus* has been previously used for the production of a cocoa butter equivalent [83, 84] and other custom-made lipids and has also been used to encapsulate lipids thereby preventing their oxidation [92, 93]. There is also a growing interest for using lipids of *C. curvatus* as a feedstock for biofuels production. Several studies have tried to improve the economic feasibility of yeast lipid production through cultivation in alternative carbon sources (Table 2).

Table 2. Alternative substrates used for the growth of and lipid accumulation in *C. curvatus.*

Substrate	Strategy	Biomass (g/L)	Lipid (%)	Ref.
Crude glycerol	FB	69	48	[30]
Crude glycerol yellow grease	Two-stage	33	52	[94]
Glycerol from yellow grease	FB	31	44	[94]
Pretreated waste active sludge	B	10	23	[95]
Hydrogen production effluent	C	9	14	[96]
Waste spent yeast from brewery	SW	50	38	[97]
Distillery waste water	B	5	25	[98]
Wheat straw hydrolysate	B	17	34	[99]
Shrimp processing waste hydrolysate	B	19	28	[100]
Sweet sorghum bagasse hydrolysate	B	11	73	[101]

B, Batch; FB, Fed-batch; C, continuous; SW, stepwise

2.3. Co-culture of algae or yeast with other species

Interactions between algae and other microorganisms (such as bacteria) have been previously studied from an ecological perspective [102, 103]; however, the effects of co-culturing algae and other organisms for commercial purposes such as oil accumulation have not been widely explored. It is assumed that bacteria and algae may establish complicated relationships (competition, syntrophy, mutualism, and antagonism) influenced by environmental conditions [104].

Previous research has assessed the co-culture effects of the microalgae *C. pyrenoidosa* with undefined contaminating bacteria in heterotrophy [104]. In that study, degradation of wastewater components was improved in co-culture without affecting algal biomass productivity. However, lipid content and productivity were significantly reduced when bacteria were present [104].

In a different study, the production of exopolysaccharides was evaluated by using a co-culture of algae and fungi (macromycetes), which originally produced different exopolysaccharides [105]. When co-cultured, the final product was different compared to those from individual algal or fungal cultures possessing mainly fungal characteristics [105].

Co-cultures have also been investigated with different microalgae in open ponds. For example, the microalgae *Chlorella* sp. has been independently co-cultured with three microalgae: *Scenedesmus obliquus, Chlamydomonas reinhardtii*, and *Selenastrum capricornutum*. With regards to lipid accumulation, *Chlorella* seemed to produce larger lipid granules when co-cultured with *S. capricornutum* and *C. reinhardtii*, but not with *S. obliquus*, presumably due to allelopathic relations (such as the excretion of specific chemicals), which also influenced growth, cell shape, and cell mobility [106].

2.4. Contamination control in algal and yeast cultures

The production of oleaginous algal or yeast biomasses requires aseptic techniques that create extra costs for the additional controls that have to be implemented before and during cultivation. The typical use of antibiotics or chemical disinfectants to control contamination in fermentations is costly and impractical for the production of microbial oils. Other strategies to combat contaminating organisms have been used, specifically for algal production in open ponds. These strategies include the use of environmental pressures (such as extreme pH or temperature), herbicides, pesticides, or other chemicals [107]. However, a novel area of growing research interest is to apply microalgal allelopathy (use of naturally occurring chemicals that can act as inhibitors) for contamination control [107]. For yeast fermentations, on the other hand, the use of analogous strategies (such as the use of bacteriocins) for biological control of contamination is under investigation for different biotechnological processes [108].

2.5. Use of Genetically Modified (GM) microbes for microbial oil

Genetically modified microbes that carry better traits for lipid accumulation or fastest growth rates are potential candidates for the production of microbial oils [109-112]. The genetic manipulation of oleaginous microorganisms has been directed towards targeting key enzymes in the lipid biosynthetic pathway mainly to boost microbial lipid accumulation or to change fatty acid profiles. The overexpression of native genes from the algal lipid biochemical pathway or the insertion of transgenes to overproduce lipids has had limited success [109, 113]. However, the alteration of the fatty acid composition of microalgae through insertion and overexpression of plant genes has been achieved [114]. Despite the promise of GM microbes for biofuels production in an industrial setting, it is important to consider that governing bodies may restrict their use. Impacts resulting from the use of GM microbial biomass in biofuels production have yet to be evaluated [115]. This specifically relates to the potential direct release of GM microbes to the environment as well as the indirect release of the same GM organisms in byproducts that may be incorporated in the food chain.

3. Nutrient recycling for microbial biomass production

Microorganisms that are used for oil production convert carbon in the culture media into biomass and energy reserves, which are stored in the form of lipids. The requirement for carbon and other essential nutrients, mainly nitrogen and phosphorus, is a major concern from an economic and environmental standpoint and will dictate whether microbial oils are a feasible feedstock for biofuel production [27]. Some strategies to address these matters are the utilization of cheap alternative carbon sources (as discussed in sections 2.1.4.2 and 2.2.3.2 of this chapter) and nutrient recycling from waste streams.

Nutrient recycling offers the possibility to improve microbial performance from an economic point of view and to reduce impact on the environment. In industrial yeast cultivation, recycling of nutrients from wastewaters has been studied mostly for economic purposes [116-118]. On the other hand, algal cultivation in wastewaters has been traditionally used as a water treatment alternative to reduce the environmental impact of industrial discharges [119]. Wastewaters can also be utilized as nutrient sources for intensive cultivation of algae for biofuels, addressing not only environmental but also economic concerns [120]. Studies suggest that the high costs associated with supplying nitrogen and phosphorous for growth represent one of the most significant obstacles to the implementation of algae in biofuels production [121]. Recent estimations indicate that the nitrogen and phosphorus demands per 1 L microalgal biodiesel are between 0.23 to 1.55 kg and 29 to 145 g, respectively [122]. Life cycle assessments have also forecasted that nitrogen supply will determine feasibility of algal use in the production of biofuels [123, 124].

A specific niche of nutrient recycling for algal cultivation has been created that uses byproducts from different industrial streams including biofuel manufacturing. This culturing alternative was originally proposed as a closed-loop cycle for resource utilization, thereby minimizing the abiotic resource requirement and maximizing value-added product yields [125].

Several recycling strategies have tried to incorporate aqueous streams generated during the hydrothermal processing of algae (discussed in section 4.3.2.4 of this chapter), which are rich in nitrogen, phosphorous, and minerals. These aqueous streams have been added to artificially illuminated algal cultures as a supplement or as a nutritional substitute (Table 3). In these studies, mixotrophy has been implemented with the aim of maximizing biomass, not for the purpose of increasing lipid or metabolite production. Although recycling of byproduct streams

has been proven viable, a purely heterotrophic process has not yet been developed. In addition, studies to optimize lipid production using aqueous waste streams have not been conducted.

3.1. Recycling of microbial spent culture media

Microbial spent culture media is another resource that can be used to recycle water and nutrients. Microbial cultures naturally stop growing at some specific time during cultivation even if nutrients are constantly provided. For microalgae, residual or spent culture media cannot be directly reused or recycled for algal cultivation. Possible explanations for this phenomenon are the depletion of a limiting nutrient and/or presence of an inhibitory compound. For example, De la Hoz [136] attempted to grow *C. protothecoides* by using spent media from a 20 day old, high density culture, which contained residual carbon and nitrogen. The author observed negligible growth in 100% spent media, but some growth was noted when 50% and lower dilutions of spent media were used in combination with nutrient supplementation.

Auto inhibition of algal cultures through allelopathy, the accumulation of excreted organic metabolites in the media, has been shown to decrease the potential for algal biomass productivity [137-139]. For instance, a recent study identified that some organic compounds accumulated in growth media of *Chlorella vulgaris* as >40 kDa biopolymers, likely polysaccharides, that were nitrogen poor. Small organic molecules between 1 to 3 kDa that were richer in nitrogen were also observed. Though the effects of such organic molecules was undetermined, they both have the potential to negatively affect algal growth [139].

The increase of counter ions (e.g. Na^+, K^+, Ca^{2+}) that are not assimilated by algae and thus accumulate in the media has been suggested as another reason why recycling of spent media is not possible for algal cultivation [140, 141]. This issue was addressed by designing a minimal mineral media that avoids accumulation of inhibitory ions during recycling [140]

Table 3. Nutrient recycling using byproduct aqueous streams derived from hydrothermal treatment of biomass

Hydrothermal Processing		**Microbial culture**		**Results**	**Ref**
Biomass	Temp. Time Pressure	Organism	Mode		
Swine manure, *Spirulina*	300°C 30 min 95 psi	Mixed algal culture and pure *C. protothecoides* and *Nannochloropsis oculata*	Mixotrophic supplementation; 10X to 200X AS	Only mixed culture survived; tolerated additions of swine manure (200X) and algal AS (50X)	[125]
Chlorella vulgaris	350°C 20 min 2600 psi	*C. vulgaris*	Mixotrophic supplementation; 300X AS, no mineral media	*C. vulgaris* grew ~1/8 in spiked culture compared to control media	[126]
Spirulina	350°C 60 min	*Chlorella minutissima*	Mixotrophic supplementation; with 10X to 500X	Biomass productivities for 500X and 100X were 0.035 and 0.027 g/L·d, respectively, compared to 0.07 g/L·d in the control	[127]
N. oculata	200°C 40 min	*C. vulgari*s	Mixotrophic supplementation; 50X, 100X, and 200X AS	Biomass concentration of 0.79 g/L for 50X compared to 0.2 g/L in control	[128]
C. vulgaris, Scenedesmus dimorphus, Spirulina platensis, Chlorogloeopsis fritschii	300°C to 350°C 60 min	*C. vulgaris, S. dimorphus, S. platensis, C. fritschii*	Mixotrophic supplementation; 50X to 600X AS	Higher biomass than control for *Chlorogloeopsis* (400X) and *Chlorella* (200X)	[129]

Desmodesmus sp.	300°C 5 min	*Desmodesmus sp.*	Mixotrophic nutrient replacement; (P or N)	50% cultivation water was substituted but minerals had to be exogenously supplied	[130]
Spirulina	300°C 30 min 92 psi	*C. protothecoides*	Mixotrophic supplementation; Initially 100X, but 50X aliquots gradually added	Culture was able to grow ($OD_{680\ nm}$ = 0.8); no biomass quantification	[131]
Nannochloropsis oculata	350°C 60 min	*Escherichia coli, Pseudomonas putida,* *S. cerevisiae*	2 g/L glucose + 2.5X to 10X AS for bacteria; 5 g/L glucose + 2.5X to 20X AS for yeast	*E. coli* and *P. putida* grew in 5X AS as the sole nutrient. *S. cerevisiae* did not grow in AS unless glucose was added	[132]
Filtered municipal wastewater + hydrothermally treated *Spirulina*	300°C 30 min 95 psi	Co-culture of several undefined species including bacteria and algae	Mixotrophic supplementation; 10X to 200X AS	700 mg/L in 10X AS vs. 300 mg/L for control. Nutrients were reduced between 63-95%	[133]
Desmodesmus sp.	300°C 5 min	*Desmodesmus* sp.	Mixotrophic supplementation; 5 cycles of AS recycling	Algal productivity similar after 5^{th} cycle. 40% N and 66% P were replaced by using AS	[134]
Saccharina latissima	500°C 5200 psi	*C. vulgaris*	Mixotrophic supplementation; 50X to 400X AS	Biomass 400 mg/L for 50X, compared to 700 mg/L in control	[135]

Note: Some dilutions originally presented as % were standardized to dilution factors (i.e. Dilution of 1% (v/v) = 100X). AS = Aqueous stream.

4. Bioconversion of microbial biomass for renewable fuels

Microbial biomass (including algae and yeast) can be used to produce biofuels through several different approaches (Figure 4). Direct production includes the use of microorganisms that transform raw materials directly into fuels, which can be isolated without further processing of the cells. Conversely, the whole biomass approach sees microbial biomass as feedstock that is transformed by other microorganisms. The production of biofuels from microbial extracts requires fractionation of the extract using a combination of processing steps to isolate a specific metabolite that can be converted to biofuels.

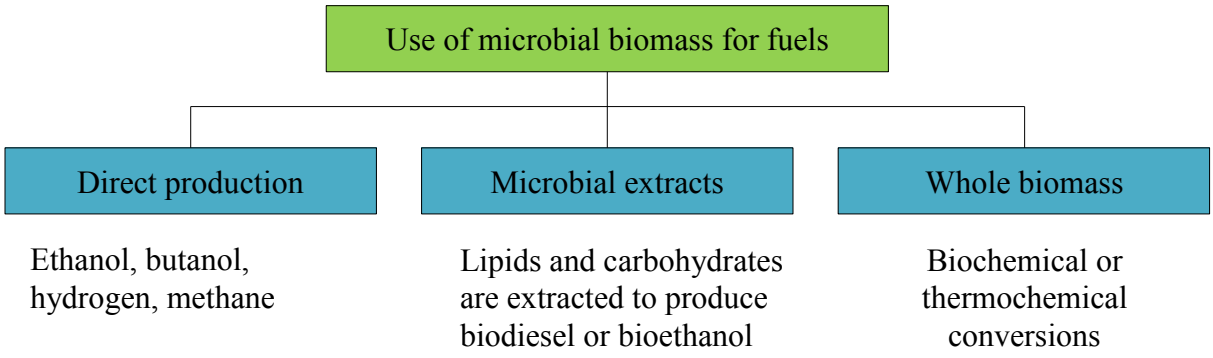


Figure 4. Overall pathways for conversion of microbial biomass to fuels [142].

4.1. Direct production fuel approach

Two of the most relevant examples of direct biofuels production are discussed below. One is the use of yeast to produce ethanol or butanol through fermentative pathways and the other is the use of green algae to produce hydrogen.

4.1.1. Production of ethanol and butanol by yeast

Yeasts are the preferred microorganisms for the production of bioethanol because of their wide substrate utilization and ethanol tolerance. The model ethanol-producing yeast is *S. cerevisiae*, which uses the EMP glycolytic pathway [143]. Different raw materials can be used as

sources of fermentable sugars, including starches from grains and lignocellulose from agricultural waste. Although most of these materials are mainly composed of glucose, other hexoses and pentoses may also be present in the raw material and used for fermentation. Although most industrial yeasts are unable to use pentoses, engineered strains capable of metabolising these sugars have been developed [144].

Production of butanol has been reported in engineered *S. cerevisiae* strains after the inclusion of exogenous enzymes and pathways [145, 146]. These results are extremely promising as butanol is a more attractive biofuel because it is less corrosive and more energy dense compared to ethanol [147]. One of the main challenges with butanol production in yeast and other organisms is the optimization of yields, which are very low compared to ethanol [147].

4.1.2. Production of hydrogen by microalgae

Some green algae such as *Chlamydomonas reinhardtii, Chlorella fusca*, or *Scenedesmus obliquus* can produce hydrogen. The two pathways used to generate hydrogen are: 1) Fixation of CO_2 into H_2-containing substrates during photosynthesis, then generation of molecular H_2 when algae is incubated under anaerobic conditions, and 2) co-production of O_2 and H_2 during photosynthesis (no CO_2 involved) [148]. Although production of hydrogen by microorganisms is well understood, more research is necessary to evaluate the real commercial potential and practicality of this technology [149, 150].

4.2. Microbial extract approach

The separation of individual components from microorganisms offers a wider range of opportunities for byproduct utilization [21, 23]. High-value metabolites and lipids for biofuels can be extracted to be sequentially or independently processed depending on the transformation technology. A typical process for lipid extraction and conversion into biofuels is presented in

Figure 5. Extraction represents 30 to 50% of the total microalgal biodiesel production cost [21, 120, 151]. Applications that use dry cells require extensive dewatering; this step is cost-intensive for large-scale production [152]. The entire process of dewatering consumes a large amount of energy and is thus considered a bottleneck for the development of industrial-scale microbe processing techniques for biofuel production [153]. Mechanical dewatering (i.e. centrifugation or filtration) is considered less expensive than other drying alternatives and is commonly used for pre-concentration of biomass before the final drying step [154]. The use of microbial extracts is associated with high infrastructure and operating costs, which are significant challenges to the commercial deployment of such technologies.

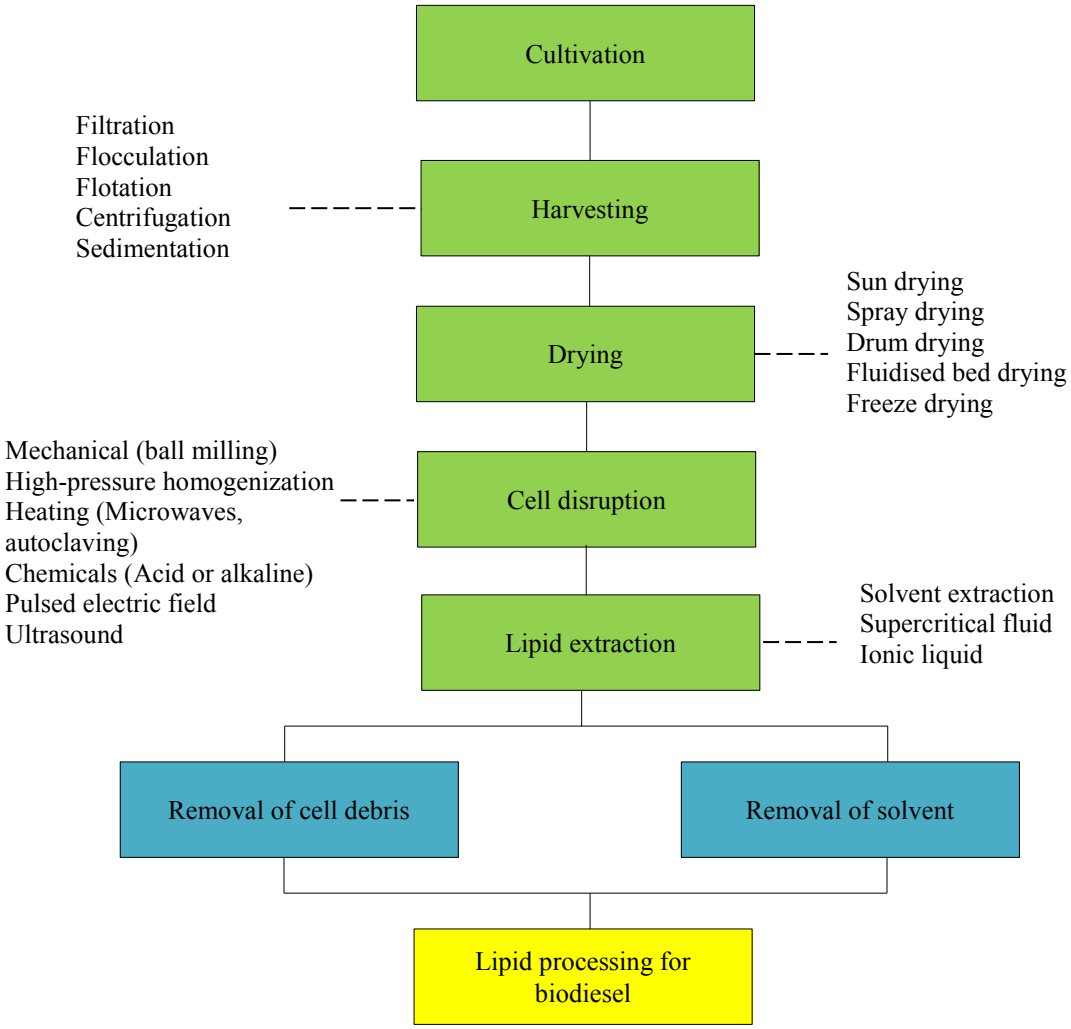


Figure 5. Downstream processing of microbial biomass extracts during biodiesel production [155].

4.2.1. Steps involved in processing microbial extracts

4.2.1.1. Harvesting

The first step in the production of biofuels from microbial extracts requires the transformation of biomass from diluted cultures (0.02 to 0.06% total solids) to concentrated pastes (5 to 25% total solids), which are much more suitable for downstream processing [153]. Most methods used for harvesting are highly energy-intensive and are thus crucial factors in the

overall feasibility of biofuel production [156]. Microbial harvesting can be achieved through filtration, flocculation, flotation, centrifugation, and sedimentation.

Filtration involves the use of a permeable medium that retains microorganisms, but allows media to pass through. This process relies on pressure from a vacuum, a centrifuge, or gravitational forces [153]. Filtration has been applied generally for larger species rather than bacterial-size microalgae [157]. The main disadvantages of using filtration are the time requirement, membrane-clogging issues, and the high costs of operation and maintenance, particularly membrane-replacement and pumping [157].

Sedimentation is a technique that uses gravitational forces to settle biomass. This process can be accelerated using pumps and sedimentation tanks [153]. Since sedimentation depends on density, several issues with the settling of small microbial cells have been reported [158]. Additionally, sedimentation is time consuming and the equipment takes up a lot of space.

Flotation is a separation technique that involves the attachment of air or gas to microorganisms, which causes them to float to the surface. Particle size, which determines contact area and particle capture, is one of the main factors that influence successful use of this technique [153]. Effective flotation occurs with particles of less than 500 μm [159]. Flotation can be achieved by saturating water with air followed by a reduction in pressure, or by injection of air coupled with the use of a high speed agitator [160]. The product of this separation method is a dense foam that is removed as slurry [142]. Currently, there is not enough evidence regarding the feasibility of this technique as a harvesting option for large-scale applications [161].

Flocculation requires the aggregation of microbial particles (cells), which occurs through collision and adhesion. Most of the time, this process is aided by the use of chemicals (flocculants) that react with the negatively charged cell surface. The most common flocculants

are inorganic and organic polymers/polyelectrolytes [153]. Inorganic flocculants such as alum, ferric chloride, and lime are used to neutralize or reduce the surface charge of cells [157]. In this method, large concentrations of inorganic flocculants are required and the final product is contaminated with salts [160]. This method is also sensitive to pH and the species of organism being used. The use of cationic flocculants is considered a better option because anionic and non-ionic polyelectrolytes do not interact with microbial cells [160]. Organic cationic polyelectrolyte flocculants (such as chitosan) are biodegradable and do not contaminate the final product [162]. These polymer flocculants physically link particles through a process called bridging [157]. The efficiency of flocculation when aided by polymer flocculants depends on polymer molecular weight, charge density of molecules, dosage, biomass concentration, broth ionic strength and pH, and mixing [157].

Centrifugation can be used as a separation technique based on particle size and density differences of the microorganisms and medium components. Although very high recoveries (>95%) can be achieved using very high acceleration speeds, this processing is highly energy intensive and thus too costly for the primary harvesting method of microbes [163]. As a consequence, centrifugation has been proposed as a secondary harvesting method to bring 1 to 2% (w/v) cultures to concentrations of 10 to 20% [156]. Also it is suitable for the recovery of high-value metabolites as other methods can result in degradation of final product [163].

4.2.1.2. Drying

Drying is commonly used to extend shelf life of the final product or, depending on the final application, as a pre-processing step. Several technologies can be applied for biomass dehydration such as sun drying, low-pressure drying, spray drying, drum drying, fluidised bed drying, or freeze drying. Sun drying is the cheapest method but requires long times and large

surfaces, and may lead to material losses [164]. The other methods can be applied in value-added product conservation where high-energy or costly operation can be justified. The appropriate drying method to use is determined by the application and the desired final product. For instance, spray drying is effective but may also damage susceptible final products (i.e. pigments) [157]. The drying temperature during lipid extraction influences lipid yield and quality [165]. Freeze drying is commonly used in a laboratory setting, but it is expensive and not feasible at an industrial scale.

4.2.1.3. Cell disruption

Cell disruption is often required for recovering intracellular products from microorganisms, such as microalgae and yeast, which are protected by extremely tough cell walls. For instance, microalgae generally possess a small but thick cell wall that can only be disrupted using harsh methods [151, 155]. Typical laboratory extraction protocols involve unit operations such as freeze drying, pulverization, cell homogenization, sonication, autoclaving, osmotic shock, or microwaving, but none are good candidates for use in industrial scale up [166].

Extraction of intracellular lipids from yeast also requires breakage of the thick yeast cell wall. This process is commonly achieved by using acids, bases, enzymes, or by applying physical and mechanical pressure. The latter includes glass shear, osmotic shock, pressing, and sonication [167].

4.2.1.4. Lipid extraction

Conventional microbial extraction and separation methods are commonly designed to obtain one product, the value-added product, with little regard for the other fractions [168]. After cell disruption, the most common way of extracting different metabolites from algae is by using solvents. The extraction solvents should meet several requirements such as being

inexpensive, volatile, pure, water-immiscible, and poor extractors of unwanted components [169]. Hexane, ethanol, chloroform, and diethyl ether are some of the most common solvents used to extract algal metabolites such as astaxanthin, β-carotene, and some specialty fatty acids [170-172]. The extraction of neutral lipids has also been reported by using a combination of polar and non-polar solvents [155].

A technique recently examined for extraction of algal metabolites is the use of supercritical fluids. The extraction fluid (i.e. CO_2) is kept at temperatures and pressures that exceed critical values, which are the suitable conditions for lipid extraction. This technique has been applied for extraction of astaxanthin, carotenes, polyunsaturated fatty acids, and other compounds [173-175]. Supercritical fluids offer some advantages such as short treatment times, adaptability of extraction conditions, and a clean, solvent free product [176]. However, some of its overall disadvantages are high installation and operation costs, a requirement for dried cells, and the occasional production of toxic substances [155].

Alternative techniques such as pulsed electric field processing, enzymatic treatment, and ultrasound application have also been studied, specifically for microalgal extractions. Although they have shown improvements in extraction yields and performance, their application in large scale production is unproven [177]. The use of ionic liquids, which are organic salts that are liquids below a threshold temperature, has been recently proposed for extraction of some microbial products. Ionic liquids are non-volatile, non-flammable, thermostable, and amenable for modification and design [178]. Microalgal biomass has been treated with hydrophilic ionic liquids (100 to 140°C at atmospheric pressure) to obtain a mixture of well-preserved algal constituents that can be easily fractionated [179]. High yields and selective lipid recoveries have been observed for different *Chlorella* strains when single or combined ionic liquids were used

[180, 181]. In addition, the selective extraction of algal isoprenoids (β-carotene, squalene, and botryococcenes) without loss of cell viability has been recently reported [182]. Although promising, the use of ionic liquids for extraction requires comprehensive databases of their basic properties, as well as further investigation of their environmental effects or use beyond lab scale.

For yeast, several specific lipid extraction technologies have been proposed in recent years. These involve the use of solvents at near-critical or supercritical conditions, or the use of pressurized liquids of different solvent mixtures [152, 183-185]. Direct extraction from fermentation broths has also been studied by using homogenizers under high pressure, microwaves and subsequent enzymatic treatments, and solvents [30, 186]. However, only some of these approaches have been scaled-up, and their feasibility and cost-effectiveness are justified only when value-added specialty lipids or metabolites are produced.

The final selection of the extraction method depends on the intended use and quality of the recovered product(s). For instance, if polyunsaturated fatty acids are the products of interest, high temperatures should not be used as this could lead to the oxidation and rancidity of the oils during their extraction.

4.3. Whole biomass approach

The use of whole microorganisms represents another option to produce biofuels and has been mainly studied in algae. This strategy intends to reduce steps and thus the costs involved in the microbial extract approach, although some pre-processing such as dewatering might still be required. The whole biomass approach can be divided into biochemical and thermochemical conversions, which are also subdivided into several processing technologies (Figure 6).

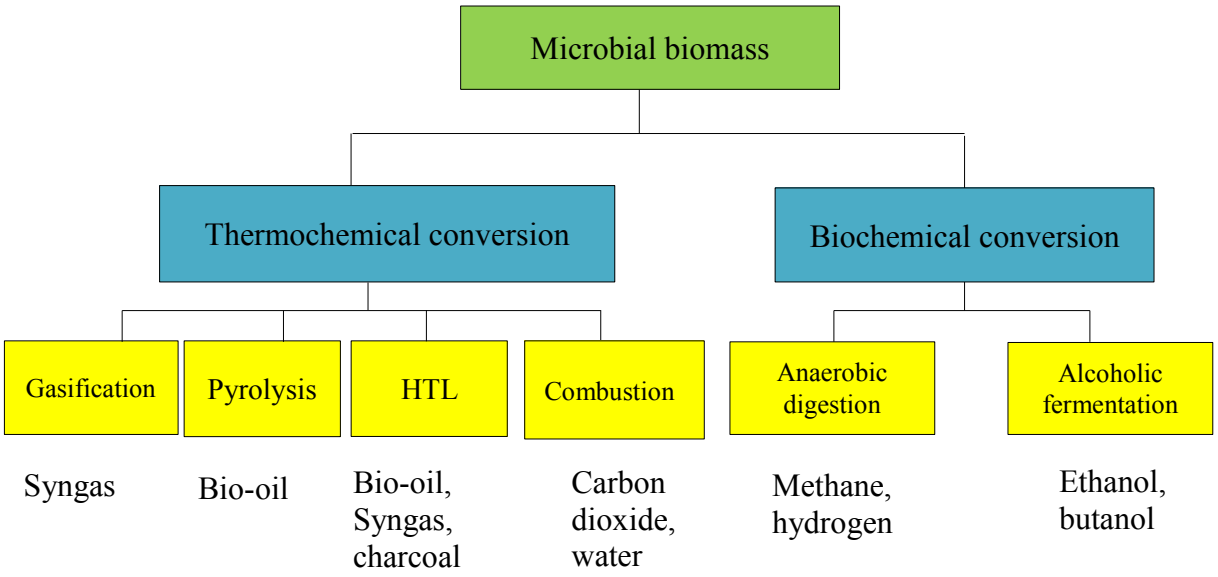


Figure 6. Processing alternatives for whole microbial biomass to biofuels [187]. HTL, hydrothermal liquefaction.

4.3.1. Biochemical conversion

Microbial biomass can serve as the main feedstock for biochemical conversions through anaerobic digestion and fermentation. Anaerobic digestion converts algal biomass into methane and carbon dioxide through a sequence of three steps. The first step of anaerobic digestion is biomass hydrolysis to release soluble sugars [161]. These sugars are used by fermentative bacteria to produce several organic compounds such as alcohols, acetic acid, and volatile fatty acids. The last stage involves methanogens that convert the organic compounds into methane and carbon dioxide. Methane production by anaerobic digestion seems to be energetically favorable for non-oleaginous algal biomass [188].

Another biochemical conversion of algae is the yeast fermentation of algal carbohydrates available after biomass hydrolysis to produce ethanol. However, little work has been done that examines production of ethanol from macro and microalgae biomass through bacterial or yeast fermentations [189-192].

4.3.2. Thermochemical conversions

This technique generally uses high temperatures in the absence or presence of specific elements to convert microbial biomass into biofuels. Overall, thermochemical processes can be separated into gasification (high temperatures with steam), pyrolysis (high temperatures in the absence of oxygen), combustion (high temperatures in the presence of oxygen), and thermochemical liquefaction (high temperatures, high pressures, with water).

4.3.2.1. Gasification

Gasification converts biomass into gases that can be used as fuel. This process uses oxygen or steam at high temperatures [193]. The desired product, which is commonly known as syngas, is composed of varying concentrations of CO, H_2, CO_2, N, and CH_4 [194]. Gasification studies using different algal biomasses have been mainly focused on optimizing conversion yields and driving the production of a particular gas, mostly H_2. Micro and macroalgae have been gasified at a wide range of temperatures, times, and pressures. For instance, temperatures from 500 to 1,000°C have been used for gasification of *Spirulina*, *Chlorella*, and gulfweed macroalgae [194-198]. Although gasification usually requires high temperatures, the use of low temperatures can also be applied when catalysts are used to drive gas production [199]. Gasification processes using milder temperatures (200 to 500°C) and catalysts (Ni, SiO_2/Al_2O_3, Ru/C, Ru/ZrO_2, Ni, PtPd, Ru, CoMo, NiMo, and Ru/TiO_2) have been investigated using a variety of different algae [126, 198, 200, 201].

4.3.2.2. Pyrolysis

Pyrolysis is a thermal process in which biomass is decomposed into gas, liquid (bio-oil), and solid (char) products. Pyrolysis takes place in the absence of oxygen at temperatures from 350 to 700°C [202]. Algal pyrolysis has been widely studied and its overall thermal behavior is

separated into three stages: 1) the evaporation of water and volatiles, 2) the major degradation of organics, and 3) the slow decomposition of solid remains [199]. Although algae have been considered as prominent biomass sources for industrial pyrolysis, other microorganisms including yeast have only been considered for analytical pyrolysis. In this case, pyrolysis of yeast biomass was coupled with mass spectrometry analysis to identify levels of specific metabolites [203].

Laboratory scale pyrolysis of several algal species has been studied in various reactors [204-214]. Most of these reports used fast pyrolysis (rapid heating), which drives products to bio-oil [202]. The highest conversion yields reported for whole algal biomass to bio-oil are about 70% dry weight [205]. Variables studied for algal pyrolysis are temperature (300 to 900°C), time (0 to 2 h), gaseous component (vacuum, N_2, Ar, CO_2), and catalysts used (Na_2CO_3, HZSM-5, AcC, CaO, SiC, S).

One of the main disadvantages of bio-oils produced by whole biomass pyrolysis is the nitrogen content of the final product. This element is undesirable in biofuel, as are other minor components such as sulphur, salts, and minerals. Biofuels with nitrogen and/or sulfur require further upgrading to meet fuel specifications [215, 216].

4.3.2.3. Direct combustion

Direct combustion implies the burning of biomass in the presence of O_2 to produce gas. During combustion, several oxidizing reactions occur including the conversions of C to CO_2 and H to H_2O [4]. In 2004, direct combustion of biomass represented 97% of the energy produced from biomass [217]. Combustion of biomass can be used to produce heat, biogas, or stream, which can drive generation of mechanical or electrical energy [4, 193]. An early estimate indicates that the net electrical efficiency for combustion of biomass ranges from 20 to 40%

[218]. Most biomass currently used for direct combustion comes from wood, municipal solid waste, or agricultural waste [4], though any type of biomass with 50% moisture or less can be used [193]. Although combustion is considered a direct method for the conversion of biomass, it requires several conditioning steps such as drying and size reduction. These costly pre-processing requirements represent a major disadvantage for the use of direct combustion, particularly when using a high moisture content biomass such as those obtained from microbial sources [202]. Another consideration for microbial biomass combustion is the emission of additional greenhouse gases, though co-firing of coal and algae may be more environmentally sound than coal burning alone [219].

4.3.2.4. Thermochemical liquefaction

Thermochemical liquefaction is a process applied to wet microbial biomass for the production of a water-immiscible biocrude. This processing requires a combination of high temperature (200 to 350°C) and pressure (5 to 20 MPa) to reach sub-critical conditions, which can be aided by use of a catalyst [202]. The product (biocrude) has to be further upgraded by catalytic hydrogenation. The goal of liquefaction is to obtain a higher-quality bio-oil than that obtained through pyrolysis, specifically related to higher heating values (30 to 35 MJ/kg) and lower oxygen content (10 to 18%) [220]. Although the processing required for liquefaction is more complex compared to pyrolysis, it allows for the use of wet materials, which eliminates the dewatering step making liquefaction an attractive alternative to pyrolysis.

Recently, an arbitrary sub-classification has been used to describe wet microbial biomass processes based on temperature and pressure. These wet processes have been termed hydrothermal carbonization (200°C, 2 MPa), hydrothermal liquefaction (280 to 370°C, 10 to 25 MPa), and hydrothermal gasification (400 to 700°C, 25 to 30 MPa) [221]. However, available

literature is not consistent in the use of this terminology and these definitions are not standardized.

Renewable fuels produced from hydrothermal reactions of different algae have been reported [216, 222-227]. Typically, the products from these processes contain high concentrations of nitrogen, sulphur, and/or salts, and thus upgrading is required to meet fuel specifications [215, 216]. An alternative approach is the treatment of algal biomass at temperatures below 300°C. This approach is known to efficiently disrupt the algal cell wall, eliminating the need for dewatering and the separating of reaction products [212, 228]. Furthermore, some nitrogen and other undesired elements remain in the aqueous byproduct stream resulting in a significantly cleaner bio-oil stream [212, 228]. However, since the bio-oil stream still contains some undesirable elements, further upgrading steps are required.

4.3.2.5. Two-step thermal lipid-to-hydrocarbon technology

A two-step thermal lipid-to-hydrocarbon technology (LTH) for the conversion of fats and oils into fuels and chemicals was recently developed in Dr. Bressler's laboratory [229]. In this process, different lipids such as edible and inedible vegetable or animal fats, as well as used cooking oils can be used as feedstocks [230]. This technology requires pre-processing of the lipid feedstock in a hydrolysis reactor to yield free fatty acids and an aqueous byproduct waste stream containing glycerol (Figure 7). Recovered fatty acids are pyrolyzed in a second reactor to produce various forms of fuels and chemicals. The major advantage of this technique is that a catalyst is not required. Also, at industrial scale, the energetic requirement in LTH is optimized by using heat exchangers between hydrolysis and pyrolysis.

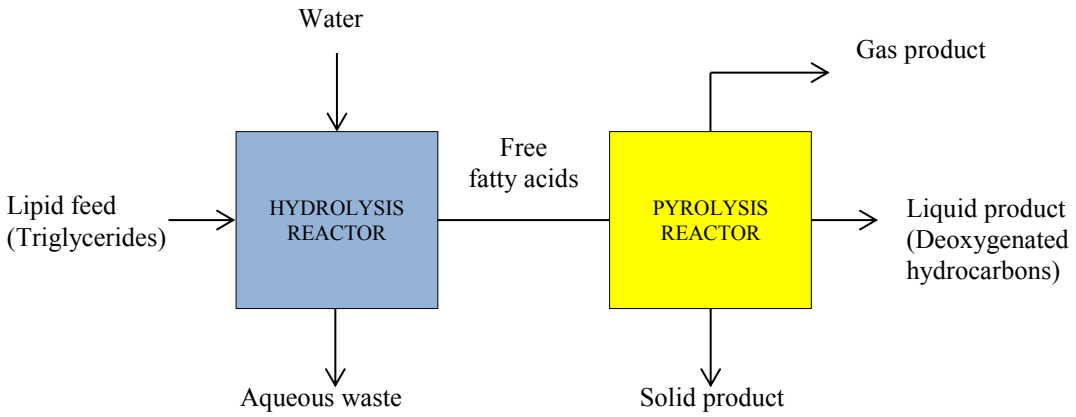


Figure 7. Two-step thermal lipid-to-hydrocarbon technology [231]

The standardized subcritical conditions of lipid hydrolysis for the LTH technology (280°C, 1 h, initial pressure of 500 psi) [232] resemble some previously reported hydrothermal treatments of algal biomasses (discussed in section 4.3.2.4). Those hydrothermal liquefaction studies of algal biomass have consistently reported two streams, bio-oil and aqueous streams, as final products. Specific differences between LTH and other hydrothermal treatments for whole biomass utilization are:

- **Purpose:** Hydrothermal processing is aimed at producing bio-oil without further thermal processing, although upgrading is strictly required to eliminate the considerable amounts of undesired nitrogen, phosphorus, sulfur, and other minerals. Conversely, the desired products of LTH are fatty acids.
- **Post hydrolysis methodology:** Previous hydrothermal treatments have not reported further separations of bio-oils into lipids, fatty acids, or other fractions. LTH requires fatty acid extraction and recovery.

- **Feedstock:** All hydrothermal reports thus far have used non-oleaginous microorganisms as biomass feedstock. In contrast, further applications of LTH strictly require high lipid content microbial biomass utilization.

5. Justification for the research conducted in this thesis

Microbial oils have been underutilized for the production of biofuels due to technological challenges in cultivation and processing. Alternative processes that offer not only different routes of transformation but also variety in product portfolio need to be developed to advance this field of biorefining. An example of unconventional processing is the LTH technology, which uses an initial pre-processing lipid hydrolysis and then recovers fatty acids for biofuel production. Although it has been proven that the LTH conversion is robust and efficiently transforms various lipids, the technological feasibility of using oleaginous microbes as lipid feedstocks is unknown due to the complex nature of these organisms (Figure 8).

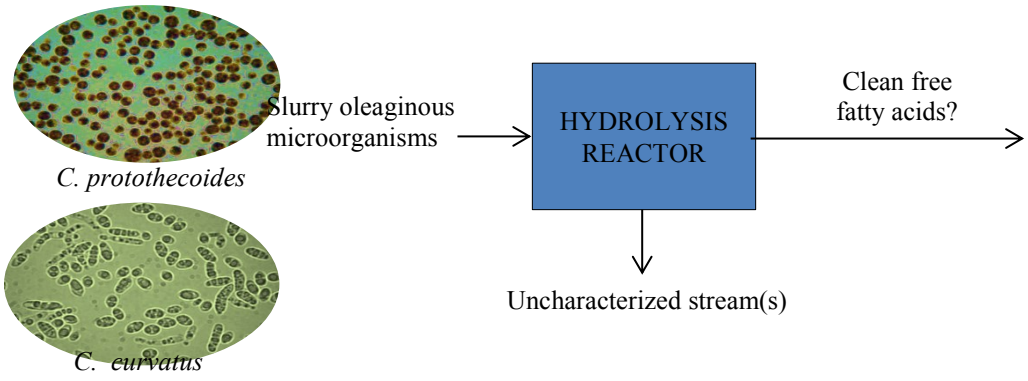


Figure 8. Hypothetical application of LTH technology to oleaginous biomass.

Factors that favour the application of LTH to oleaginous microorganisms are the direct use of microbial slurries without dewatering and the generation of an aqueous byproduct containing not only glycerol, but also other potentially useful nutrients for cultivation. However, such application of LTH requires further investigation with regards to: 1) if intracellular lipids in

oleaginous organisms would be released as free fatty acids after applying standardized lipid hydrolysis conditions of LTH, 2) if decomposition products from the microbial biomass would be mixed with free fatty acids following hydrolysis, 3) the composition and properties of post-hydrolysis stream(s), and 4) the potential for incorporation of byproduct stream(s) in cultivation of oleaginous microorganisms through recycling.

6. Research aims

The general research aim of this thesis is to prove that LTH conversion is a feasible technological alternative that can be used to generate desired products from biomass. Furthermore, this thesis attempts to demonstrate that aqueous byproduct streams produced through the LTH technology and other industries can be used to cultivate oleaginous microorganisms. These aims will be addressed through analysis of the following questions:

i. Can application of LTH pre-processing effectively release fatty acids from oleaginous algal and yeast biomasses and if so, can these free fatty acids be efficiently recovered?
ii. Are the fatty acids recovered from LTH pre-processing of oleaginous biomass drop-in compatible with current methods of biofuel production?
iii. Can byproduct streams from LTH and other industries be incorporated into nutrient recycling programs where they will be used to promote growth of and lipid accumulation in oleaginous microorganisms?

The first two questions are addressed in Chapter II: Two-step thermal conversion of oleaginous microorganisms into renewable hydrocarbons. The final question is examined in Chapter III: Cultivation of oleaginous microorganisms using aqueous fractions derived from hydrothermal pretreatments of biomass and Chapter IV: Heterotrophic growth and lipid

accumulation of *C. protothecoides* in whey permeate, a dairy byproduct stream, for biofuel production.

II. Two-step thermal conversion of oleaginous microorganisms into renewable hydrocarbons[a]

1. Introduction

The production of third generation lipid-based renewable fuels from microbial biomass represents a promising option to meet future energy demands. Use of oleaginous microorganisms, especially algae and yeast, overcomes several major challenges associated with other lipid sources, particularly those that are edible products or compete with such products for resource utilization [233, 234]. Some oleaginous organisms can be cultivated at high cell densities using various nutritional feedstocks and can accumulate lipids in amounts up to 70% dry weight [51]. One of the most influential factors for promoting lipid accumulation in oleaginous microorganisms is the carbon: nitrogen (C:N) ratio of the growth medium [51]. Cultures having an excess of carbon and limited nitrogen (i.e. a high C:N ratio) undergo a shift in biosynthesis from proteins to lipids, which are then stored in the cells (as lipid droplets) as an energy reservoir [235-237]. The recovery and utilization of these microbial lipids to produce biofuels are currently faced with some technological complications.

Typical lipid-based fuel (biodiesel) is produced through transesterification reactions of triglycerides with methanol and a catalyst to yield fatty acid methyl esters along with glycerol [238, 239]. The utilization of alternative feedstocks for biodiesel production, such as oleaginous microbial biomasses, would require modification of the traditional process and may require the use of novel catalysis to avoid undesired saponification reactions [240]. Another major challenge is the extraction of lipids from oleaginous microorganisms. Intracellular lipids must first be freed

[a] Parts of this chapter have been previously published:
Espinosa-Gonzalez I., J. Asomaning, P. Mussone, D.C. Bressler, Two-step thermal conversion of oleaginous microalgae into renewable hydrocarbons. Bioresource Technology, 2014.158: p.91-97.
Espinosa-Gonzalez I., A. Parashar, D.C. Bressler, Hydrothermal treatment of oleaginous yeast for the recovery of free fatty acids for use in advanced biofuel production. Journal of Biotechnology, 2014. 187:p.10-15.

from microalgae and yeast through breakage of thick cell walls. Extraction of microbial lipids from cell lysates is commonly achieved by using mechanical, chemical, or enzymatic methodologies, which are difficult to implement at an industrial scale [172]. Furthermore, some lipid extraction methods demand the use of dry cells, which requires extensive cost-intensive dewatering [152]. Successful implementation of oleaginous microorganisms in biofuel production requires cost-effective strategies to deal with these technological concerns.

The hydrothermal treatment of biomass is an alternative processing approach that disrupts microbial cell walls and also eliminates the need for dewatering [241]. Different hydrothermal processes (hydrothermal liquefaction) have been previously applied to microalgae biomass during the production of bio-oil, which is a complex thermal decomposition product of biomass that can be further upgraded to biofuel [212, 228]. LTH technology, which converts fats and oils into fuels and chemicals [229], consists of two main steps: 1) hydrolysis of fats and oils to yield fatty acids, and 2) pyrolysis of the recovered fatty acids to produce biofuel. Since the hydrolysis of fats and oils using LTH and hydrothermal liquefaction methodologies occurs under similar processing temperature and pressures, we wondered if hydrothermal liquefaction could be used to simultaneously break down cell walls and generate fatty acids that could be recovered and used directly in the second step of the LTH process. If so, the current major issues of adapting existing biodiesel production to microorganisms use would be eliminated.

This study evaluated the implementation of oleaginous microbial biomasses as a feedstock for the LTH technology. Lipid-rich biomasses from model algal and yeast were subjected to a hydrothermal treatment. Following hydrolysis, the aqueous stream was separated from the solid stream, and the latter was subjected to hexane extraction to recover fatty acids. The recovered fatty acids were subsequently used in a pyrolysis reaction for the production of

biofuels. This study demonstrates the direct conversion of oleaginous microalgal and yeast biomass into valuable platform chemicals and fuels that are compatible with the existing industrial hydrocarbon infrastructure.

2. Materials and methods

2.1. Materials

Microalgae *C. protothecoides* (UTEX 256) was directly obtained from a culture collection (University of Texas, Austin, TX). Axenic stocks were stored at room temperature (23 ± 2°C) with 12/12h light/dark cycles (light intensity of 25 μmol/m^2s) as the long-term storage conditions. New agar slants were made every 4 weeks. Base mineral media used for algal growth was composed of potassium phosphate monobasic (99%; 2.8 g/L), potassium phosphate dibasic (99%; 1.2 g/L), magnesium sulfate heptahydrate (99%; 1.2 g/L), iron(II) sulfate heptahydrate (99%; 48 mg/L), boric acid (99.5%; 11.6 mg/L), calcium chloride dehydrate (99%; 10 mg/L), manganese(II) chloride tetrahydrate (99%; 7.2 g/L), zinc sulfate heptahydrate (99%; 0.88 mg/L), copper(II) sulfate (99%; 0.32 mg/L), molybdenum(VI) oxide (99.5%; 72 μg/L), and thiamine hydrochloride (40 μg/L) [242].

Yeast *Cryptococcus curvatus* (ATCC 96219) was obtained from the American Type Culture Collection Centre (Manassas, VA). For long term storage, glycerol (15%) stocks were kept at -20°C. Yeast Extract Peptone Dextrose (YEPD) agar was used for short term agar plate stocks, which were transferred to fresh YEPD media every 4 weeks. Colonies from these plate stocks were used to inoculate small amounts of YEPD broth that were grown for 24 h at 30°C to produce starter cultures. Base mineral media used for yeast cultivation (1X) contained ammonium chloride (99%; 0.645 g/L), potassium phosphate monobasic (99%; 7.0 g/L), sodium phosphate dibasic dodecahydrate (99%; 2.0 g/L), magnesium sulfate heptahydrate (99%; 1.5

g/L), calcium chloride dihydrate (99%; 0.1 g/L), iron(III) chloride hexahydrate (98%; 24 mg/L), zinc sulfate heptahydrate (99%; 5 mg/L), manganese(II) sulfate monohydrate (98-101%; 2 mg/L), copper(II) sulfate (99%; 7 mg/L), and thiamine hydrochloride (99%; 40 μg/L) [243].

All chemicals used for media preparation were purchased from Sigma-Aldrich (St. Louis, MO). Sulfuric acid and hexane (HPLC grade) were obtained from Fisher Scientific (Fairlawn, NJ) and nitrogen (99.998%) was obtained from Praxair (Mississauga, ON).

2.2. Methods

2.2.1. Algal biomass production

C. protothecoides was grown in a fed-batch regime in a 10 L bioreactor (Biostat B. Sartorius, Germany). Media used for growth was composed of a mineral base [242] with glucose and yeast extract as the main carbon and nitrogen sources, respectively. Lipid accumulation in the microalgae was promoted by maintaining a high carbon-to-nitrogen mass ratio in the media [244]. The reactor was inoculated at 1.5 OD_{600nm} from a shake flask culture entering stationary phase. Concentrated nutrients (400 g/L glucose, 16 g/L yeast extract) were added to the fermentation at irregular intervals to maintain glucose concentration between 10-30 g/L thereby avoiding substrate inhibition [244]. Aeration rate and stirrer speed were varied between 1-2 vvm and 100-200 rpm, respectively, to keep air saturation over 20% [244]. When necessary, pure O_2 was mixed with air for the same purpose. At harvesting time, algal biomass was pelleted at 5,000 x *g* for 10 min in an Avanti, Beckman Coulter centrifuge (Brea, CA) and the concentrated slurry stored in a fridge (2°C) before being used for the hydrolysis experiments. Fermentations were conducted in triplicate to produce enough biomass for the experiments. Algal slurries were pooled and homogenized for use in hydrolysis reactions. This slurry had 27 ± 1 % total solids.

2.2.2. Yeast biomass production

2.2.2.1. Batch

C. curvatus was cultured in 250 mL Erlenmeyer flasks with 100 mL base mineral media [243]. Glucose was used as the main carbon source and was added at a final concentration of 30 g/L (1 C mol/L). Yeast extract was used as the main nitrogen (N) source and was added at a final concentration of 1.2 g/L (9.2 E^{-3} mol/L) to establish a final carbon to nitrogen ratio of 100:1. The pH of the media was initially adjusted to 5.4 with 2 M KOH. Flasks were inoculated with a starter culture (5% v/v) in exponential growth phase (24 h). Cultures were grown at 30°C with agitation (200 rpm) for a total of 72 h. All experiments were done in triplicate and assessed for contamination microscopically.

2.2.2.2. Fed-batch small scale

C. curvatus was grown in fed-batch mode in 500 mL flasks with 200 mL mineral media. Flasks were inoculated with 5% (v/v) starter culture grown to exponential phase (24 h). 8.5 mL of a concentrated glucose solution (200 g/L glucose) and 0.50 mL of a concentrated yeast extract solution (140 g/L) were added to the fermentation as needed to maintain a glucose concentration of approximately 10 g/L. The experiment was conducted in triplicate.

2.2.2.3. Fed-batch large scale

C. curvatus was grown in fed-batch mode in 5 L Infors-HT bioreactors (Bottmingen, Switzerland). Media used for growth was the same as described above for batch cultures (section 2.2.2.1). Bioreactors were inoculated with 5% (v/v) starter culture in exponential growth phase (24 h). Concentrated glucose and yeast extract solutions (750 g/L glucose, 30 g/L yeast extract) were added to the fermentation as needed to maintain a glucose concentration below 60 g/L.

Aeration rate and stirrer speed were varied between 1-2 vvm and 100-200 rpm, respectively, to keep air saturation over 30% [245]. When necessary, pure O_2 was mixed with air for the same purpose. At harvest, yeast biomass was concentrated by centrifugation (5,000 x *g* for 10 min) in an Avanti, Beckman Coulter centrifuge (Brea, CA) and the slurry was stored in a fridge (2°C) until further use. Fermentations were done in triplicate. Yeast slurries were pooled and homogenized before hydrolysis reactions.

2.2.3. Analysis of fermentations

2.2.3.1. Glucose

Glucose concentration was monitored using the glucose oxidase/peroxidase (GOPOD) method (Megazyme test kit, Wicklow, Ireland) in a microtiter format. Briefly, GOPOD reagent (200 µL) was added to wells of microtiter plates (96 flat bottom well plate, Corning, NY) along with the glucose-containing samples (7 µL). Microplates were incubated in a microplate reader (Biotek, Winooski, VT) at 40°C for 20 min. After incubation, absorbance (510 nm) was measured and glucose levels were determined through comparison with the reagent blank and the glucose standard. Five replicates were done for each measurement.

2.2.3.2. Growth

Growth of algal and yeast cultures was assessed using OD_{600nm} readings and standard curves for dry biomass. To create standard curves, several dilutions of stationary algal or yeast cultures were made (OD_{600nm} from 0.2 to 0.8). A fixed volume of each dilution was filtered through pre-weighed 0.22 µm membranes. Dry weight of cultures was then measured and correlated with optical density readings. Experiments were done in triplicate.

For algae: $y = 2.2899\ x + 0.0523$ ($R^2 = 0.9944$)

For yeast: $y = 1.9734 x + 0.0955$ ($R^2 = 0.9947$)

where $y = OD_{600nm}$ and x = dry weight (g/L).

2.2.3.3. Microbial lipids

Levels of microbial lipids were determined gravimetrically. Briefly, freeze-dried algal or yeast biomass was crushed with a mortar and pestle followed by several hexane washes of the resulting paste (as needed). Lipid percentage was determined gravimetrically from hexane supernatants. For algal samples, lipid accumulation was measured by fluorescence with Nile Red [136]. For this measurement, algal biomasses of known lipid content (6.3 - 48.9% dry weight basis) were used to produce a standard curve (fluorescence signal vs. lipid percentage) for each experiment.

2.2.4. Hydrolysis

Hydrolysis experiments were conducted in two different scale reactors in order to amass quantities required for overall product distribution and mass balance determination for the large scale reaction. In the first case, 15 mL stainless steel batch reactors were heated in a Techne model SBS-4 fluidized sand bath with a Techne TC-8D temperature controller (Burlington, NJ) [246]. Large-scale reactions were conducted in a 2 L or 5.5 L batch stainless steel reactor (Parr Series 4530 and 4582, Parr Instrument Co., Moline, IL) for algae and yeast, respectively.

Hydrolysis reactions in both cases were conducted at 280°C for 1 h with an initial pressure of 500 psi. These conditions were selected based on lipid hydrolysis standardization studies conducted in our laboratory (personal communication). The reactors were loaded with 10 g of algal or yeast slurries (15 mL reactor), 600 g of algal slurry (2 L reactor), or 2.4 kg of yeast slurry (5.5 L reactor). After loading, the reactors were purged three times at 500 psi, and then pressurized to the desired initial pressure with nitrogen. Hydrolysis time started when the set

temperature was reached. The reactions were stopped by quenching in a water bath or by using an external glycol cooling unit for the small and large scale reactors, respectively.

2.2.5. Post-hydrolysis treatment

2.2.5.1. 2 L and 5.5 L reactors

Samples of the gas fraction were collected during the depressurization cycle following completion of the reaction. Samples of liquid and solid products were collected using plastic containers. A Büchner funnel with glass fiber Whatman GF/A filter (Whatman, Maidstone, Kent) was used for initial separation of the aqueous fraction from the paste. The paste was then successively washed with de-ionized water and hexane and the filtrates were collected in separate containers. Hexane solubles were recovered by evaporating hexane in a Rotovapor® (Büchi, Flawil, Switzerland), and were further dried in a convection oven for 2 h at 105°C to enable determination of their mass gravimetrically. The insoluble paste retained on the filter was dried in a convection oven at 105°C to a constant weight and this fraction was considered as insoluble solids (Figure 9).

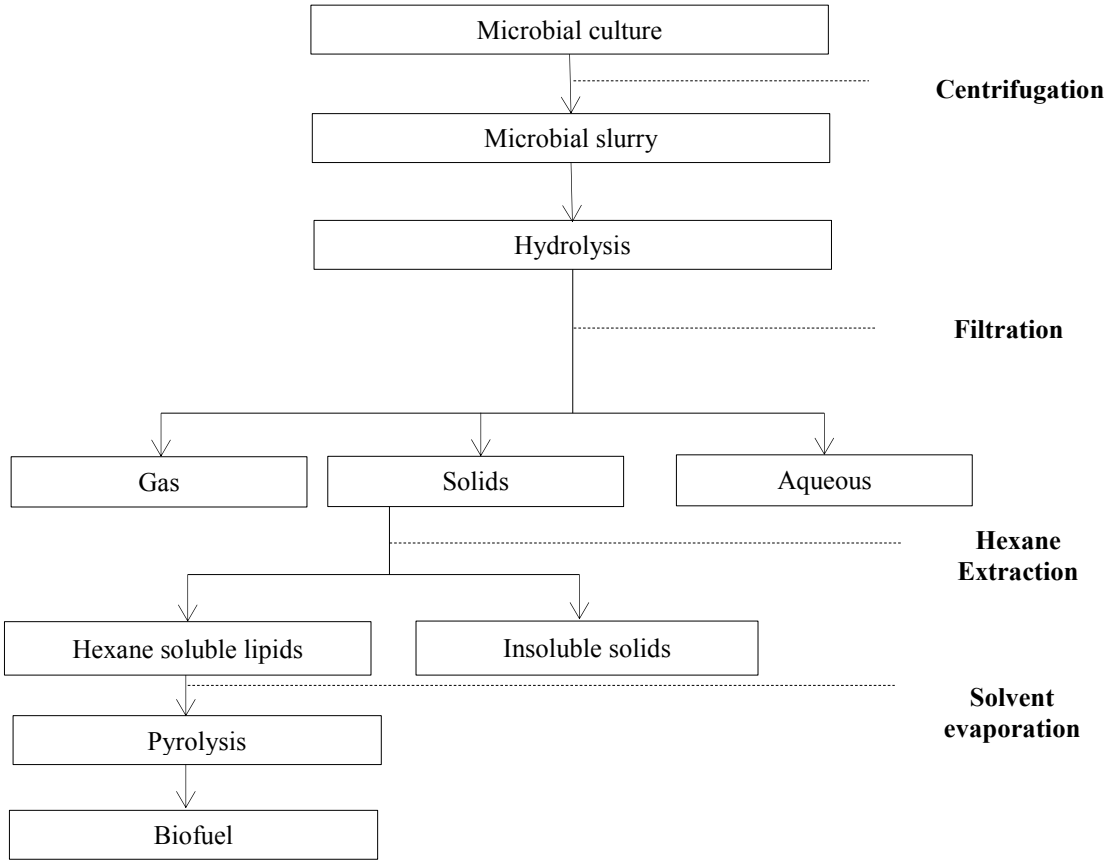


Figure 9. Production of biofuels from fatty acids recovered from oleaginous *C. protothecoides* and *C. curvatus.*

2.2.5.2. 15 mL reactor

The mass of gas was determined by measuring the weight of the reactor before and after venting [246]. The composition of the gas fraction was analyzed using GC-TCD and GC-FID following the method described by Asomaning *et al.* [247]. The reaction product was collected in pre-weighed 50 mL plastic conical centrifuge tubes (Fisher Scientific, Fairlawn, NJ), frozen at -80°C, and freeze-dried for 48 h. The weight of the freeze-dried material was used to calculate the mass of the aqueous and soluble products by difference.

2.2.6. Pyrolysis of hexane solubles from algal samples

Pyrolysis was conducted in 15 mL batch stainless steel microreactors as described by Maher *et al.* [246]. Briefly, approximately 1 g of hexane soluble lipids was weighed into a clean and dry microreactor which was closed, checked for leaks, purged with nitrogen, and sealed. The microreactor was heated at 410°C under constant agitation for 2 h and immediately quenched in a bucket of water at room temperature. The surface of the microreactor was cleaned and dried using compressed air to ensure complete removal of sand residues.

2.2.7. Analysis of products

2.2.7.1. Proximate

Moisture content was determined gravimetrically by drying samples in a convection oven at 105°C to a constant weight. Total lipid content was determined gravimetrically following the extraction method described by De la Hoz *et al.* [248]. Ash determination was conducted gravimetrically by weighing residues after complete combustion in a muffle furnace at 550°C. Total nitrogen was measured by the Dumas Combustion Method (using a protein estimation factor of 6.25) at the Natural Resources Analytical Laboratory (NRAL), University of Alberta.

2.2.7.2. Chemical

The glycerol content of the aqueous fraction was determined using an Agilent 1200 series HPLC, with a refractive index detector, Bio-Rad Aminex HPX87H column at 60°C (300 mm x 7.8 mm), and 5 mM sulfuric acid as mobile phase, at a flow rate of 0.5 mL/min. Water-soluble phosphorus (PO_4^{3-}), ammonium, and nitrate were determined colorimetrically using a SmartChem Discrete Wet Chemistry Analyzer at NRAL.

2.2.7.3. Elemental

Elemental analysis (C, H, N, S, and O by difference) was done using a Carlo Erba EA1108 Elemental Analyzer at the Analytical and Instrumentation Laboratory at the Chemistry Department, University of Alberta. Total phosphorous was measured by the Kjeldahl method at NRAL.

Metal cations were measured by Atomic Absorption Spectrophotometry in a Varian 880 Atomic Absorption Spectrometer by aspiration into a high temperature air/acetylene or nitrous oxide/acetylene flame at NRAL.

2.2.7.4. Lipids

Qualitative analysis of lipid classes was performed on a thin layer chromatography Whatman aluminum silica plate (Maidstone, Kent) with standards of triolein, diolein, monoolein, and oleic acid using hexane and hexane:ether:acetic acid (80:20:1) as the mobile phases.

Total fatty acid composition in freeze-dried algal biomass slurry and hexane soluble products was determined by esterification with 3 N methanolic HCl (Sigma-Aldrich, St. Louis, MO) according to the manufacturer instructions, including use of nonadecanoic acid methyl ester as an internal standard, followed by GC–FID analyses. Fatty acid quantification analysis was done using a 30 m X 0.25 mm X 0.25 μm column from SGE Analytical Science (Melbourne, Australia). The initial injection temperature of 50°C was held for 0.2 min before ramping up to 230°C using the following program: increase of temperature from 50°C to 170°C over 20 min, hold at 170°C for 5 min, increase of temperature to 230°C at a rate of 10°C/min, then hold at 230°C for 13 minutes. The injection volume was 1μL in splitless mode. Statistical analysis (differences in fatty acid composition before and after) was done using the unpaired T test (GradPad software, La Jolla, CA). FTIR analysis of the hexane soluble fractions was carried out

in a Shimadzu 8400S (Tokyo Japan). Liquid and gas pyrolysis products were analyzed as previously reported [247].

3. Results and discussion

3.1. Growth and lipid accumulation of *C. protothecoides*

C. protothecoides was grown in a 10 L bioreactor in a mineral medium with glucose and yeast extract as the principal carbon and nitrogen sources, respectively. The growth, substrate consumption, and lipid accumulation of *C. protothecoides* in a bioreactor is presented in Figure 10. The growth and lipid accumulation over time showed that the optimum levels of production were achieved at 168 h. A limited increase in both cell density and lipid accumulation was recorded thereafter. A high density culture (32 g/L at the end point) with 40% cell dry weight neutral lipid was harvested, in agreement with data previously reported [244].

The proximate composition of *C. protothecoides* from the slurry used in hydrolysis was 40 ± 1% lipids, 10 ± 1% ash, 8.8 ± 0.1% protein, and 41 ± 2 % carbohydrates, on a dry weight basis. Note that the protein percentage was calculated by using total nitrogen multiplied by a factor of 6.25 and the amount of carbohydrates was calculated by difference.

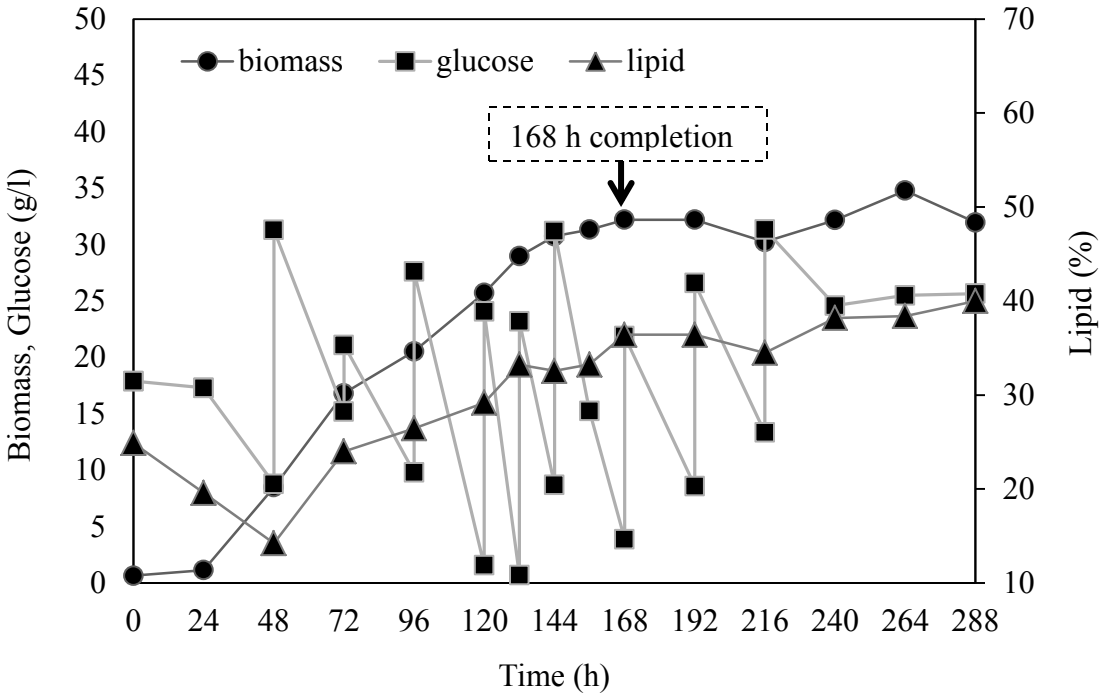


Figure 10. Sugar consumption, growth, and lipid accumulation of *C. protothecoides* in 10 L fed-batch bioreactors for 288 h using glucose and yeast extract.

3.2. Growth of *C. curvatus*

3.2.1. Batch mode

To evaluate biomass production and lipid accumulation of *C. curvatus* grown in glucose, batch cultures were grown using monomeric sugar and yeast extract with a C to N ratio of 100:1. Sugar utilization curves (Figure 11) showed that the yeast consumed the glucose after 68 h. At this point, the biomass was 10 ± 1 g/L with a lipid content of 59 ± 2% dry weigh basis. No contamination was observed in cultures during the entire growth period or harvesting.

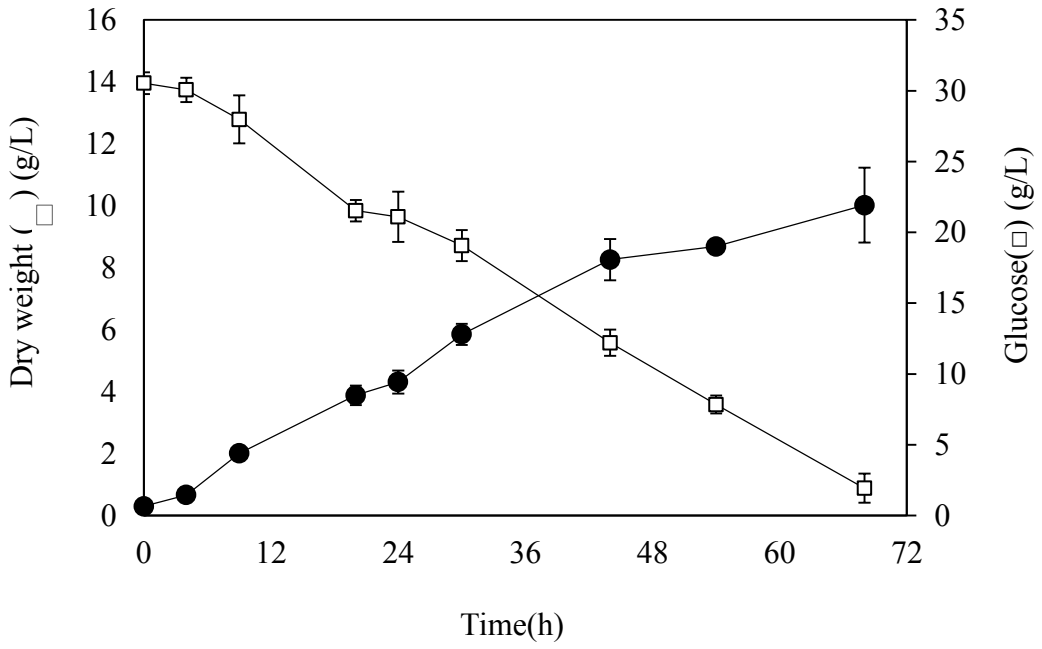


Figure 11. Sugar consumption and growth of *C. curvatus* in batch cultures for 72 h using glucose as the main carbon source at a final concentration of 30 g/L and yeast extract at 1.2 g/L for a C to N ratio of 100:1. Results express the means of replicate experiments (n=3).

3.2.2. Fed-batch mode

3.2.2.1. Small scale

Biomass production and lipid accumulation of *C. curvatus* were evaluated with increasing amounts of glucose and yeast extract during the course of the fermentation. For this purpose, cultures started under the same conditions as described in section 3.2.1 were supplemented with glucose and yeast extract prior to full glucose consumption (68 h); these additions were repeated at 100 and 140 h for a total of 11.1 g glucose loaded. The culture was monitored for a total of 168 h; the final volume of the culture was 230 mL at harvesting time. At this point, biomass was 13 ± 1 g/L with a lipid content of 55 ± 3% in dry weigh basis and cultures had residual glucose levels of 6.6 ± 1 g/L (Figure 12A).

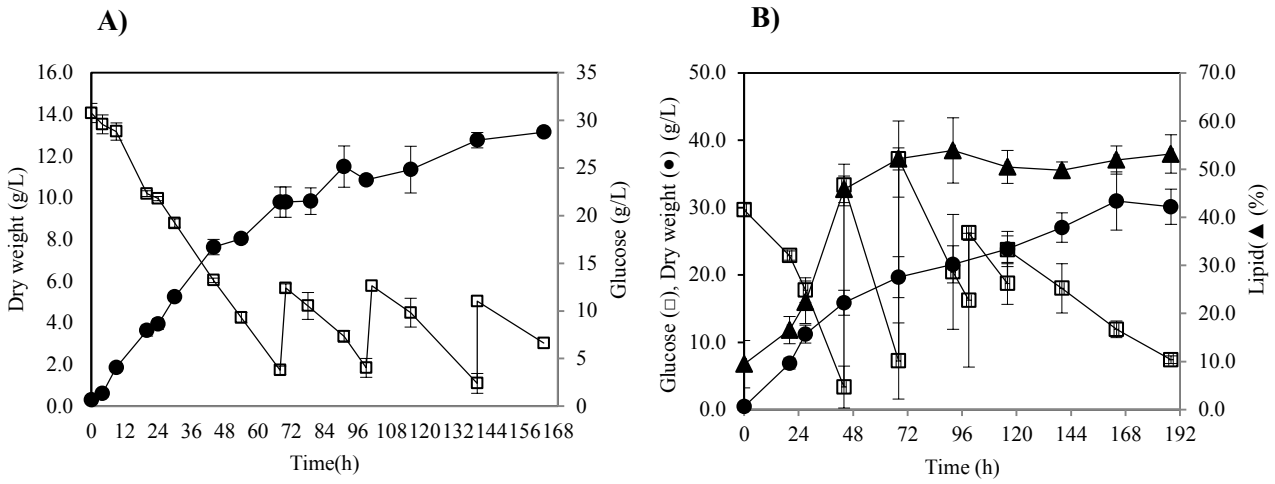


Figure 12. Biomass production and substrate consumption of *C. curvatus* in fed-batch cultures grown using glucose and yeast extract for 168 in 500 mL shake flasks (A) and 192 h in 5L reactor (B). Results express the means of replicate experiments (n=3).

3.2.2.2. Large scale

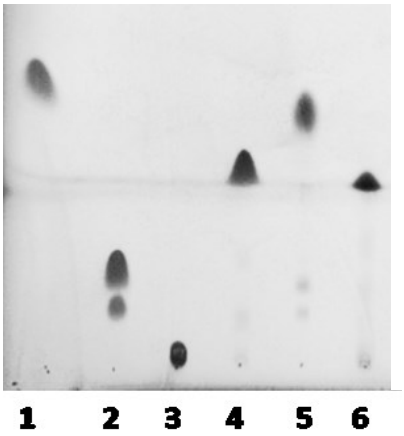
To produce large amounts of biomass, fed-batch cultures were scaled up to 5 L reactors. These cultures were fed with 120 mL of glucose and yeast extract solution (750 g/L glucose, 30 g/L yeast extract) after 44 and 68 h. Subsequent feedings of 40 and 20 mL of glucose and yeast extract solution (750 g/L glucose, 30 g/L yeast extract) were done at 100 and 116 h, respectively. Glucose concentration over the course of the fermentation was kept around 40 g/L (315 g total mass maximum), which is below the 60 g/L previously reported as the inhibition limit [245]. The culture was monitored for a total of 192 h; the final volume of the culture was 3.5 L at the time of harvest. At this point, biomass was 30 ± 3 g/L with a lipid content of 53 ± 4% in dry weight basis and cultures had residual glucose levels of 7.4 ± 0.6 g/L (Figure 12B). Yeast biomass from repeated fed-batch fermentations was pooled and the resulting slurry had a total solid content of 31 ± 1%. The lipid content of *C. curvatus* used in this study is comparable to previous reports of high-density cultures grown in glycerol (48%) and glucose (65%) [30, 245]. The proximate composition of the yeast biomass (expressed in dry weight basis) was found to be 53 ± 4% lipids,

8.6 ±1% protein (calculated from total nitrogen), 3.2 ± 0% ash, and 35.2 ± 5% carbohydrates (calculated by difference).

3.3. Hydrolysis of *C. protothecoides* slurry

After hydrolysis, the product distribution was 71 ± 8% of volatiles, 27 ± 1% total solids, and 8.5 ± 3.3% of gas. Elemental and mineral compositions of the solid fraction are reported in sections 3.3.2 and 3.3.3 of this chapter, respectively. Carbon dioxide was the main component of the gas product stream. Other compounds present were C1 to C4 hydrocarbons.

Hexane soluble lipids, recovered by washing the paste produced by the hydrolysis reaction, represented 42 ± 5% (w/w) of the original dry algal biomass, which was within the range of the initial lipid content of 40 ± 1% (w/w). A preliminary analysis of this fraction was conducted using thin layer chromatography on a silica plate. A sample of the extracted total lipids from the freeze dried raw algal biomass was compared with hexane soluble products after hydrolysis. The composition of the total lipids before hydrolysis was found to be mainly triacylglycerols while in the hexane soluble products only fatty acids were visualized (Figure 13). The absence of the initial lipid or partially hydrolyzed products in the final extract demonstrated the complete conversion of algal lipids to free fatty acids.



1. Triolein
2. Diolein
3. Monoolein
4. Oleic acid
5. Hexane extracted lipids before hydrolysis
6. Hexane extracted lipids after hydrolysis

Figure 13. Thin layer chromatography (silica plate) of algal lipids before and after hydrolysis. A mobile phase of hexane:ether:acetic acid 80:20:1 was used.

FTIR spectra of the hexane solubles together with an oleic acid standard and a 5-hydroxymethylfurfural (a thermal degradation product of structural components of microalgae) clearly show that the hexane solubles fraction is composed predominantly of fatty acids (Figure 14). Since the hexane solubles FTIR spectrum did not present other functional groups than those observed in the oleic acid standard, it is presumed that this fraction is almost entirely composed of fatty acids.

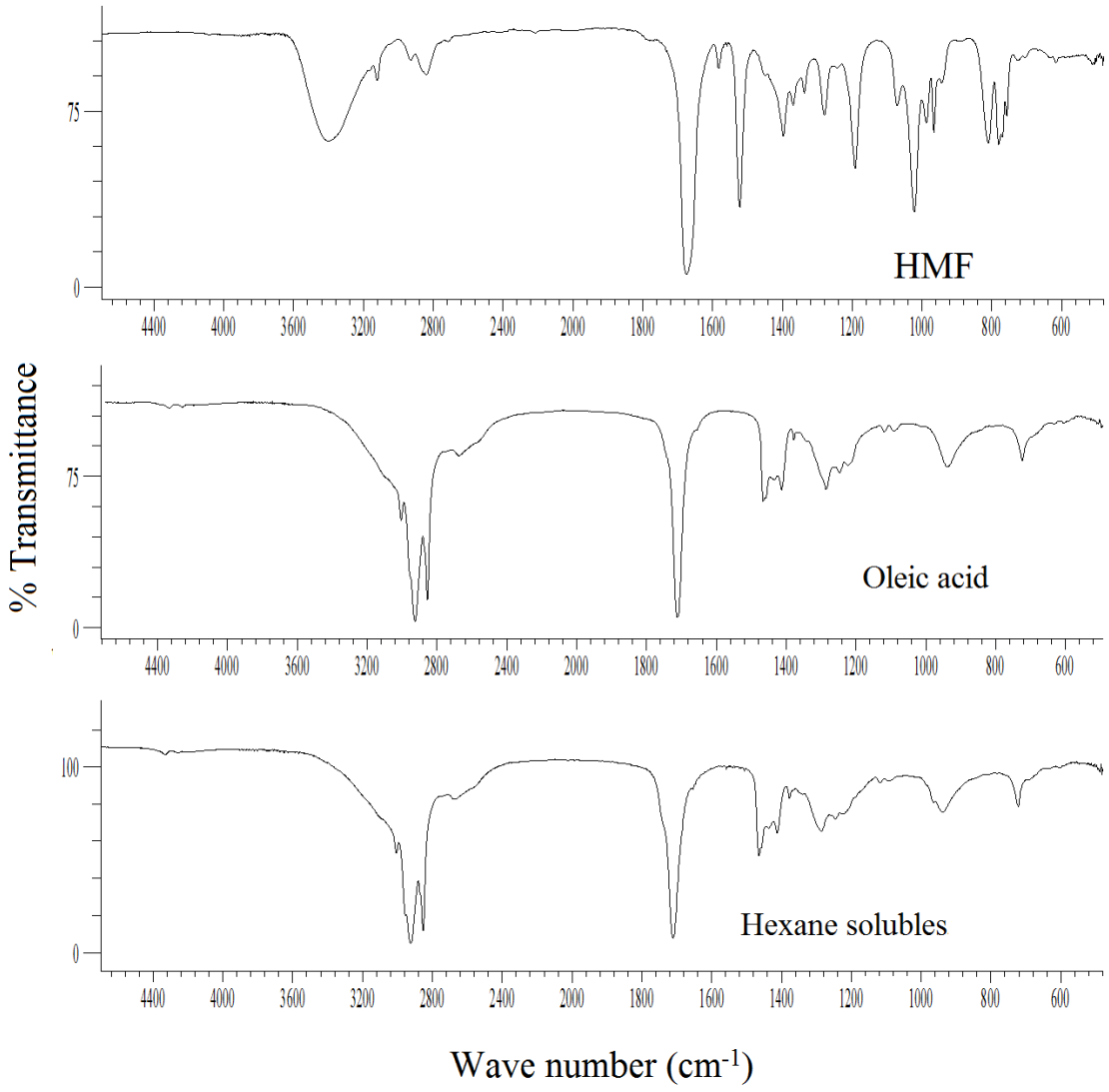


Figure 14. FTIR spectra of hydroxymethylfurfural (HMF), oleic acid, and hexane solubles.

3.3.1. Total fatty acid composition of *C. prototheocoides* before and after hydrolysis

The amount of fatty acids from the hexane extracted products and the raw algal biomass were quantified by GC-FID and normalized to express their composition relative to the total. The fatty acids of *C. prototheocoides* before hydrolysis were found to be oleic (52%), linoleic (23%), stearic (13%), along with lower levels of palmitic, myristic, linolenic, palmotoleic, and arachidic acids (Figure 15). The hydrolysis reaction did not alter the original total fatty acid profile

quantified in the hexane solubles fraction. The thermal stability of pure stearic, oleic, and linoleic acid in hydrolysis reactions below 300°C for 30 min has been previously discussed [249]. Long chain fatty acid models do not undergo degradation in these hydrolytic conditions but may isomerize [249]. Small variations in the relative composition of the total fatty acids may be justified by differences in time and pressure conditions used in this study and by the fact that the fatty acids used here are derived from a biological complex system rather than a purified chemical source.

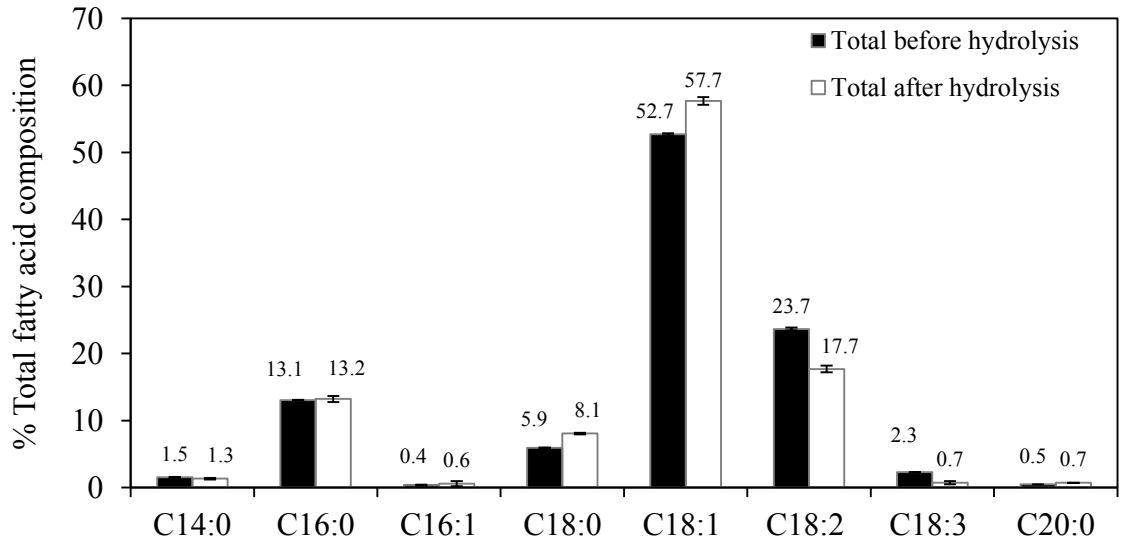


Figure 15. Fatty acid composition of *C. protothecoides* grown in a 10 L bioreactor with glucose and yeast extract. Results express the means of replicate experiments (n=3).

3.3.2. Elemental analysis of *C. protothecoides* hydrolysis products

The distribution of elements from the raw biomass in the streams produced after hydrolysis treatment is presented in Table 4. Carbon was enriched from 56% in the original algal biomass to 75% in the hexane solubles; this represented approximately 67% of the total carbon available in the system. The second major carbon-containing product was the insoluble residue (approximately 29%) followed by the aqueous stream (<1%). The carbon enrichment in the

hexane soluble lipids fraction was expected and likely resulted from the concentration of the lipids, which have a higher carbon to other elements ratio than the original biomass.

The initial nitrogen load was distributed among the various streams after hydrolysis with the aqueous and solid fractions being the main acceptors. This element represented only 0.3% in mass of the hexane soluble lipids. It is important to note that the initial nitrogen in the algal biomass from heterotrophic growth was lower (1.4% w/w; dry weight basis) compared to other algal systems from autotrophic regimes (generally >5%). In this context, the total nitrogen in hexane solubles was significantly lower than any other process from hydrothermal treatments using whole algal biomass or the paste from hydrolysis where total nitrogen was reported in the range of 5% to 40% of the initial value [216, 222-224, 226-228, 250].

Sulphur content represented only 0.1% of algal slurry (dry weight basis). After hydrolysis, the presence of this element in each of the streams was below detection limit of the analytical method used (10 ppm). For other studies, sulphur has been detected in the range of 0.5 to 2.3% (wt) in the distinct biocrude fractions produced from the thermal treatment of the algae *Spirulina platensis* [225].

Table 4. Elemental analysis of *C. protothecoides* biomass products after hydrolysis. Results express the means of replicate experiments (n=3).

	Ash (%)	**N (%)**	**C (%)**	**H (%)**	**O *(%)**	**S (%)**
Algal biomass	8.6 ± 0.2	1.4 ± 0.0	55.9 ± 0.2	8.3 ± 0.2	25.6 ± 0.5	0.1 ± 0.0
Hexane solubles	1.7^{E-2} ± 3^{E-3}	0.3 ± 0.0	75.2 ± 0.7	11.6 ± 0.4	12.9 ± 1.0	BDL
Aqueous	5.6 ± 0.48	0.4 ± 0.2	3.4 ± 0.2	11.2 ± 0.9	79.4 ± 1.0	BDL
Insolubles	11.2 ± 1.8	3.3 ± 0.3	66.2 ± 0.2	6.2 ± 0.2	13.0 ± 0.2	BDL

*Calculated by difference %O = 100-(N+C+H+S+Ash). BDL, Below Detection Limit (10 ppm).

3.3.3. Mineral composition of *C. protothecoides* hydrolysis products

The mineral composition of the raw biomass and each of the streams after hydrolysis is presented in Table 5. The major trace mineral in the algal slurry was magnesium followed by sodium, iron, calcium, zinc, manganese, potassium, and copper, presumably as a result of bioaccumulation from the culture mineral media. After hydrolysis, most of the initial phosphorous and calcium were washed out into the aqueous stream. The insoluble fraction was the major acceptor of magnesium, iron, and the remaining minerals.

In the hexane soluble lipids fraction, the element present in highest concentration was sodium (75 ppm). Calcium, copper, iron, and magnesium were detected in concentrations lower than 10 ppm. Hexane solubles were found to be virtually free of potassium, manganese, phosphorous, and zinc. The presence of minerals passed to the biofuels from hydrothermal reactions has been previously reported. Specifically, iron, copper, nickel, chromium, zinc, sodium, magnesium, and calcium have been found in higher concentrations than those in typical biocrudes (i.e. iron >800 ppm) [224, 225].

Table 5. Composition of minerals in *C. protothecoides* biomass and products after hydrolysis. Results express the means of replicate experiments (n=3).

	Algal biomass (ppm)	Hexane solubles (ppm)	Aqueous (ppm)	Insolubles (ppm)
Ca	133 ± 10	9 ± 4	46 ± 13	245 ± 40
Cu	1 ± 0	5 ± 3	2 ± 0	2 ± 1
Fe	158 ± 15	4 ± 2	13 ± 3	1,454 ± 84
K	2 ± 0	0 ± 0	1 ± 0	2 ± 1
Mg	2,418 ± 105	2 ± 1	33 ± 1	10,611 ± 1,315
Mn	5 ± 1	0 ± 0	1 ± 0	369 ± 22
Na	546 ± 9	75 ± 5	479 ± 72	490 ± 70
P (%)	2 ± 0	0 ± 0	1 ± 1	2 ± 0
Zn	22 ± 3	0 ± 0	2 ± 1	94 ± 8

3.3.4. Chemical characterization of *C. protothecoides* aqueous stream

The aqueous stream isolated after hydrolysis had a pH of 4.2 ± 0.1, similar to algal pre-treatments at subcritical conditions [228]. However, this stream was significantly different from the typical aqueous streams from hydrothermal treatments of algae (>300°C). Those streams have a basic pH due to the high levels of ammonia produced from prolonged protein degradation [216, 222, 223, 225, 226].

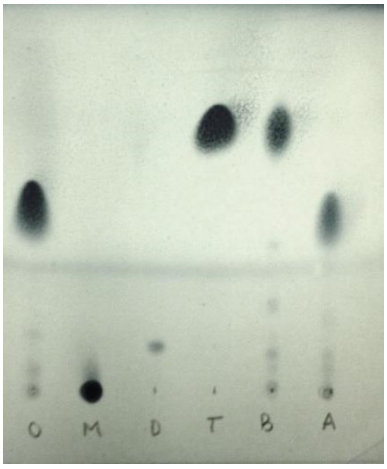
Glycerol, a byproduct of hydrolysis of triacylglycerols to free fatty acids, was found at a concentration of 22 ± 4 g/L. Phosphates were quantified at a concentration of 1.6 ± 0.1 g/L, representing around 23% of the original total phosphorous in the aqueous stream (6.6 g/L). This is significant considering that recovering soluble phosphorous enables the recycling of this finite resource, which currently challenges the feasibility of producing biofuels from microalgae [251].

Total nitrogen (2.29 ± 0.48 g/L), was composed mostly of organic forms, since no nitrates were detected and ammonium was only present at 1.1 ± 0.1 mg/L. On the other hand, hydrothermal reactions typically report half of the total nitrogen (initially loaded) as ammonia and other organics in the aqueous streams [216, 222, 223, 225, 226].

The aqueous stream from our study contained nitrogen, phosphorous, salts, and most importantly, glycerol from lipid hydrolysis. Glycerol can be used as a carbon source by the same algae at a concentration similar to that observed in the aqueous stream [252]. Thus, supplementation of media for algal growth with the aqueous stream produced from thermal hydrolysis represents a promising byproduct recycling alternative [228]. This is discussed further in Chapter III.

3.4. Hydrolysis of *C. curvatus* slurry

C. curvatus slurry was hydrolyzed and the product distribution was found to be 70 ± 2% volatiles, 26 ± 3% total solids, and 2 ± 1% gas. After hexane washing, 43 ± 4% of the solids were found in the hexane soluble fraction. Thin layer chromatography showed that the total yeast lipids before hydrolysis were mainly triacylglycerides, while the hexane soluble products after hydrolysis were exclusively fatty acids (Figure 16). This result demonstrated the success of the hydrolysis reaction in achieving complete lipid hydrolysis.



1. Oleic acid
2. Monoolein
3. Diolein
4. Triolein
5. Hexane extracted lipids before hydrolysis
6. Hexane extracted lipids after hydrolysis

Figure 16. Thin layer chromatography (silica plate) of yeast lipids before and after hydrolysis. A mobile phase of hexane:ether:acetic acid (80:20:1) was used.

Previous reports of hydrothermal treatments of oleaginous yeasts are rare. In one study, a combination of different hydrothermal conditions (temperature and reaction time) was studied to develop a method for lipid extraction from the yeast *Yarrowia lipolytica* [253]. In this approach, freeze dried yeast was thermally treated (175°C for 20 min), recovered, and freeze-dried again. This treatment improved lipid yields of pretreated biomass compared with non-treated biomass when using typical extraction methods with solvents. However, the pretreated yeast biomass was

used for the production of traditional biodiesel through transesterification [253], which is a different method than pyrolysis used in the LTH process.

A recent study proposed a sequential hydrothermal processing of the yeast *C. curvatus* that includes a mild treatment (180°C for 30 min) to solubilize and separate sugar and proteins in the aqueous streams, followed by a more intense treatment (240°C for 30 min) for bio-oil production [254]. Even though this methodology resembles the sequential approach of LTH, the conditions of the treatments were optimized for the production of bio-oil (without further separation) and not for fatty acid release as is the case for the LTH methodology [254].

Thermal treatment of the non-oleaginous industrial spent baker's yeast has been previously performed to achieve protein hydrolysis for amino acid release [255]. In this case, temperatures from 100 to 250°C at different time intervals were studied to assess the quality of amino acids released. A different study processed non-oleaginous baker's yeast at under-critical conditions (330-450°C with pressures of 20-30 MPa) using reducing agents for the purpose of using whole biomass to produce liquid biofuels [256]. Similarly, the hydrothermal treatment (350°C for 60 min) of the non-oleaginous yeast *Saccharomyces cerevisiae* was recently reported [226]. The purpose of this treatment was to obtain a high yield of an O and N-enriched biocrude. Although these approaches tried to produce biofuels, their overall strategy was significantly different from our hydrothermal processing, which prevents a direct comparison of performance. Specifically, the operational hydrothermal conditions required higher temperature and pressures, the feedstock selected had a limited fat content, and the final product was intended for direct application as bio oil without prior fractionation.

3.4.1. Total fatty acid composition of *C. curvatus* before and after hydrolysis

Fatty acids from the raw yeast biomass and the hexane-extracted fraction were quantified by GC-FID and normalized to express their composition relative to the total. Fatty acids of *C. curvatus* before hydrolysis consisted of oleic (42 ± 1%), palmitic (24 ± 0%), stearic (19 ± 0%), linoleic (14 ± 0%), and traces of linolenic (1 ± 0%; Table 6). The fatty acid distribution and profile of *C. curvatus* used for hydrolysis is consistent with previous reports. Slight differences in the profile distribution and accumulation of specific fatty acids may be a consequence of differences in substrates, nitrogen sources, or culture conditions [30, 245, 257]. Some changes in the original fatty acid distribution of the raw biomass were found in the fatty acids recovered after hydrolysis; they had a smaller proportion of palmitic and linoleic acids and a higher proportion of oleic acid.

Table 6. Fatty acid profile of *C. curvatus* biomass before and after hydrolysis. Results express the means of replicate experiments (n=3).

Fatty acid	Composition (%) Before hydrolysis	Composition (%) After hydrolysis
C16:0*	23.9 ± 0.4	21.3 ± 0.1
C18:0	19.0 ± 0.2	20.0 ± 0.4
C18:1*	42.0 ± 0.5	50.3 ± 0.3
C18:2*	14.3 ± 0.1	8.4 ± 0.1
C18:3*	0.8 ± 0.0	0.0 ± 0.0

*Significantly different (α=0.05).

3.4.2. Elemental composition of *C. curvatus* hydrolysis products

The distribution of elements in each byproduct stream before and after hydrolysis was characterized (Table 7). Carbon was enriched from 61% in the original algal biomass to 76% in the hexane solubles due to the concentration of the lipids from the original biomass. An estimate of the total carbon available in the system indicates that it was mainly distributed between

hexane solubles and the remaining solid. The initial nitrogen load was distributed among the various streams after hydrolysis. Sulfur content in the original yeast biomass and in each of the analyzed fractions after hydrolysis was below detection limits (10 ppm).

As previously discussed, nitrogen is an undesirable element [215, 216] and the hexane solubles fraction had a 10 times lower concentration than the value reported from a previous thermal treatment of whole yeast [256]. This may be explained by differences in the initial nitrogen load as the oleaginous yeast in this study were grown in limiting nitrogen conditions (100:1 C:N ratio). Previous studies found that *C. curvatus* grown in high C:N ratios contained approximately three times less nitrogen than cells grown in unrestricted media [258]. Thus, this represents a significant technical modification that will simultaneously increase lipid yields and decrease contaminating nitrogen species.

Table 7. Elemental analysis of *C. curvatus* biomass products after hydrolysis.

	Ash (%)	**N (%)**	**C (%)**	**H (%)**	**O* (%)**	**S (%)**
Yeast biomass	3.16 ± 0.12	1.37 ± 0.01	61.33 ± 0.03	9.62 ± 0.03	22.24 ± 0.15	BDL
Hexane solubles	0.03 ± 0.00	0.23 ± 0.82	75.52 ± 0.18	12.23 ± 0.05	9.53 ± 0.25	BDL
Aqueous	0.70 ± 0.23	0.18 ± 0.00	1.98 ± 0.01	10.83 ± 0.20	83.43 ± 0.29	BDL
Insolubles	3.24 ± 0.49	3.33 ± 0.08	72.28 ± 0.02	8.09 ± 0.10	10.89 ± 0.16	BDL

*Calculated by difference %O =100-(N+C+H+S+ Ash). BDL, Below Detection Limit (10 ppm)

3.4.3. Mineral composition of *C. curvatus* hydrolysis products

Mineral analysis was conducted on raw yeast biomass and each byproduct stream after hydrolysis to assess mineral distribution (Table 8). Raw yeast contained high levels of potassium (7 E^3 mg/kg), phosphorus (6 E^3 mg/kg), as well as magnesium, sodium, and calcium (all close

to1 E^3 mg/kg), while iron, copper, and manganese were found in small concentrations. Zinc was not detected in any of the fractions. Most of the minerals from the system were recovered in the insoluble fraction, as reflected in its high value in ash (Table 7). The elements that were washed out in the aqueous stream were mainly sodium (210 ppm), magnesium (167 ppm), calcium (41 ppm), and phosphorus (33 ppm) with traces (<10 ppm) of the other elements. The hexane solubles fraction was found to have a small concentration (<100 mg/kg) of each of the analyzed minerals, which is relevant considering further applications of these fatty acids as feedstock for fuel and chemical production where the presence of minerals is undesirable [215, 216].

Table 8. Mineral analysis of *C. curvatus* biomass and byproducts after hydrolysis

	Yeast biomass (ppm)	**Hexane solubles (ppm)**	**Aqueous (ppm)**	**Insolubles (ppm)**
Ca	865 ± 5	19 ± 13	41 ± 1	3849 ± 128
Cu	8 ± 1	18 ± 25	0 ± 0	69 ± 26
Fe	129 ± 3	15 ± 4	6 ± 2	563 ± 17
K	7227 ± 15	63 ± 22	0 ± 0	567 ± 74
Mg	1328 ± 6	17 ± 2	167 ± 9	3095 ± 82
Mn	8 ± 0	1 ± 0	1 ± 0	29 ± 1
Na	881 ± 4	49 ± 37	210 ± 19	178 ± 12
P	5634 ± 32	87 ± 4	33 ± 0	5249 ± 139
Zn	BDL	BDL	BDL	BDL

BDL, Below Detection Limit

3.4.3. Chemical characterization of the *C. curvatus* aqueous stream

The aqueous stream recovered after filtration of the solid paste was found to have a slightly acidic pH of 5.5. Glycerol, from the hydrolysis of triacylglycerides, was quantified at 19.9 g/L. Total nitrogen was found to be 1.08 ± 0.01 g/L while the ammonium concentration was 28.4 ± 0.4 mg/L. No nitrates were found in this aqueous stream suggesting that organic nitrogen compounds comprise the vast majority of total nitrogen in the system. Total phosphorus from mineral analysis was 32.5 ± 0.07 mg/L, while this element in the phosphate form was quantified

as 397 ± 2 mg/L. Considering that nitrogen and phosphorus are essential for any fermentation for biomass production, the aqueous stream from *C. curvatus* hydrolysis has potential applications in nutrient recycling. In addition to nitrogen and phosphorus, the presence of glycerol, which is a good carbon source for several organisms including *C. curvatus* [30, 257], makes recycling of the aqueous stream even more attractive. Recycling of nutrients in the aqueous stream will be discussed in chapter III.

3.5. Pyrolysis of *C. protothecoides* hexane soluble lipids

The pyrolysis of fatty acids extracted from hydrolysis of *C. protothecoides* resulted in the formation of both gaseous and liquid products. No solid formation was observed under the conditions used in this study. The weight of the liquid product was measured while the gas product yield was calculated by difference. The liquid and gaseous pyrolysis products were quantified at 80 ± 1% and 20 ± 1% by weight of feed, respectively.

Analysis of the gas product showed the presence of both CO (38 ± 2 vol%) and CO_2 (27 ± 1 vol%; Figure 17). These species are deoxygenation products conventionally attributed to decarbonylation and decarboxylation of fatty acids, respectively [259, 260]. The gas product also contained hydrocarbons (Figure 18). C1-C4 alkanes were the predominant fraction (24 ± 0 vol%) while C2-C4 alkenes and C5 and higher hydrocarbons were present in smaller amounts, 5.4 ± 0.0 vol% and 0.8 ± 0.0 vol%, respectively. The formation of the bulk of the hydrocarbons in the gas phase is known to occur through C-C bond cleavage through β-scission and ethylene elimination [261, 262].

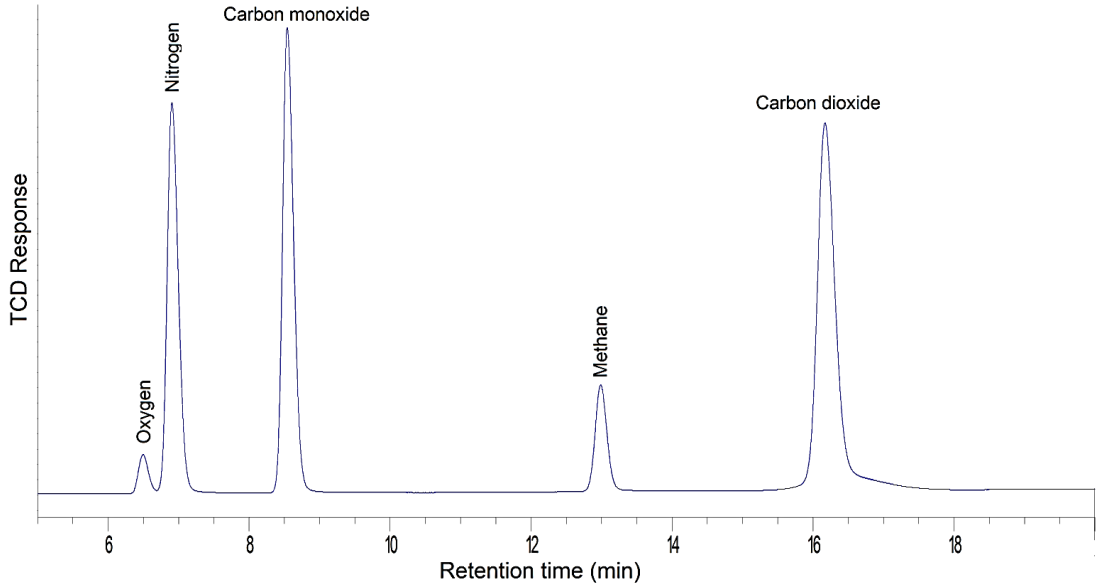


Figure 17. GC-TCD chromatogram of the gas fraction produced through pyrolysis.

The hydrocarbons in the gas fraction consisted predominantly of saturated hydrocarbons (Figure 18) even though the feed for pyrolysis was predominantly unsaturated, presumably as a result of saturation reactions. The presence of hydrogen was detected in the gas fraction (5.9 ± 1.3 vol%). Hydrogen formation is attributed to the formation of cyclic compounds and subsequent dehydrogenation of the cyclic compounds to form aromatic compounds [261]. The presence of dienes (mostly 1,3-butadiene) in the gas product is significant because they are important precursors in the formation of cyclic compounds through the Diels-Alder reaction of dienes and dienophiles [262, 263].

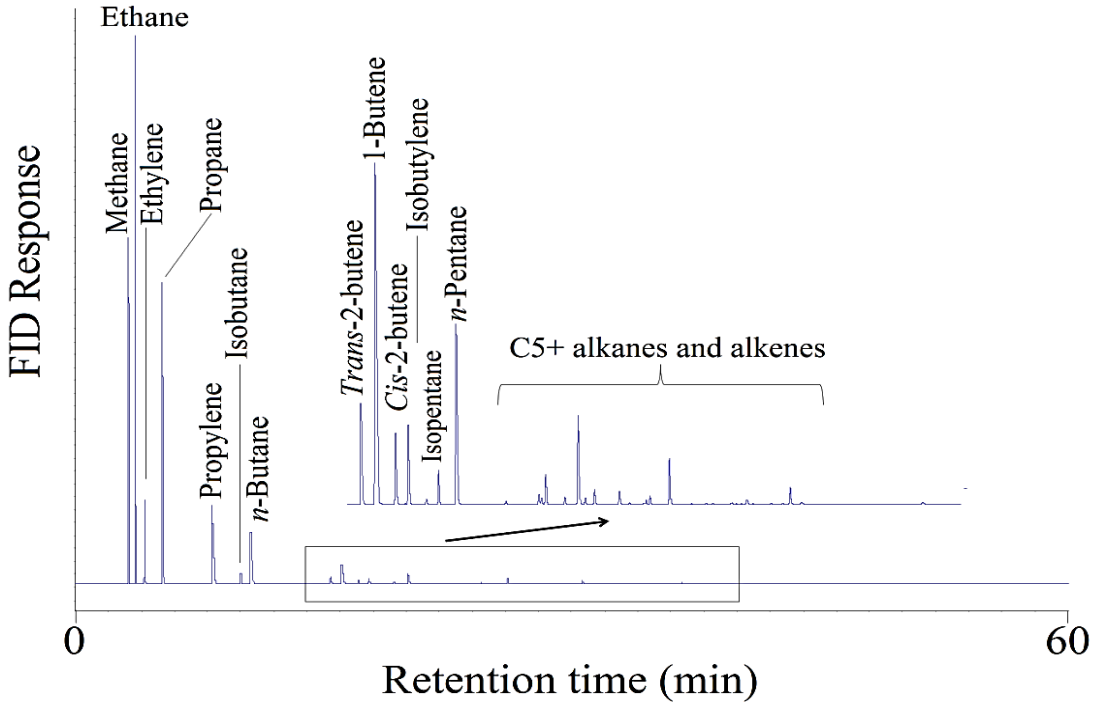


Figure 18. GC-FID chromatogram of the gas fraction produced through pyrolysis.

In the chromatogram of the liquid pyrolysis product (Figure 19), only compounds with molecular weights higher than C5 (pentane) were included in the quantification as the solvent used in the derivatization of fatty acids to their methyl ester equivalents overlapped with early eluting peaks.

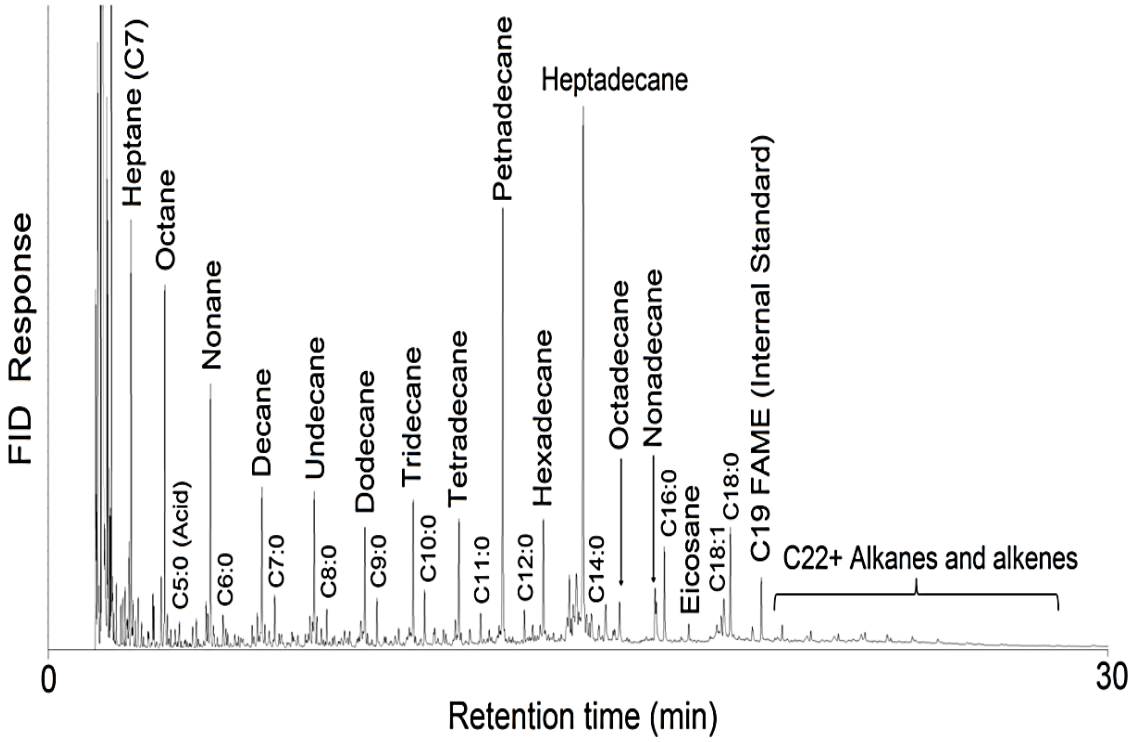


Figure 19. GC-FID chromatogram of derivatized liquid products from the hexane soluble lipids generated through pyrolysis (410°C, 2 h, N_2 atmosphere).

The classes of compounds found in the liquid pyrolysis product are shown in Table 9. The main product class was C6 to C26 *n*-alkanes, which accounted for 39 ± 2 wt% of the liquid product. Linear alkenes, both internal and alpha olefins, accounted for approximately 14 wt% of the liquid product while branched and cyclo alkanes and alkenes collectively constituted 13 wt% of the liquid product. The liquid product also included aromatic compounds (mostly monoaromatics) that accounted for approximately 3.9 wt%. Unreacted free fatty acids (such as C14:0, C16:0, and C18:0) as well as low molecular weight fatty acids produced through pyrolysis (such as C5:0 to C12:0) were observed in the liquid product and together they accounted for 8.4 wt% of the liquid product.

Table 9. Classes of compounds in the liquid products from the hexane soluble lipids generated through pyrolysis. Results express the means of replicate experiments (n=3).

Compound Class	Weight % of liquid product
n-Alkanes	38.7 ± 1.6
1-Alkenes	3.41 ± 0.14
Internal alkenes	10.7 ± 1.0
Branched (linear)	5.02 ± 0.06
Cyclic alkanes and alkenes	8.37 ± 0.27
Aromatics	3.92 ± 0.26
Fatty acids	8.38 ± 0.10
Unidentified	12.4 ± 0.5
Unaccounted (balance)	9.01 ± 1.83

It is important to note that while the fatty acids in the feedstock were predominantly unsaturated, the liquid fraction consisted mostly of saturated compounds as a result of well-known saturation reactions occurring in this temperature regime [264]. The product distribution and class of compounds found in this study are in agreement with previous studies involving model saturated [246] and unsaturated [247] fatty acids. The majority of products in these studies were linear alkanes and alkenes accounting for up to 50% of the liquid product. The distribution of linear hydrocarbon with respect to carbon number was more consistent with the model unsaturated fatty acids study where there is a general decrease in amounts as carbon number increases from C6, with the exception of C15 and C17. The higher amount of C15 and C17 are likely the result of direct deoxygenation of C16:0 and C18:0 in the fatty acid feed (Figure 15). Another possible reason for the higher C17 is the deoxygenation of C18:0 formed as a result of C18:1 and C18:2 saturation reactions as reported earlier [247]. This phenomenon also explains the relatively higher amount of C17 alkane than C15 alkane even though the fatty acid feed contains a higher amount of C16:0 than C18:0 (Figure 15). The distribution of the alkanes with

respect to carbon number is influenced by the presence of the double bonds in the thermal cracking of fatty acids as described earlier [247].

The hexane solubles fraction from yeast processing has similar characteristics (such as the sole presence of fatty acids, fatty acid profile similar to vegetable oils, and the low content of nitrogen and minerals) as the same algal fraction (sections 3.3 and 3.4). This suggests that yeast fatty acids may also be a suitable feedstock for pyrolysis in LTH process.

4. Conclusion

The oleaginous yeast *C. curvatus* and the heterotrophically-produced *C. protothecoides* were thermally hydrolyzed under subcritical conditions. The hydrolysis of high lipid content biomass slurries offers the advantage of extracting oils from minimally-processed microbial cultures without extensive dewatering. The hydrolysis pretreatment yielded a solid stream and an aqueous byproduct. The latter carried nutrients that can be potentially integrated in microbial cultivation. The solid stream contained fatty acids derived from microbial lipids, which were subsequently recovered by hexane extraction. These microbial fatty acids were virtually free of sulphur and low in salts and nitrogen. This is desired for biofuel production because the presence of such elements in the biofuel represents contamination that requires additional upgrading steps.

Recovered fatty acids from microalgae were used as feedstock for the production of renewable deoxygenated hydrocarbons via pyrolysis. The pyrolysis product was composed of hydrocarbon molecules indistinguishable from conventional petroleum. Short chain fatty acids present in the pyrolysates may be recovered and used as platform chemicals for other applications such as the development of cosmetics and detergents. Considering the similar profile and quality of algal and yeast fatty acids recovered following hydrothermal treatment, it is likely that pyrolysis of fatty acids extracted from yeast biomass will yield a similar hydrocarbon

profile. This work demonstrates the viability of a novel biological and thermal hybrid technology for the conversion of biomass into conventional hydrocarbons.

III. Cultivation of oleaginous microorganisms using aqueous fractions derived from hydrothermal pretreatments of biomass[b]

1. Introduction

The development of cost-effective processing technologies is necessary to integrate microbial oils into biofuel production. Some of the current challenges for using oleaginous microbes in fuel production include: 1) the identification of suitable and inexpensive carbon sources for their cultivation, 2) a large demand for limited resources such as nitrogen and phosphorus that are required for growth, and 3) the development of methods to valorize byproducts generated during conversion of biomass to biofuels [265]. Although microorganisms are renewable sources of lipids, their cultivation requires non-renewable inputs such as nitrogen and phosphorus. Therefore, the use of those elements needs to be optimized by the implementation of recycling strategies.

The LTH methodology for the transformation of lipids into biofuels, which was applied to oleaginous biomass feedstocks in Chapter II, produced an aqueous byproduct stream that is rich in glycerol. Since glycerol can be used as a carbon source by oleaginous yeasts and microalgae [29, 252, 266], this byproduct stream is a good candidate for recycling strategies. However, some of the additional compounds in this stream, such as the degradation products of proteins and carbohydrates, may serve as microbial nutrients or inhibitors. Thus, further investigation into the composition of hydrothermal byproduct aqueous streams and the ability of these streams to be recycled as substrates for yeast and microalgal cultivation is required.

[b] A version of this chapter has been submitted for publication.
Espinosa-Gonzalez I., A. Parashar, M. Chae, D.C. Bressler, Cultivation of oleaginous yeast using aqueous fractions derived from hydrothermal pretreatments of biomass. Bioresource Technology.

An opportunity to address nutrient requirements for the cultivation of oleaginous microorganisms lies in the utilization of low-value carbon sources that do not have applications as food [120]. Some potential carbon sources include lignocellulosic raw materials or industrial waste streams [267]. In particular, the use of industrial waste streams as substrates for growing oleaginous microorganisms is economically attractive as such feedstocks are not likely to be expensive and would create value from waste.

This study investigated the recycling potential of crude glycerol from waste streams of the thermal hydrolysis of fats and oils as well as the aqueous byproduct stream from the thermal hydrolysis of oleaginous microorganisms in the cultivation and lipid accumulation of the oleaginous yeast *C. curvatus* and the oleaginous microalgae *C. protothecoides*. This work seeks to simultaneously address two main demands of microbial biofuels: the use of cheap nutrient sources for cultivation of oleaginous microorganisms and valorization of microbial bioprocessing waste streams.

2. Materials and methods

2.1. Materials

Microalgae *C. protothecoides* (UTEX 256) and yeast *C.curvatus* (ATCC 96219) were used. Storage conditions, mineral media, and inoculum preparation were done as described in section 2.1 of chapter II. Algal and yeast hydrolysates from hydrothermal treatment of oleaginous biomasses used were obtained as described in section 2.2.4 of chapter II.

Sunflower, soybean, canola, camelina, and peanut oils were purchased from a local retail store and used “as is”. Beef and poultry tallow were obtained directly from rendering industries and used “as is”. Potassium hydroxide (85-90%), phthaldialdehyde (99%), amino acids in solution (2.5 µM/mL), β-amino-n-butyric acid (97%), yeast extract (11% nitrogen), and

hydrochloric acid (37%) were obtained from Sigma-Aldrich (St. Louis, MO). Sodium meta-bisulfite (97%), hexane (HPLC grade), acetonitrile (HPLC grade), methanol (HPLC grade), dichloromethane (HPLC grade), and sulfuric acid (2 N), were obtained from Fisher Scientific (Fairlawn, NJ). Nitrogen (99.998 %) was obtained from Praxair (Mississauga, ON).

2.2. Methods

2.2.1. Microalgal and yeast cultures in glycerol

C. protothecoides and *C. curvatus* were cultured in 250 mL Erlenmeyer flasks with 100 mL of algal or yeast base mineral media (described in section 2.1 of chapter II). Crude glycerol recovered from hydrothermally processed byproduct streams or commercially purchased glycerol at a final concentration of 20 g/L (0.65 C mol/L) was used as the main carbon source. To promote lipid accumulation, a low concentration of nitrogen (yeast extract at 0.8 g/L; 6.0 mmol/L) was used in the growth media to establish a carbon to nitrogen ratio of 100:1. The pH of the media was initially adjusted to 6.4 or 5.4 (for algae and yeast cultures, respectively) with 2 M KOH. Flasks were inoculated with a starter culture (5% v/v) in exponential growth phase (72 h and 24 h for algae and yeast, respectively). Algal cultures were grown at 25°C with agitation (150 rpm) for a total of 264 hours. Yeast cultures were grown at 30°C with agitation (200 rpm) for a total of 168 hours. Small samples were taken at the beginning and end of the experiments to monitor growth. All experiments were done in triplicate and assessed for contamination microscopically; algal cultures were also analyzed for bacterial contamination by streaking on Luria Bertani (LB) agar plates with subsequent incubation at 37°C.

2.2.2. Hydrolysis of fats and oils

Hydrolysis of fats and oils was conducted in a 5.5 L batch stainless steel reactor (Parr Series 4582, Parr Instrument Co., Moline, IL). The reactors were loaded with 2 kg of fats or oils, and 2 kg of water, which was standardized to a water ratio of 1:1 (by mass) to balance hydrolysis with product recovery and separation. Reactors were purged three times at 500 psi, and then pressurized to 500 psi with nitrogen. Hydrolysis time started when the temperature reached 280°C. The reaction was stopped after 1 h using an external glycol cooling unit. After cooling, organic and aqueous phases were separated using a separatory funnel. Aqueous fractions were stored at 2°C until needed.

2.2.3. Liquid-liquid organic extractions

Liquid-liquid extraction of the organic fraction of the algal and yeast hydrolysates was conducted as reported by Pham *et al*. [131]. Dichloromethane (DCM) was the organic solvent used in sequential extractions of the algal and yeast hydrolysates. The pH of each aqueous fraction was adjusted to 12 with 5 M KOH prior to extraction with DCM, and then to 5 with 6 M HCl followed by another DCM extraction. Organic extracts were pooled, concentrated, and re-suspended in distilled water to 10X and 1X (v/v) concentrations, with respect to the volume of the original aqueous fraction.

2.2.4. Growth of algae and yeast in byproduct streams

2.2.4.1. Screening experiments

The tolerance of *C. protothecoides* and *C. curvatus* to algal and yeast hydrolysates was assessed in microtiter plates (sterile 96 round bottom well plate with low evaporation lid, Corning, NY) in a procedure adapted from Blaise and Vassieur [268]. Briefly, previously

neutralized and filtered (0.22 μm) algal or yeast hydrolysate was added to individual lanes of a microplate (n=6; final dilution factors of 2, 4, 8, 16, 32, 64,128 and 256). 15 μL of concentrated (13.5X) base mineral media were also added to the experimental lanes along with glycerol and yeast extract at final concentrations of 10 g/L and 0.4 g/L, respectively. A lane without yeast hydrolysate was used as a control. The volume in each well was brought up to 190 μL with sterile double distilled (Milli-Q) water. Microplate wells were inoculated with 10 μL of exponentially growing (24 h) yeast cultures. Because of the potential for elevated rates of evaporation in the outer wells of the microplate, they were not used for experimental samples and filled only with sterile Milli-Q water. Microplates were incubated in a microplate vibratory shaker (Heidolph, Schwabach, Germany) at 25°C and 750 rpm for a total of 72 h. Initial and final optical density (OD_{600nm}) measurements were done in a separate flat bottom microplate using a microplate reader (Biotek, Winooski, VT).

2.2.4.2. Batch culture

C. protothecoides and *C. curvatus* were grown in 250 mL Erlenmeyer flasks with a total of 100 mL of media prepared as follows: Each flask initially contained 10 mL of a concentrated 10X base mineral media, 0.8 g/L yeast extract, and 50 mL of water. Previously neutralized and filtered (0.22 μm) yeast or algal hydrolysate (volume used was determined by screening experiments) and glycerol (standardized to a final concentration of 20 g/L) were added and the total volume of each culture was brought to 100 mL with sterile Milli-Q water. The pH of flasks was adjusted to 6.4 or 5.4 (microalgae or yeast, respectively) using 2 M KOH. Flasks were inoculated and incubated as previously described for algal or yeast glycerol cultures (section 2.2.1 of this chapter). The OD_{600nm} of samples taken at 24 h intervals was assessed and growth was calculated using a standard curve for dry biomass (section 2.2.3.2. of chapter II). Each

experimental replicate was monitored for contamination by microscopic examination; all experiments were done in triplicate.

2.2.5. Analytical methods

The glycerol content of the aqueous streams recovered from hydrothermally processed fats and oils, and glycerol consumption of *C. protothecoides* and *C. curvatus* were determined as previously described (section 2.2.7.2 of chapter II). Total organic carbon in hydrothermally processed byproduct streams (from fats and oils) was analyzed with a Shimadzu TOC-V at the Natural Resources Analytical Laboratory, University of Alberta.

Volatile fatty acids in algal and yeast hydrolysates were analyzed using an Agilent 78908 GC-FID with an Agilent 7693 series autosampler and injector and a Restek Stabilwax-DA column (30 m X 0.53 mm, film thickness 0.5 μm; Bellefonte, PA). The injector and detector temperatures were kept constant at 230°C and 250°C, respectively. The temperature of the GC oven was initially set at 90°C for 0.1 min, increased at a rate of 10°C per min to 190°C, held for 1.9 min, increased at 10°C per min to 220°C, and held for an additional 1 min for a total run time of 16 min. The carrier gas was helium at a constant pressure of 7.5 psi. A 10:1 split injection with a volume of 1 μL was used.

Total amino acid quantification of freeze-dried algal and yeast biomasses and free amino acids in the algal and yeast hydrolysates were analyzed using HPLC (Agilent 1200 series, Agilent Technologies, Inc., CA, USA) with an Agilent 1260 fluorescence detector (excitation at 340 nm and emission at 450 nm). Free amino acids in hydrolysates were directly derivatized using O-phthaldialdehyde (Agilent ZORBAX Eclipse AAA, protocol 23). A 3.5 μm ZORBAX Eclipse AAA column (4.6 X 150 mm) equipped with a ZORBAX guard column (4.6 X 12.5 mm) was used for separation. The derivatized samples were eluted with a gradient composed of

40 mM NaH_2PO_4, pH 7.8 (eluent A) and acetonitrile:methanol:water (45:45:10; v/v/v; eluent B) according to the manufacturer's instructions for a total analysis time of 35 min. The external standard was composed of 2.5 μmol/mL of each amino acid in solution, while 25 μmol/mL β-amino-n-butyric acid (BABA) was included in each sample as an internal standard.

Total amino acids (excluding cysteine and methionine) were quantified by treating 100 mg of freeze dried yeast with 3 mL of 6 M HCl in a glass test tube, then sparging with nitrogen gas to exclude oxygen. Samples were hydrolyzed at 110°C for 24 h and then derivatized and processed for total amino acid quantification as described for free amino acid analysis above.

Quantification of cysteine and methionine was done by adding 1 mL of cold performic acid to 100 mg of freeze dried yeast biomass, followed by incubation of the samples at 4°C for 16 hours. Cold sodium meta-bisulfite solution (0.35 mL of a 0.5 g/mL aqueous solution) was added every 15 minutes for a total of 1 h with vortexing after each addition. 3 mL of 6 M HCl was then added and the samples were hydrolyzed at 110°C for 24 hours. Samples were derivatized and processed for cysteine and methionine quantification as described for free amino acid analysis above.

Total lipid content was determined gravimetrically following extraction as previously explained in section 2.2.3.3 of chapter II. Total fatty acids were analyzed as previously described (section 2.2.7.4 of chapter II).

2.2.6. Calculations

Statistical analysis was done using the unpaired T test (GradPad software, La Jolla, CA). Biomass, lipid production, and fatty acid composition of control cultures grown with pure glycerol were compared with those of cultures grown using crude glycerol sources or hydrolysate supplementation.

3. Results and discussion

3.1. Hydrolysis of fats and oils

The thermal hydrolysis of different fats and oils has been applied as a pre-processing step in the patented LTH technology [229] in which the desired product recovered is the light fraction (fatty acids). For this study, the leftover heavy fraction (byproduct aqueous stream) was characterized to evaluate its use in any further applications. Various model fats and oils (sunflower oil, soybean oil, canola oil, camelina oil, peanut oil, beef tallow, and poultry tallow) were combined with an equal mass of water (2 kg) and individually hydrolyzed at 280°C for 1 h at an initial pressure of 500 psi. After hydrolysis, the heavy phase was separated and analyzed (Table 10). All aqueous fractions had an acidic pH ranging from 3.6 to 4.8. In these hydrolysates, glycerol was quantified at 80.7 to 91.0 g/L. Total carbon was measured to investigate the presence of carbon sources other than glycerol in each hydrolysate. The measured amount of total carbon was compared with the calculated carbon from glycerol to determine the percentage of carbon in hydrolysates emanating from sources other than glycerol (Table 10). Virtually all of the carbon present in the various hydrolysates examined exists as a constituent of glycerol.

Table 10. Characterization of byproduct aqueous streams from hydrolysis of fats and oils. Fats and oils were mixed with water (1:1 by mass) and individually hydrolyzed (280°C, 1 h, 500 psi initial pressure). The difference between the measured total carbon and the calculated total carbon from glycerol was divided by measured total carbon to determine the % carbon from sources other than glycerol.

Hydrolysate	pH	Glycerol (g/L)	Total carbon			
			Measured (g/L)	Calculated total carbon from glycerol (g/L)	Difference (g/L)	% carbon from other source than glycerol
Sunflower oil	4.1	87.1	34.6	34.0	0.6	1.7
Soybean oil	4.0	84.7	32.9	33.1	-0.2	-0.6
Peanut oil	3.8	87.5	34.6	34.2	0.4	1.3
Camelina oil	3.6	80.7	31.4	31.5	-0.1	-0.3
Canola oil	4.6	88.5	34.1	34.6	-0.5	-1.5
Poultry tallow	4.8	81.9	32.4	32.0	0.4	1.1
Beef tallow	3.8	91.0	35.9	35.6	0.3	0.9

3.2. Cultivation of oleaginous microalgae and yeast in glycerol

To evaluate the recycling potential of crude glycerol recovered from hydrothermally processed byproduct streams, growth and lipid accumulation of *C. protothecoides* and *C. curvatus* using these crude glycerol sources were directly compared with cultures supplemented with pure glycerol. In all cases, glycerol was added to a final concentration of 20 g/L (0.65 C mol/L). To facilitate accumulation of lipids in *C. protothecoides* and *C. curvatus*, a limiting amount of nitrogen was used in the growth media to achieve a carbon to nitrogen ratio of 100:1 [51]. No contamination was observed in cultures throughout the entire growth process.

3.2.1. Cultures of *C. protothecoides*

The biomass concentration after 264 h of growth in pure glycerol was 5.77 ± 0.2 g/L. Alternative glycerol sources that produced significantly greater (α= 0.05) biomass from the control were: sunflower oil, soybean oil, peanut oil, beef tallow, and poultry tallow (Figure 20). Biomass obtained using canola or camelina oil was not significantly different (α= 0.05) from the control. The lipid content of the microalgae grown in pure glycerol was 26.4 ± 2.4% dry weight, which was significantly lower (α= 0.05) than that obtained using any of the crude glycerol sources (Figure 20). Thus, all of the crude glycerol sources promote lipid accumulation, which can likely be attributed to other components within the byproduct stream.

Biomass and lipid yields per substrate utilized were calculated by dividing total biomass (g) or lipid (g) by the total glycerol consumed (Table 11). The biomass yield of 0.49 ± 0.02 g biomass/g glycerol in the pure glycerol grown culture was not significantly different (α=0.05) from the yields generated when *C. protothecoides* was cultured using other crude glycerol sources with the exception of sunflower oil (0.56 ± 0.01g biomass/ g glycerol). These results suggest that the apparent higher growth of *C. protothecoides* in some crude glycerol sources compared to the control is likely due to higher glycerol consumption.

The lipid yield of *C. protothecoides* grown in pure glycerol culture was not significantly different (α=0.05) from the yield obtained using crude glycerol from hydrolysis of canola oil, but significantly lower than those observed using the other crude glycerol sources. These results demonstrate that the byproduct aqueous streams from the thermal hydrolysis of fats and oils can be directly incorporated as a source of glycerol and other micronutrients in the cultivation of the microalgae *C. protothecoides*. Since our main goal for microalgal growth is lipid accumulation, use of these crude glycerol sources is a promising method of nutrient recycling.

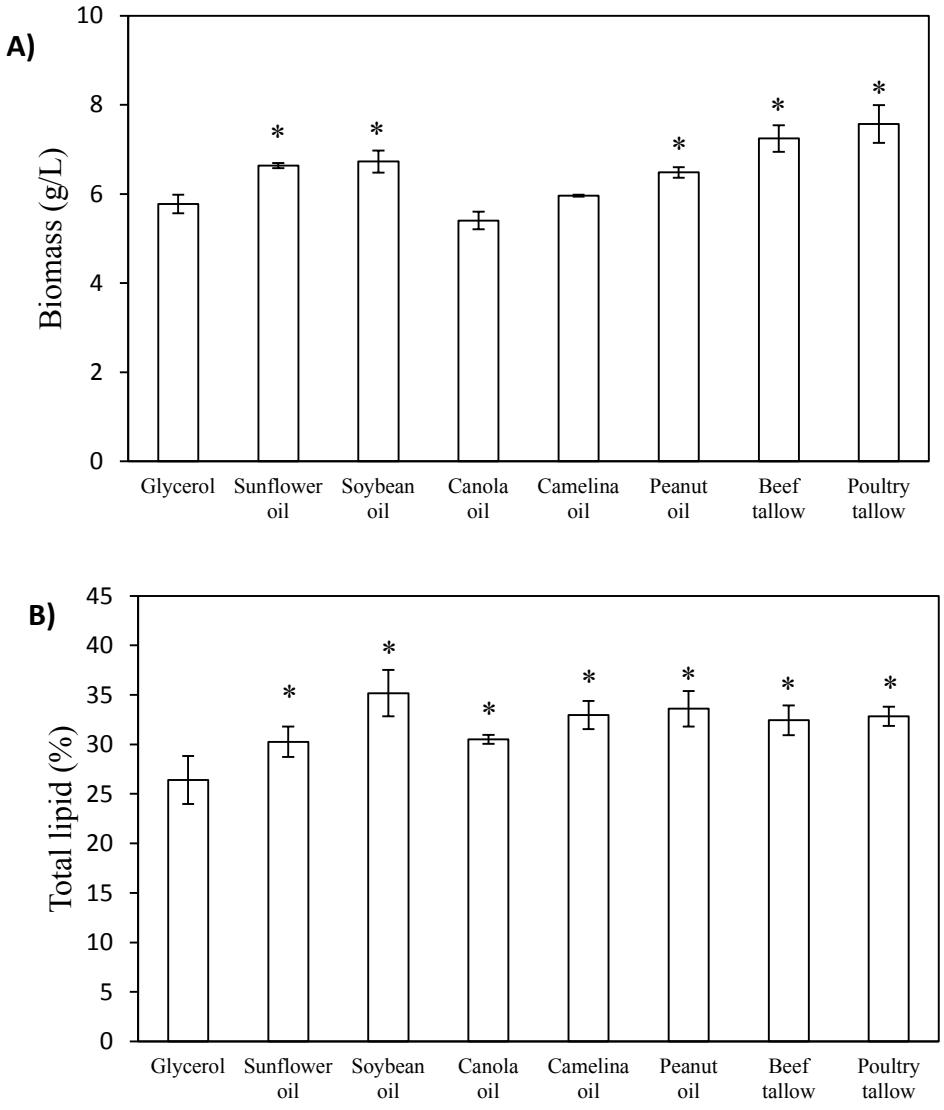


Figure 20. Performance of *C. protothecoides* cultures grown in glycerol from various sources. Algal cultures were grown at 25°C for 264 hours with shaking at 150 rpm. The amount of glycerol in the growth media was standardized at 20 g/L regardless of the source. The total biomass (A) and lipid accumulation (B) was determined based on dry weight. Results where the difference from the control condition (Pure glycerol) is statistically significant (α=0.05) are indicated with an asterisk (*). Results express the means of replicate experiments (n=3).

Table 11. Performance of *C. protothecoides* cultures grown in glycerol from various sources. Algal cultures were grown at 25°C for 264 hours with shaking at 150 rpm. The amount of glycerol in the growth media was standardized at 20 g/L regardless of the source. Total biomass (Figure 20A) and lipid accumulation (Figure 20B) values were divided by the amount of glycerol used for comparison of glycerol conversion rates. Results where the difference from the control condition (Pure glycerol) is statistically significant (α=0.05) are indicated with an asterisk (*).

Glycerol source	Glycerol used (g/L)	Biomass yield (g biomass/ g glycerol)	Lipid yield (g lipid/ g glycerol)
Pure glycerol	11.86 ± 0.21	0.49 ± 0.02	0.13 ± 0.01
Sunflower oil	11.85 ± 0.21	0.56 ± 0.01*	0.17 ± 0.01*
Soybean oil	12.94 ± 0.48	0.52 ± 0.00	0.18 ± 0.01*
Canola oil	11.78 ± 0.07	0.46 ± 0.02	0.14 ± 0.01
Camelina oil	11.54 ± 0.08	0.52 ± 0.00	0.17 ± 0.01*
Peanut oil	12.18 ± 0.42	0.53 ± 0.02	0.18 ± 0.01*
Beef tallow	13.95 ± 1.35	0.52 ± 0.04	0.17 ± 0.01*
Poultry tallow	14.69 ± 2.25	0.52 ± 0.11	0.17 ± 0.03*

3.2.2. Cultures of *C. curvatus*

The biomass concentration of *C. curvatus* after growth for 168 h in pure glycerol was not significantly different (α= 0.05) from cultures grown using the other crude glycerol sources with the exception of poultry tallow (Figure 21A). The lipid content of yeast grown in pure glycerol was comparable to that obtained from growth of yeast in crude glycerol sources (Figure 21B). The biomass yield of 0.51 ± 0.1 g biomass/g glycerol of the pure glycerol grown culture was not significantly different (α=0.05) from the yields generated when *C. curvatus* was cultured using any of the crude glycerol sources (Table 12). Similarly, the lipid yield of cultures grown in commercially purchased glycerol was not significantly different (α=0.05) from cultures obtained using crude glycerol sources.

Poultry tallow, which was beneficial not only for yeast but also algal performance, and its derivatives have been previously used for some biotechnological applications [270-271]. For instance, specific filamentous fungi and yeast were able to convert poultry fat into monoacylglycerols through enzymatic glycerolysis [271]. Curiously, in that study the fat utilization percentage for all microorganisms screened was higher when poultry tallow was used compared to beef tallow [271]. Another report examined growth of three fungi in media supplemented with beef and poultry tallow and found higher growth rates using poultry tallow [270]. The authors suggested this was because poultry fat could be dispersed in the medium more efficiently. In our study, the glycerol byproduct of poultry tallow promoted better biomass accumulation for yeast and microalgae compared with the other glycerol sources. This suggests that poultry tallow may be one of the best lipid feedstocks for the LTH pretreatment if recycling of its aqueous stream in microbial cultivation is intended.

The amount of glycerol used for our batch cultivation (20 g/L) was within the previously reported optimal range for *C. curvatus* growth (16-32 g/L) using the same culture protocol [29]. However, based on previous work (section 3.2, chapter II), nitrogen was limited by using considerably less yeast extract to promote lipid accumulation in *C. curvatus*. Although this seemed to limit full glycerol consumption, the purpose of this study was to compare the overall performance of the yeast when grown in pure glycerol versus alternative crude glycerol sources.

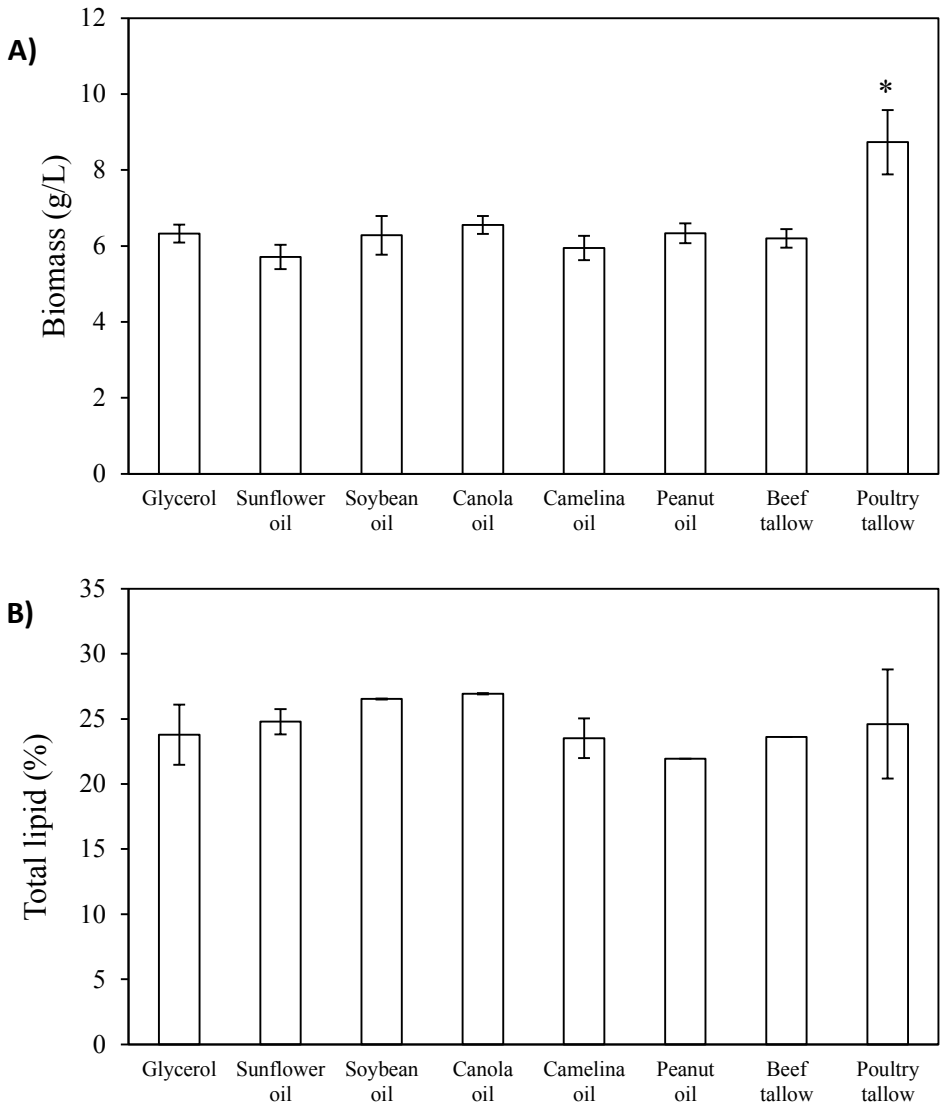


Figure 21. Performance of *C. curvatus* grown in glycerol from various sources. Yeast cultures were grown at 30°C for 168 hours with shaking at 200 rpm. The amount of glycerol in the growth media was standardized at 20 g/L regardless of the source. The total biomass (A) and lipid accumulation (B) was determined based on dry weight. Results where the difference from the control condition (glycerol) is statistically significant (α=0.05) are indicated with an asterisk (*). Results express the means of replicate experiments (n=3).

Table 12. Performance of *C. curvatus* grown in glycerol from various sources. Yeast cultures were grown at 30°C for 168 hours with shaking at 200 rpm. The amount of glycerol in the growth media was standardized at 20 g/L regardless of the source. Total biomass (Figure 21A) and lipid accumulation (Figure 21B) values were divided by the amount of glycerol used for comparison of glycerol conversion rates.

Glycerol source	Glycerol used (g/L)	Biomass yield (g biomass/ g glycerol)	Lipid yield (g lipid/ g glycerol)
Pure glycerol	10.4 ± 0.87	0.51 ± 0.01	0.13 ± 0.00
Sunflower oil	8.2 ± 1.67	0.54 ± 0.04	0.13 ± 0.01
Soybean oil	9.0 ± 0.07	0.56 ± 0.05	0.13 ± 0.01
Canola oil	11.1 ± 0.39	0.50 ± 0.01	0.14 ± 0.00
Camelina oil	9.2 ± 0.46	0.53 ± 0.04	0.13 ± 0.01
Peanut oil	9.8 ± 0.45	0.52 ± 0.01	0.13 ± 0.00
Beef tallow	9.3 ± 0.52	0.53 ± 0.02	0.13 ± 0.01
Poultry tallow	18.3 ± 0.45	0.47 ± 0.04	0.11 ± 0.01

The results from the cultivation of *C. protothecoides* and *C. curvatus* demonstrate that the aqueous streams from the thermal hydrolysis of different fats and oils support growth and lipid accumulation of these microorganisms. Some of the algal cultures grown with glycerol from the studied sources produced statistically significant improvements in growth and lipid accumulation compared to cultures of pure glycerol. Yeast cultures grown with alternative glycerol sources performed in a manner comparable to that observed using pure glycerol (except in cultures using glycerol from poultry tallow, which were improved). Diverse lipid sources including edible or inedible vegetable oils, used cooking oils and animal fats have been previously used as source of fatty acids for the LTH technology [230, 246, 247]. This study demonstrated the use of some of these byproduct aqueous streams for yeast or heterotrophic algal cultivation. Since two of the

model fats used in this work are inedible resources (beef and poultry tallow), we would expect their preferential utilization in order to addresses carbon feedstock concerns of microbial cultivation. Further investigation is required to assess the suitability for growing microorganisms using aqueous streams from different inedible fats and oils processed in the LTH technology.

3.3. Characterization of microbial hydrolysates

Algal and yeast hydrolysates were recovered as aqueous byproduct streams from the hydrothermal treatment of oleaginous *C. protothecoides* and *C. curvatus* slurries in a 2 L or a 5.5 L batch stainless steel reactor, respectively, at 280°C for 1 hour with an initial pressure of 500 psi (section 2.2.4 of chapter II). To further characterize these streams, volatile fatty acid and amino acid contents were determined as described below.

3.3.1. Volatile fatty acids

The production of low molecular weight fatty acids resulting from decomposition of algal or yeast biomass during hydrothermal treatment was analyzed by GC-FID. Volatile fatty acids have been previously reported as decomposition products of various organic materials, specifically proteins and lipids, at high temperatures [272-274].

Acetic and propionic acids were found in algal (2.73 ± 0.23 g/L and 0.21 ± 0.03 g/L, respectively) and yeast (0.23 g/L and 0.30 g/L, respectively) hydrolysates as degradation products of saccharides and amino acids [241]. Acetic acid has been previously found in the aqueous stream from the hydrothermal treatment (300°C for 5 min) of the microalgae *Desmodesmus* [130]. Acetic and propionic acids can be metabolized by different microalgae including some from the genus *Chlorella* [275, 278]. While some volatile fatty acids have been reported as inhibitors of yeast growth [277], acetic acid has shown to be a growth substrate for *C.*

curvatus in a two-stage cultivation process following depletion of the primary carbon source (glucose) [278].

3.3.2. Amino acids composition

Although the use of oleaginous algae and yeast in LTH technology is focused on lipid hydrolysis and recovery, it is also important to evaluate the effects of hydrothermal treatment on other biomass constituents. Proteins are of particular interest since they represent the third major component in oleaginous microalgae and yeast biomass after lipids and carbohydrates (proximate composition in sections 3.1 and 3.2.2.2 of chapter II, respectively). Proteins are broken down into amino acids during hydrothermal processes, but the severity of this treatment may lead to degradation of amino acids [274]. Previous analyses (sections 3.3.4 and 3.4.4 of chapter II) demonstrated that inorganic nitrogen sources, ammonium, and nitrate, account for an extremely small fraction of total nitrogen present in algal and yeast hydrolysates. These data suggest the presence of organic nitrogen, which may exist as part of amino acids.

3.3.2.1. Total amino acids in *C. prototheocoides* and *C. curvatus* biomass

The amino acid composition of the oleaginous microalgae *C. prototheocoides* and the yeast *C. curvatus* was analyzed to help assess if hydrothermal treatment affects individual and total amino acid recovery in the aqueous stream. The amino acid composition of *C. prototheocoides* and *C. curvatus* biomass prior to hydrothermal treatment was determined using HPLC and the results are shown in Figure 22. The sum of all amino acids quantified represents 6.3 ± 0.4% and 5.0 ± 0.0% of *C. prototheocoides* and *C. curvatus* biomass (dry weight), respectively.

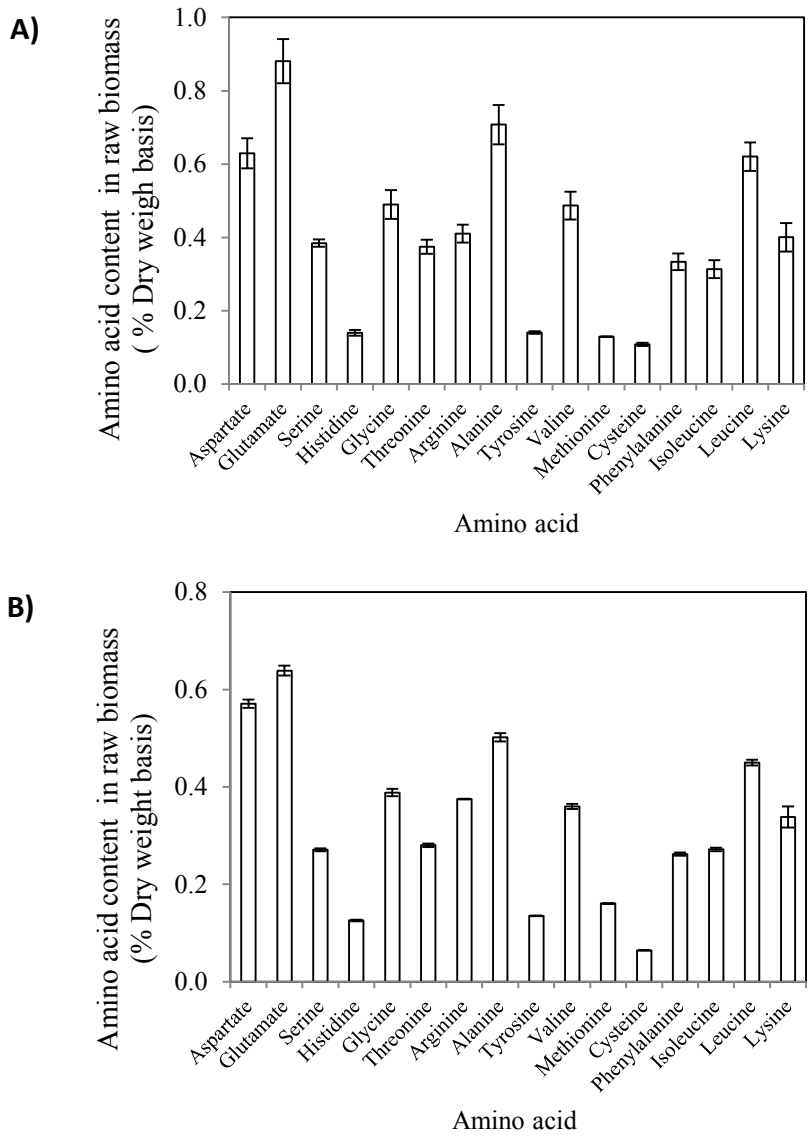


Figure 22. Amino acid analysis of *C. protothecoides* (A) and *C. curvatus* (B) biomasses. Individual amino acids in oleaginous microalgae prior to hydrothermal treatment (expressed as % dry biomass). Results express the means of replicate experiments (n=3). Note: aspartate reflects both asparagine and aspartic acid and glutamate reflects both glutamine and glutamic acids.

3.3.2.2. Free amino acids in *C. protothecoides* and *C. curvatus* hydrolysates

Ideally, amino acids in hydrolysates would exist in their free forms, which are a favoured nitrogen source for the microalgae and yeast [136, 279, 280]. However, the expected yields of amino acids from protein are very low above 230°C due to amino acid degradation [241]. The free amino acids identified in the algal and yeast hydrolysates are shown in Figure 23. The sum of all free amino acids (132.4 ± 7.1 mg/L and 32.8 ± 0.8 mg/L, respectively) in the aqueous streams following hydrolysis represents less than 1% of the total amino acids initially present in the *C. protothecoides* and *C. curvatus* biomasses. Amino acids from biomasses were most likely degraded by decarboxylation (producing ammonia and organic acids) and deamination (producing carbonic acid and amines) reactions [241, 281]. The highest recovery in the algal hydrolysate was observed for methionine (7.3%) followed by alanine (1.3%) while the other amino acids displayed recovery rates of less than 1% (Figure 23A). The highest recovery in the yeast hydrolysate was observed for methionine (26.3%), followed by tyrosine (4.2%), alanine (1.8%), and glycine (1.2%). All other amino acids examined in the yeast hydrolysates displayed recovery rates of less than 1% (Figure 23B).

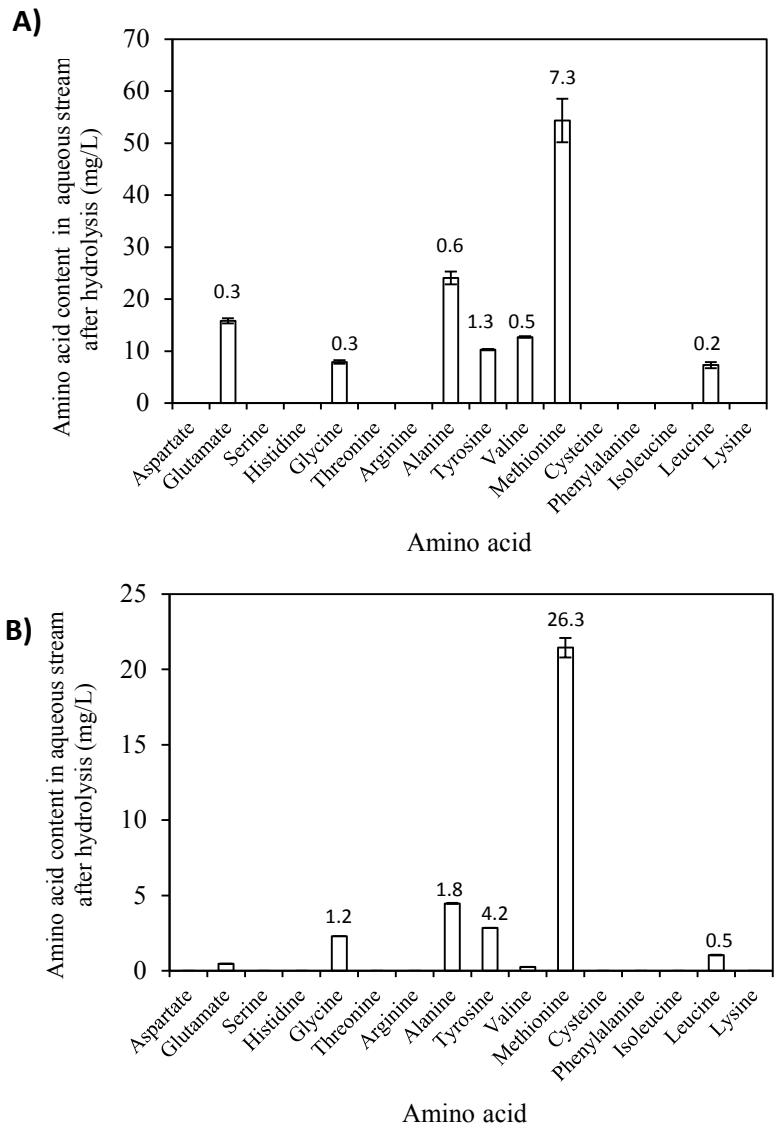


Figure 23. Amino acid analyses of *C. protothecoides* (A) and *C. curvatus* (B) hydrolysates. Free amino acids in the aqueous stream recovered after hydrothermal treatment (280°C, 1 h, 500 psi initial pressure), expressed as mg/L in hydrolysate. Percentage above the bars represent the recoveries of each amino acid and were determined using the following formula:
% amino acid recovery = CV/MP × 100%, where C = concentration of the amino acid in the hydrolysate (shown in Figure 22), V = volume of hydrolysate produced, M = mass of the biomass used to produce the hydrolysate, and P = percentage of amino acid in dry biomass weight (shown in Figure 22). Results express the means of replicate experiments (n=3). Note: aspartate reflects both asparagine and aspartic acid and glutamate reflects both glutamine and glutamic acids.

Previous research has discussed the effect of hydrothermal processing conditions on the degradation of diverse proteinaceous materials into amino acids [255, 274, 282]. One of the main factors influencing protein and amino acid degradation is temperature. A study of fish-derived wastes showed that low molecular weight amino acids obtained via hydrothermal processing at constant time (60 min) and pressure (2,900 psi) significantly decreased when the temperature was increased from 272°C to 300°C [282]. For thermal degradation of specified risk material, the highest level of free amino acids was found at 240°C and severely diminished (~50%) at 260°C [274]. Lamoolphak *et al.* [255] reported that the lowest yield of amino acids from thermal decomposition of baker's yeast occurred at the highest temperature and time studied (250°C for 30 min).

Only one previous study quantified total amino acids in an algal hydrolysate. In that study, the hydrolysates of the microalgae *Desmodesmus* contained amino acids at a concentration of 152 ± 35 mg/L [130]. Other studies have only estimated amino acids in algal hydrothermal aqueous streams by subtracting inorganic nitrogen from total nitrogen quantified [212]. In this thesis, algal and yeast streams were analyzed at the level of amino acid content to help to elucidate how these byproducts could be better utilized.

Amino acids are value added products used in food, pharmacy, and cosmetic industries. From the current amino acid production methods (extraction, synthesis, fermentation, and enzymatic catalysis), extraction from protein hydrolysates is the least used [283]. However, it is still employed for the production of serine, proline, and tyrosine, which cannot be easily obtained by other methods [283]. The algal and yeast hydrolysates produced in this study had marginal total amino acid amounts which limits their potential recovery; however, these amino acids in solution can be directly consumed by microorganisms.

Algal and yeast strains are able to use amino acids as nitrogen sources. In autotrophically-grown *Chlorella*, several amino acid uptake systems (neutral-acidic, short-chain, basic, acidic, methionine, glutamine, and threonine) have been found [284]. Each of these uptake systems have different mechanisms of induction, such as the absence or presence of inorganic nitrogen or glucose [285]. On the other hand, yeast for ethanolic fermentation has shown differential amino acid consumption for 7 (serine, asparagine, alanine, arginine, tyrosine, glycine, and cysteine) out of the 20 amino acids [286]. The amount of amino acids consumed by yeasts is determined by the strain although there is preference for cysteine, tyrosine, glycine, and alanine consumption [286]. Thus, the amino acids in the hydrolysates are suitable for algal or yeast consumption as they contain relatively high amounts of tyrosine, glycine, and alanine.

3.4. Cultivation of oleaginous algae and yeast in microbial hydrolysates

3.4.1. Screening

3.4.1.1. Growth of *C. protothecoides* on various dilutions of hydrolysates

Preliminary screening for the tolerance of *C. protothecoides* to algal and yeast hydrolysates was conducted by exposing exponentially growing algal cultures to serial dilutions of the hydrolysates mixed with algal background medium containing a constant amount of nutrients (minerals, glycerol, and yeast extract) for 72 h in 96 well microtiter plates. Compared to control cultures grown without algal hydrolysate, *C. protothecoides* seemed to be inhibited by higher concentrations of algal hydrolysate (from 1:2 to 1:16; Figure 24). Supplementation of algal hydrolysate at lower concentrations resulted in significantly improved algal growth compared to the control (1:32 to 1:256). Similarly, growth inhibition of *C. protothecoides* relative to the control was observed when cells were supplemented with high concentrations of yeast

hydrolysate (1:2 to 1:16; Figure 24). Lower concentrations of yeast hydrolysate (1:32 to 1:256) did not affect algal growth. These results suggest that algal and yeast hydrolysates contain inhibitory compounds that have negligible efficacy when the hydrolysates are used at concentrations lower than 1:16. Furthermore, it is anticipated that the supplementation of algal cultures with sufficiently diluted algal hydrolysate will improve growth. Conversely, significant improvements of algal growth are not expected in cultures supplemented with yeast hydrolysate. However, it should be noted that these small-scale experiments were performed for screening purposes only and results from larger scale experiments may be different and more reliable.

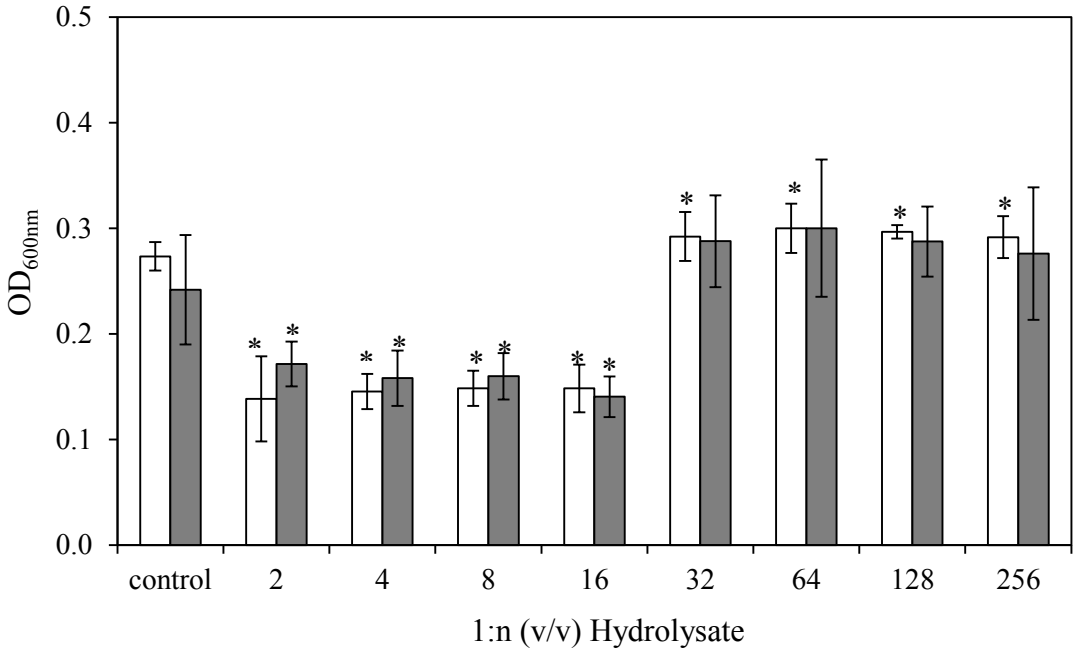


Figure 24. Growth of *C. protothecoides* with algal or yeast hydrolysate supplementation. Cultures were grown in the absence (control) or presence of various algal (white bars) or yeast (gray bars) hydrolysate concentrations. Algal cells were grown in 96-well plates at 25°C for 72 hours while shaking at 750 rpm. Optical density (OD_{600nm}) was used to quantify the amount of growth. Optical density readings were obtained from samples composed of 60 µL of culture and 180 µl of water. Differences in growth (relative to the control) that were statistically significant (α=0.05) are indicated with an asterisk (*).

3.4.1.2. Growth of *C. curvatus* on various dilutions of hydrolysates

C. curvatus was also grown for 72 h in 96 well microtiter plates with different volumes of algal or yeast hydrolysate in order to assess the effect of these supplements on yeast growth. *C. curvatus* growth was significantly diminished in cultures supplemented with higher concentrations of algal and yeast hydrolysates (1:2 and 1:4; Figure 25). Conversely, growth enhancement was observed when lower dilutions of algal (1:8 to 1:64) or yeast (1:8) hydrolysates were used. Further dilutions of hydrolysates (1:128 to 1:256 for algal hydrolysate and 1:16 to 1:256 for yeast hydrolysate) did not have a significant impact on growth relative to the control culture. These data suggest that algal hydrolysates supplemented at final dilutions lower than 1:4 but greater than 1:128 improve yeast growth. Also, the final ratios (v/v) of the yeast hydrolysate lower than1:4 and higher than 1:16 (v/v) may enhance growth of *C. curvatus*.

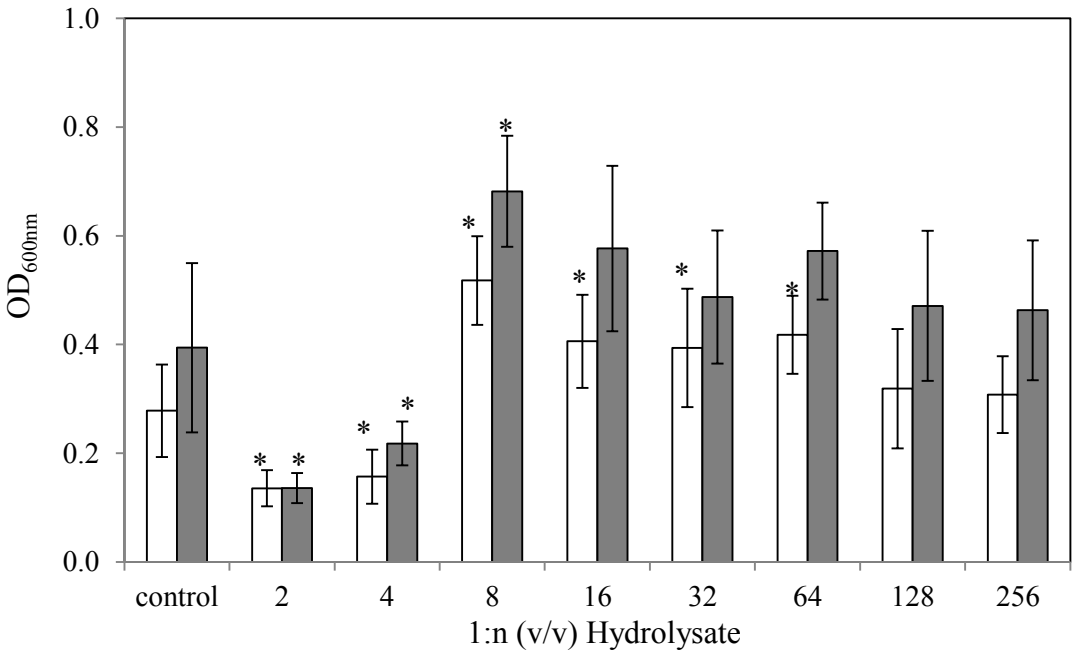


Figure 25. Growth of *C. curvatus* with algal and yeast hydrolysate supplementation. Cultures were grown in the absence (control) or presence of various algal (white bars) or yeast (gray bars) hydrolysate concentrations. Yeast cells were grown in 96-well plates at 25°C for 72 hours while shaking at 750 rpm. Optical density (OD_{600nm}) was used to quantify the amount of growth. Optical density readings were obtained from samples composed of 60 µL of culture and 180 µl of water. Differences in growth (relative to the control) that were statistically significant (α=0.05) are indicated with an asterisk (*).

3.4.1.3. Toxicity of organic material contained in hydrolysates

Growth inhibition observed in *C. protothecoides* or *C. curvatus* cultures supplemented with higher concentrations of algal and yeast hydrolysates may be caused by organic molecules contained within the hydrolysates. To assess the effects of organics from the hydrolysates, further screening was carried out by supplementing cultures with organic extracts obtained through liquid-liquid extraction with DCM. The specific components and concentrations of organic molecules in thesesolutions were not determined due to technical and analytical

challenges [131]. The effect of supplementing algal or yeast growth media with 1X or 10X organic extract is shown in Figure 26.

Compared to the controls, growth of *C. protothecoides* was significantly lower (α=0.05) when supplemented with the 1X organic extract from yeast hydrolysates and the 10X organic extract from algal hydrolysate. These results suggest that the algal hydrolysate contains toxic organic molecules, but their levels in the hydrolysate (i.e. 1X) are not high enough to drastically influence algal growth. Furthermore, although the decreased growth using 10X organic extract from algal hydrolysates was significant, the culture still grew fairly well. The results using organic extracts from yeast hydrolysates were more puzzling. It is unlikely that the 10X organic extract would be less toxic than the 1X. However, this fluctuation might also be influenced by the intrinsic limitations of this screening methodology. Regardless, *C. protothecoides* grew relatively well regardless of which dilution of organic extract from yeast hydrolysates was used.

C. curvatus was negatively affected (α=0.05) when supplemented with organic extracts from either yeast (10X) or algal (1X and 10) hydrolysates (Figure 26). This suggests that *C. curvatus* is sensitive to organic compounds within the algal and yeast hydrolysates, particularly when compared to results using *C. protothecoides*. However, it is likely that in the dilutions of hydrolysate used in further experimentations with *C. curvatus*, these organic molecules are present at concentrations too low to have an effect on growth.

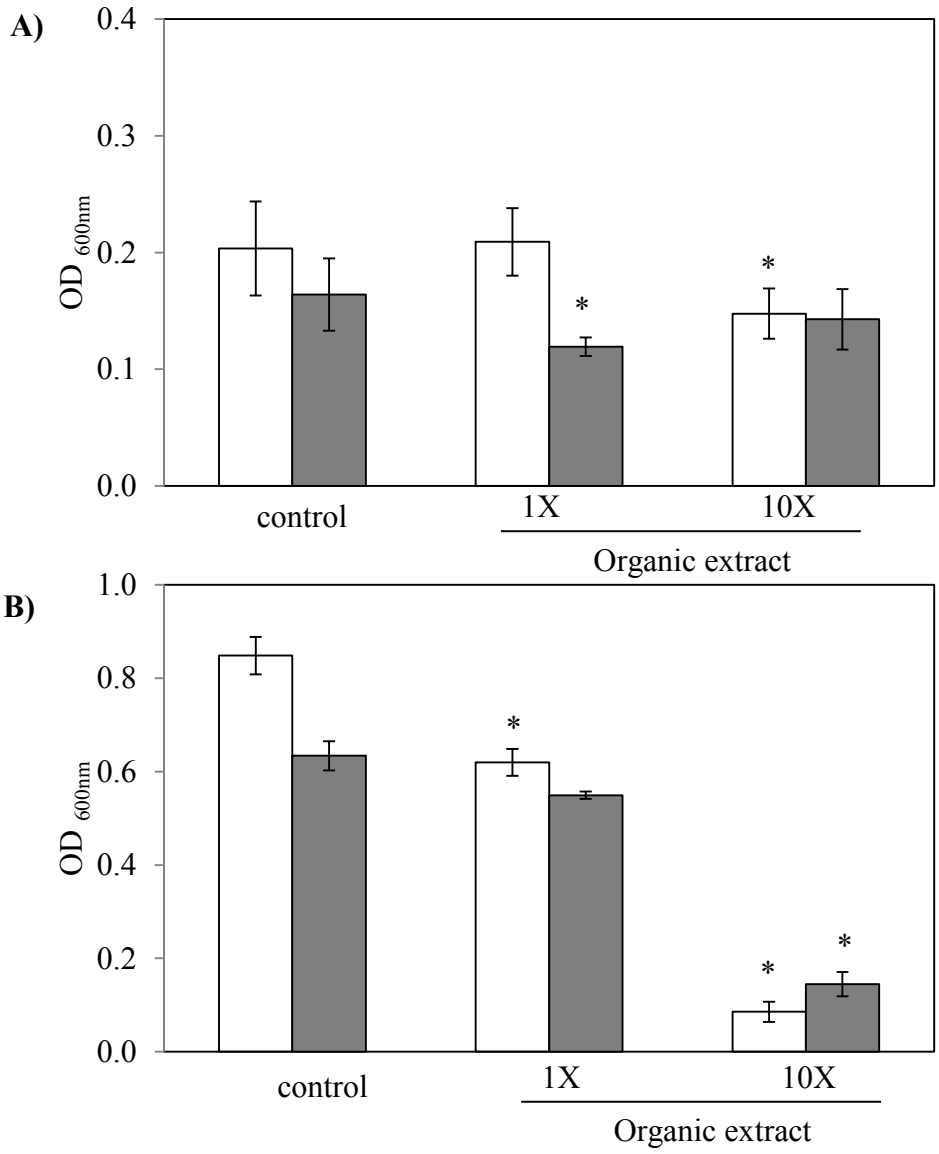


Figure 26. Growth of *C. protothecoides* (A) and *C. curvatus* (B) supplemented with organic material extracted from hydrolysates. Organic extracts obtained through DCM-extraction of algal (white bars) or yeast (gray bars) hydrolysates were added to (10X or 1X concentration) or omitted from (control) the growth media. The experiment was performed as described in Figure 25, but using 30 µL of culture and 150 µl of water to determine Optical density. An asterisk (*) was used to indicate growth that was significantly different (α=0.05) from control samples.

A wide range of organic compounds have been previously reported in aqueous streams from hydrothermal treatment of different biomass sources [131, 287]. Some of these components

can be metabolized as nitrogen sources (specifically by green algae such as *Chlorella*), but the vast majority are a great environmental concern due to their associated toxicity [131, 288, 289]. The assessment of toxicity and tolerance of yeast to decomposition products found in biomass acid hydrolysates has been previously reported [290, 291]. Most toxicity studies have evaluated the growth and ethanol production of yeast strains in response to inhibitory compounds of lignocellulosic hydrolysis, such as weak acids, furan derivatives, and phenolics [290, 291]. One study also studied the performance of the oleaginous yeast *Rhodosporidium toruloides* grown in the presence of these representative components [292]. The growth of this yeast was negatively affected by most inhibitors (by additive and synergistic effects) although its lipid accumulation was basically unmodified [292]. These studies were specific to lignocellulosic feedstocks and their acid hydrolysis, which are extensively characterized [293]. Conversely, detailed characterization and studies of products generated from microbial biomass in hydrothermal treatment as described in this work are scarce.

Some algal species have been widely recognized for their ability to bio-accumulate and remove pollutants from wastewaters (aromatic hydrocarbons, phenolics, organic solvents, and heavy metals). However, the level of pollutants that these algal species can tolerate are compound and concentration dependent [294]. Some *Chlorella* strains, commonly used in water treatment, have been adapted to tolerate high concentrations of pollutants by sequentially increasing the exposition of the microalgae to greater amounts of toxic components [294-296].

3.1.1.4 Minerals in hydrolysates

In order to evaluate if the trace minerals contained in algal or yeast hydrolysates (sections 3.3.3 and 3.4.3 of chapter II) are sufficient for algal or yeast growth, mineral base media was excluded from wells while maintaining constant glycerol and yeast extract levels. For *C.*

protothecoides, algal or yeast hydrolysate (1:25 and 1:50 v/v) was then added to the wells. These concentrations were selected to further define the growth characteristics obtained in the preliminary screening (section 3.4.1. of this chapter). Compared to the control, *C. protothecoides* growth in the presence of minerals was higher when algal hydrolysate was added at a concentration of 1:50 (Figure 27A). Conversely, supplementation with 1:25 algal hydrolysates displayed growth comparable to the control. However, when minerals were excluded, both concentrations of algal hydrolysate supplementation resulted in significantly lower growth. This indicates that minerals likely have to be exogenously supplied when algal hydrolysates are used to grow *C. protothecoides.* No effect was observed in cultures supplemented with different ratios of yeast hydrolysate including cultures without minerals. This suggests that the yeast hydrolysate might supply a significant amount of minerals to the algal cultures. However, extensive studies are required to determine at which extent those minerals would be sufficient to support algal growth, and also to determine individual limiting nutrients in the context of a complete microbial culture.

For *C. curvatus*, algal or yeast hydrolysate (1:5 and 1:10 v/v) was added to the wells. These concentrations were chosen in an attempt to add more results to the screening data (section 3.4.1.2 of this chapter) and better resolve the ideal hydrolysates concentrations to support *C. curvatus* growth. Yeast cultures supplemented with 1:5 (v/v) algal hydrolysate were significantly different than control (α=0.05); a lower dilution (1:10 v/v) did not affect yeast performance. (Figure 27B). The performance of cultures supplemented with 1:5 (v/v) algal hydrolysate corresponded to the trend previously observed in the initial screening (Figure 25), indicating that this dilution was still too high to promote growth of *C. curvatus*. Supplemented cultures at both

concentrations without minerals did not grow as well as the control, which demonstrates the importance of keeping minerals constant to support baseline yeast growth.

When 1:5 (v/v) yeast hydrolysate was used (Figure 27B), growth of *C. curvatus* was not well supported without mineral supplementation. This suggests that for this amount of hydrolysate, minerals have to be exogenously supplied. Relative to the control, use of 1:10 (v/v) yeast hydrolysate led to a lower growth of yeast cultures regardless of if minerals were supplemented.

The growth observed using algal or yeast hydrolysate at a ratio of 1:10 (v/v) did not correspond with data from the initial screening experiments (section 3.4.1.2). Based on the trend uncovered in those experiments, it was expected that a 1:10 (v/v) ratio to promote better (or at least similar) yeast growth relative to the control sample. These discrepancies likely resulted from the small cultures used (200 μL), which were more sensitive to small changes in the growth media. However, the experiments carried out in 96-well plates were used only as a preliminary screening process in order to obtain a starting point with regards to the appropriate concentration of algal or yeast hydrolysates to use in larger scale experiments (discussed in section 3.4.2).

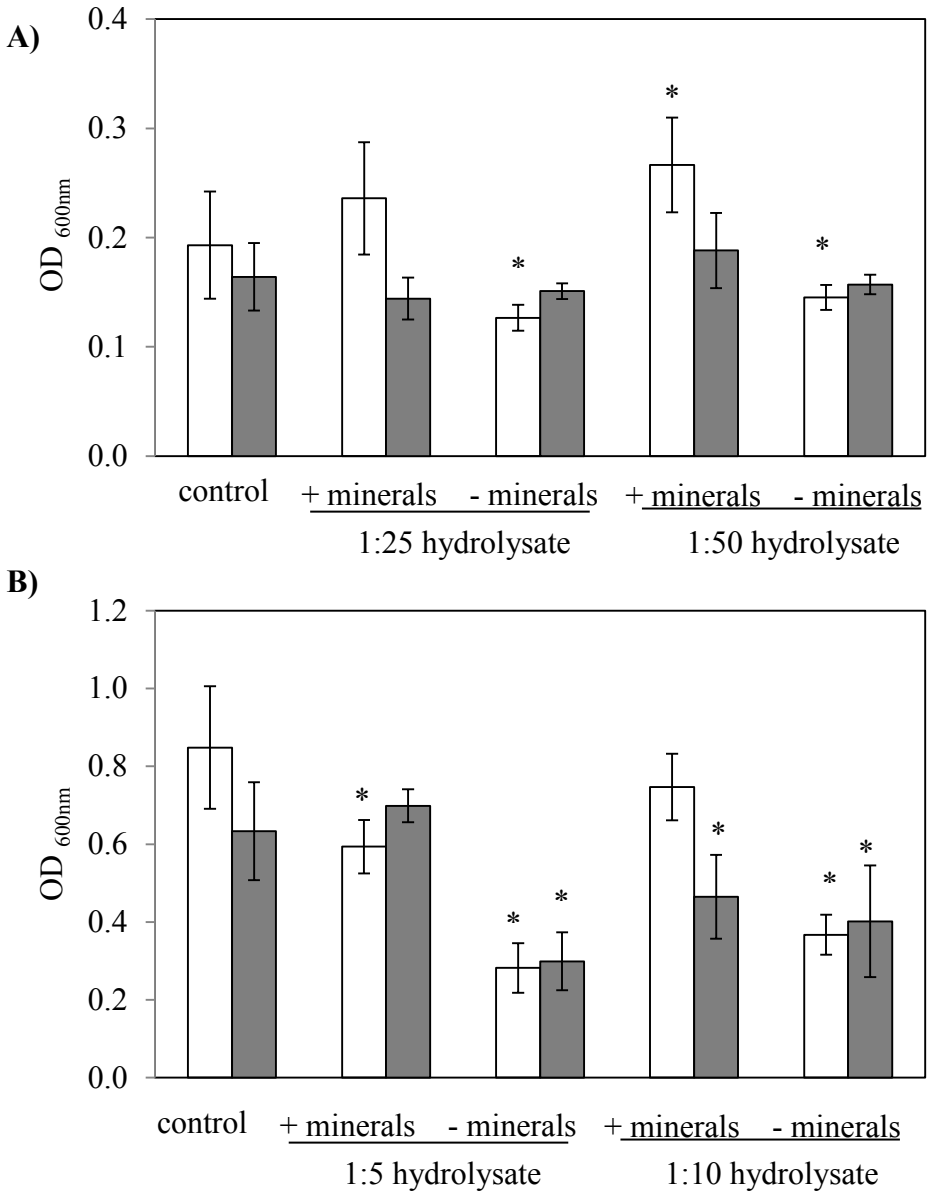


Figure 27. Growth of *C. protothecoides* (A) and *C. curvatus* (B) using yeast hydrolysates with and without mineral supplementation. Algae and yeast were cultured in the absence (control) or presence of algal (white bars) or yeast (gray bars) hydrolysates (1:5 or 1:10; v/v). Yeast or algal mineral media was added as indicated. The experiment was performed as described in Figure 25. Samples displaying significantly (α=0.05) different growth compared to the control are indicated with an asterisk (*).

3.4.2. Batch cultures

Batch cultures of *C. protothecoides* and *C. curvatus* grown in pure glycerol (control) were compared to those supplemented with algal or yeast hydrolysates with respect to biomass, lipid production, and fatty acid profile. In *C. protothecoides* cultures, algal or yeast hydrolysate was supplemented at a ratio of 1:25 (v/v). Specifically for this experiment, starter cultures used for inoculation were centrifuged and re-suspended in Milli-Q water to avoid any carryover of yeast extract into the new cultures.

In *C. curvatus* supplemented cultures, algal or yeast hydrolysate was used at a ratio of 1:5 (v/v). Dilutions of hydrolysates to add in algal or yeast cultures were chosen since they were presumably the highest concentrations of hydrolysates that did not have inhibitory effects on growth (Figures 24 and 25).

The total amount of glycerol in both growth media was standardized (20 g/L) and a limiting amount of yeast extract (0.8 g/L) was used. Contamination was not observed in cultures throughout the entire growth period and harvesting.

3.4.2.1. Growth of *C. protothecoides* supplemented with hydrolysates

Biomass production and glycerol consumption are presented in Figure 28. Total biomass after 264 h in control culture was 2.94 ± 0.09 g/L, which was significantly lower (α=0.05) than the 5.8 ± 0.14 g/L or 3.4 ± 0.08 g/L in cultures supplemented with 1:25 (v/v) of algal or yeast hydrolysates, respectively. Algal hydrolysate supplementation represented almost a 2 fold increase in final biomass concentration. Yeast hydrolysate supplementation also enhanced growth at roughly 15% compared to control cultures. Based on preliminary screening (Figures 24 and 27A), we expected that algal hydrolysates supplementation would promote similar or increased growth relative to the control while addition of yeast hydrolysates would not have a

significant effect. These expectations were confirmed in batch cultures, though we should point out that batch cultures were grown for 264 hours whereas the screening cultures were only incubated for 72 hours.

The poor consumption of glycerol in control cultures could be due to a limiting nutrient that might be partially provided by the supplemented hydrolysates, particularly the algal hydrolysate (since yeast hydrolysate had a less pronounced effect). One of the main differences between the hydrolysates is their total amino acid content (Table 3), which is almost 3 times higher in algal than yeast hydrolysate. *C. protothecoides* might have grown better by using the higher amount of free amino acids supplied in the algal hydrolysate compared to the yeast hydrolysate. Acetic and propionic acids in algal or yeast hydrolysates were not consumed during growth (data not shown).

Aqueous streams from diverse hydrothermal treatments of algae have been previously characterized. These byproduct streams had a highly variable composition depending on the algal biomass used (species, cultivation mode, and proximate composition) as well as processing conditions (time and temperature). For example, algal pretreatments at subcritical conditions have produced streams with acidic pH [228], while higher temperatures (>300°C) have resulted in basic pH as a result of ammonia production [216, 222, 223, 225, 226]. Minerals, chemicals, and other potential nutrients or inhibitors reported in those aqueous streams were qualitatively and quantitatively variable and generalizations of these streams are not possible.

Despite their heterogeneity, several studies have attempted the supplementation of hydrothermally-treated algal biomass byproduct aqueous streams in the mixotrophic cultivation of different algae. Generally, microalgal growth was moderately improved when autotrophic cultures were supplemented with aqueous streams (dilution ratios of 1:50 to 1:400 v/v) [126,

129, 131, 212]. However, negative effects on the growth of *Chlorella minutissima* were observed when cultures were supplemented with an aqueous stream from hydrothermally treated (350°C for 60 min) *Spirulina* [225]. Recycling has also been reported by using other approaches such as the substitution of specific nutrients [130], the co-culture of algae with other microorganisms [133], and the repetition of cultivation cycles [134]. The recycling strategy proposed in this thesis is unique since it provides the opportunity to establish an algal heterotrophic culture by providing the carbon source and all other nutrients required for cultivation by supplementation of different waste streams.

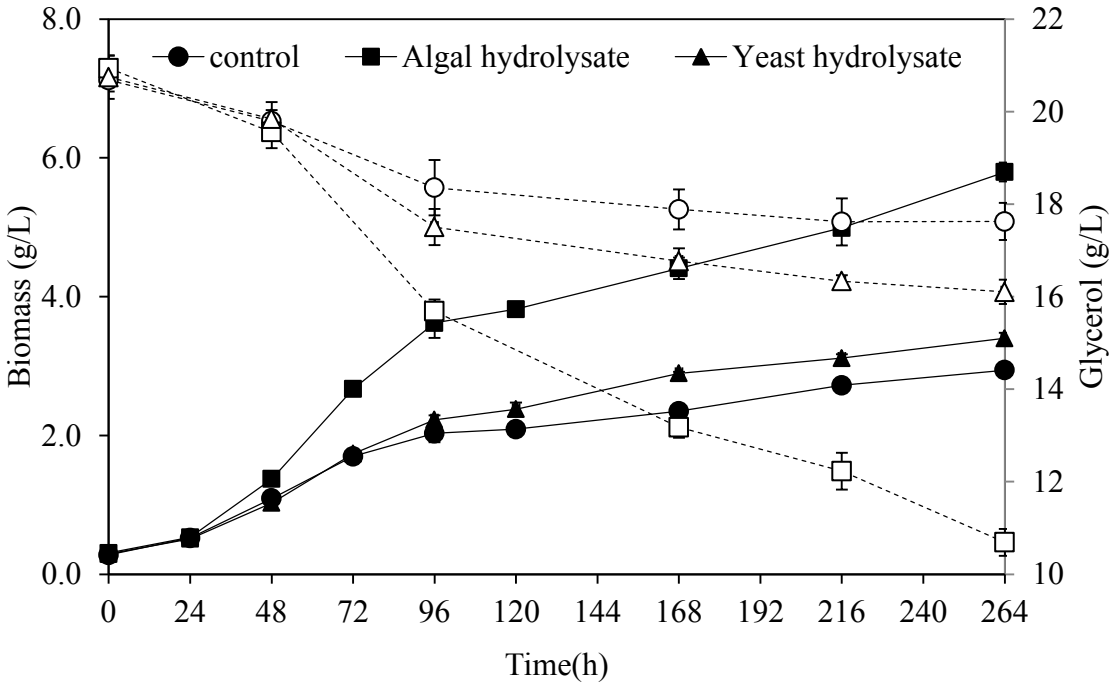


Figure 28. Biomass production and glycerol consumption of *C. protothecoides* in batch cultures grown at 20°C for 264 hours with shaking at 150 rpm. Algal or yeast hydrolysate (1:25; v/v) was added to the growth media as indicated. Biomass (g/L; solid line) and glycerol concentrations (g/L; dotted line) are plotted relative to time (h). Results express the means of replicate experiments (n=3).

3.4.2.2. Growth of *C. curvatus* supplemented with hydrolysates

Biomass production and glycerol consumption curves are presented in Figure 29. The total biomass after 168 h of the control culture was 6.6 ± 0.5 g/L, which was lower than the 9.3 ± 0.4 g/L and 9.8 ± 0.5 g/L seen in cultures supplemented with 1:5 (v/v) algal and yeast hydrolysates, respectively. These correspond to 40% and 47% increases in the final biomass concentrations for cultures supplemented with algal and yeast hydrolysates, respectively. These data clearly show that supplementation of both hydrolysates at a ratio of 1:5 (v/v) promotes superior growth of *C. curvatus*. It is of interest to note that at 72 h, which was the incubation time for the screening cultures, there was no significant difference in the amount of accumulated biomass.

Control cultures showed poor glycerol consumption most likely due to the lack of a specific nutrient such as nitrogen as suggested by the linear trend of glycerol consumption. In contrast, cultures supplemented with hydrolysates had extra nutrients supplied in various inorganic and organic forms, which may have favoured full glycerol consumption and promoted an overall improvement in the growth of the cultures. Acetic and propionic acids present in algal or yeast hydrolysates were not consumed during growth (data not shown).

Only one study has reported the use of byproduct aqueous streams from hydrothermal treatment of biomass for yeast cultivation [132]. In that work, the non-oleaginous yeast *Saccharomyces cerevisiae* was supplemented with 20% (v/v) of the aqueous stream from thermal treatment of the microalgae *Nannochloropsis oculata*. It is important to mention that an external carbon source, glucose, was strictly required in those yeast cultures. This thesis proposes the alternative culturing of oleaginous yeast (intended for biofuel production) by using waste streams from different bioprocessing technologies.

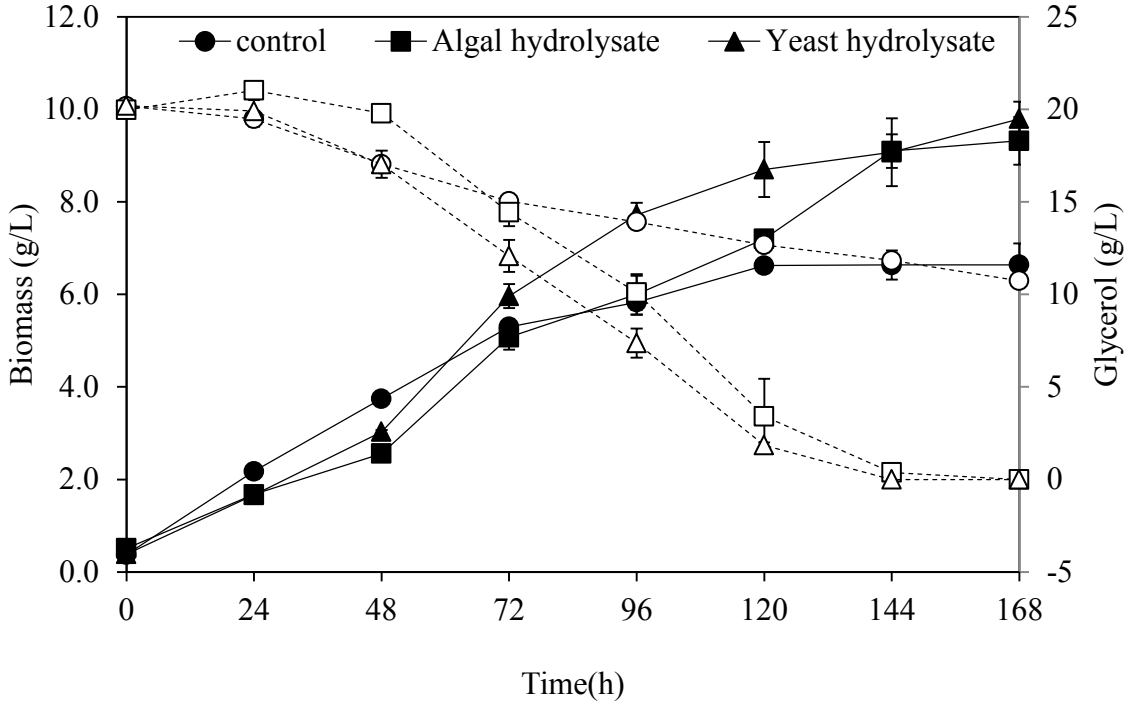


Figure 29. Biomass production and glycerol consumption of *C. curvatus* in batch cultures grown at 30°C for 168 hours with shaking at 200 rpm. Yeast or algal hydrolysate (1:5 (v/v)) was added to the growth media as indicated. Biomass (g/L; solid line) and glycerol concentrations (g/L; dotted line) are plotted relative to time (h). Results express the means of replicate experiments (n=3).

3.4.2.3. Lipids in *C. protothecoides* supplemented with hydrolysates

Compared to control cultures, total lipid content in *C. protothecoides* (32.3 ± 1.5% dry weight) was significantly different (α=0.05) from cultures supplemented with algal hydrolysate (39.2 ± 1.8%), but not yeast hydrolysate (31.8 ± 1.7%). This result suggests that even though nitrogen (via amino acids) was present in relatively high amounts in the algal hydrolysates and thus had the potential to alter C:N ratios, lipid accumulation was significant indicating that algal cultures were still growing under nitrogen limitation [23, 165].

The fatty acid profile of *C. protothecoides* grown in glycerol was composed of oleic, linoleic, palmitic, linolenic, and stearic acids. The specific amounts of fatty acids present in the

microalgal cultures supplemented with algal hydrolysate were significantly different (α=0.05) than the control. An increase in oleic (44.2 ± 0.6 to 55.8 ± 0.4%) and stearic (2.1 ± 0.0 to 3.7 ± 0.1 %) acids was accompanied by a decrease in palmitic (14.8 ± 0.2 to 10.8 ± 0.2%), linoleic (35.3 ± 0.3 to 28.9 ± 0.4%), and linolenic (from 3.6 ± 0.2 to 0.9 ± 0.0%) acids (Figure 30A). *C. protothecoides* cultures supplemented with yeast hydrolysate also changed their fatty acid profile; there was an increase in linoleic acid (35.3 ± 0.3 to 42.8 ± 1.7%) and a decrease in oleic acid (44.2 ± 0.6 to 38.3 ± 2.0%; Figure 30A). Alteration of the relative proportion of fatty acids in microalgae has been previously demonstrated when culture conditions as well as the type or concentration of nitrogen or carbon sources are changed [78, 297]. The fatty acids obtained from algal supplemented cultures did not change qualitatively compared to control; this suggests that there was no affectation in the biosynthesis of lipids.

The composition of algal fatty acids strongly influences the properties of typical biodiesel produced through transesterification [297]. For this type of biodiesel, the fatty acid profile should have a prevalence of oleic acid, which imparts some of the desired characteristics of biofuel, such as cetane number, kinematic viscosity, and oxidative stability [298]. Other fatty acids that are also desirable for the same purpose are palmitic and decanoic acids [299]. However, the biofuel produced under the LTH technology does not have the same requirements as typical biodiesel because fatty acids for LTH undergo further intensive thermal treatment (pyrolysis). In LTH, the liquid pyrolysis product distribution also depends on the composition of the feedstock fatty acids; more saturated compounds are expected when feedstocks have predominance of unsaturated fatty acids [230]. However, the robustness of the pyrolysis stage using a variety of lipid sources has been previously demonstrated [230]. Our data suggest that the microbial fatty acids obtained from algal cultures, regardless of if they have been supplemented with

hydrolysates, can serve as drop in raw material for the LTH process (as previously demonstrated in section 3.5. of chapter II).

3.4.2.4. Lipids in *C. curvatus* supplemented with hydrolysates

Total lipid content in *C. curvatus* biomass from control cultures was 21.4 ± 1% dry weight, which was not significantly different (α=0.05) from the values of 22.1 ± 2.5% or 21.1 ± 1.7% obtained from cultures supplemented with algal or yeast hydrolysates, respectively. This indicates that the increase in biomass observed when *C. curvatus* cultures are supplemented with yeast hydrolysate is not accompanied with a decrease in lipid production, which further highlights the value of yeast and algal hydrolysate supplementation. Total lipid content of *C. curvatus* grown on glycerol was similar to the previously reported 25% observed by Meesteres *et al.* [29].

The fatty acid profile of *C. curvatus* grown in glycerol was composed of oleic, linoleic, palmitic, stearic, and linolenic acids (Figure 30B). When grown with supplementation of algal hydrolysate, only the amount of oleic acid (37 ± 0 %) was significantly different (α=0.05) from the control (32.7 ± 2%). *C. curavtus* grown in the presence of yeast hydrolysates also showed an altered fatty acid profile; palmitic acid decreased from 27 ± 1 to 24 ± 0% and stearic acid increased from 7 ± 1 to 11 ± 2%.

Major differences in the fatty acid distribution of *C. curvatus* grown in glycerol were found when compared with the previously reported profile from glucose grown cultures (section 3.4.1, Chapter II), such as lower amounts of stearic and oleic acids and higher amounts of linoleic acid. Minor changes in the fatty acid distribution of glycerol grown *C. curvatus* were found compared to previous reports [30], having lower and higher proportions of oleic and linoleic acids, respectively. These changes in fatty acid distribution are not surprising; an increase in the

synthesis of linoleic acid has been previously found in long-incubated and nitrogen-limited yeast cultures [300]. Also, changes in fatty acid profile are common when different carbon sources [301], nitrogen amounts [243], and culture conditions (media and time) [300] are used. As previously discussed for algal cultures (section 3.4.2.3.), yeast fatty acids from glycerol grown cultures are suitable feedstock for LTH.

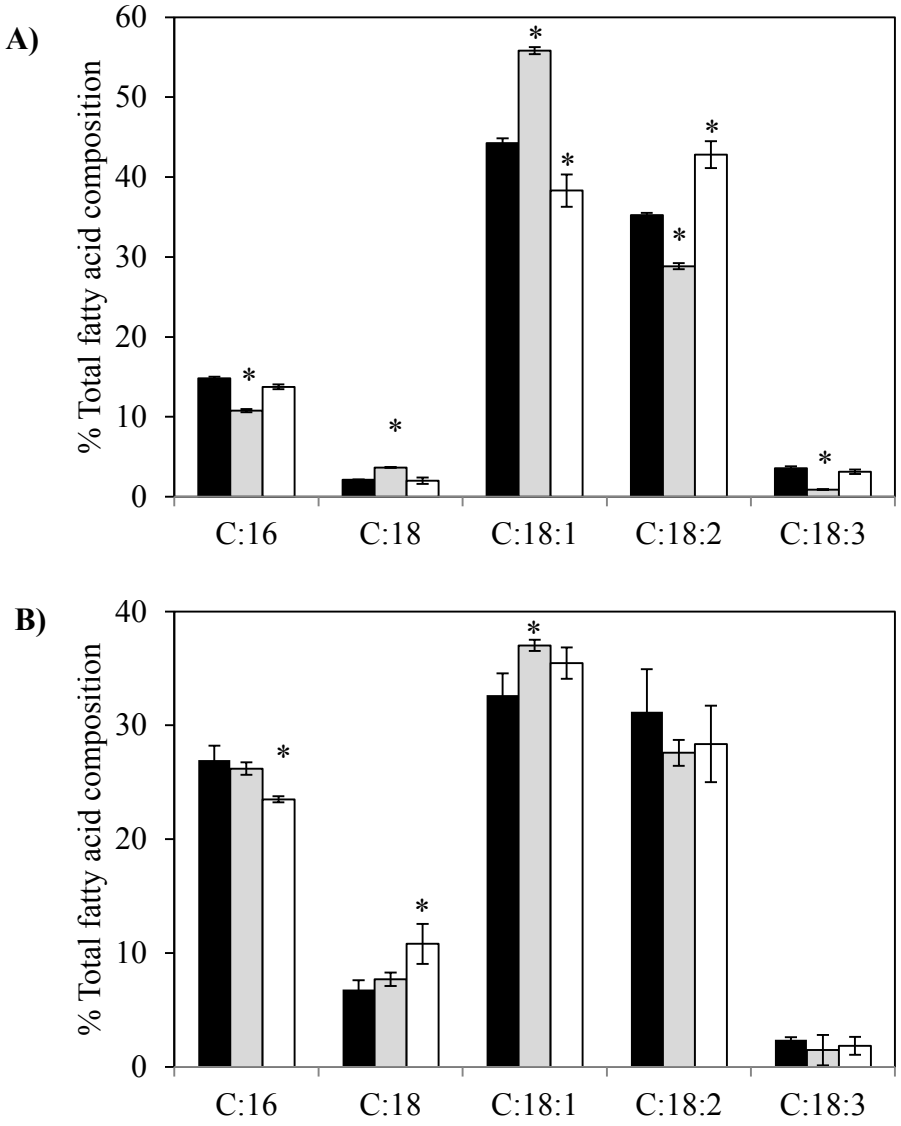


Figure 30. Fatty acid composition of *C. protothecoides* (A) and *C. curvatus* (B) grown in 20 g/L glycerol and 0.8 g/L yeast extract. Fatty acid content was determined for yeast cultures grown in the absence (control; black bars) or presence (algal hydrolysates = gray bars; yeast hydrolysate = white bars) of hydrolysates (1:25 (algal) or 1:5 (yeast) v/v). Fatty acid levels that differ significantly (α=0.05) between the control culture and the culture supplemented with hydrolysate are indicated with asterisks (*). C:16, palmitic acid; C:18, stearic acid; C:18:1, oleic acid; C:18:2, linoleic acid; C:18:3, linolenic acid. Results express the means of replicate experiments (n=3).

4. Conclusions

Recycling of different byproduct streams was successfully implemented in the cultivation of oleaginous microalgae and yeast. The hydrolysis of various fats and oils using the patented LTH technology [229] was conducted to create byproduct aqueous streams that contain glycerol. It was demonstrated that crude glycerol from different feedstocks can serve as the main carbon source for algal and yeast growth. This strategy valorizes a readily available biorefinery byproduct by directly integrating it in microbial cultivation (for biofuel production) without the need for purification or other further processing. Aqueous streams from the thermal hydrolysis of *C. protothecoides* and *C. curvatus* were also supplemented in yeast and heterotrophic microalgal cultures. It was shown that the hydrolysates of both oleaginous microorganisms, which contain several nutrients such as glycerol, organic and inorganic nitrogen, phosphates, and minerals, can be recycled and used to promote growth of future microbial cultures. Optimization of the appropriate concentration of yeast and algal hydrolysates to use during cultivation still needs to be conducted. The potential integration of the different bioprocessing waste streams described in this chapter serves to decrease feedstock costs and to minimize environmental impacts, such as the limitation of non-renewable resources, of intensive microbial culturing for biofuels.

IV. Heterotrophic growth and lipid accumulation of *Chlorella protothecoides* in whey permeate, a dairy byproduct stream, for biofuel production[c]

1. Introduction

One of the drawbacks of using heterotrophic processes to cultivate oleaginous algae is the requirement for expensive organic carbon feedstocks required for cultivation. Some of the carbon sources used by heterotrophic algae are hexoses (glucose, maltose, mannose, galactose, and fructose), organic acids (acetate and lactate), alcohols (methanol and ethanol), and glycerol [47, 302]. One of the best lipid producing microalgae examined to date is *Chlorella protothecoides* [303], which prefers glucose as a carbon source. The favoured consumption of glucose is also common for other members of the *Chlorella* genus [304, 305]. Another hexose consumed by *Chlorella* is galactose, though to a lesser extent [304, 305]. Despite the individual utilization of glucose and galactose, *Chlorella* cannot utilize the disaccharide lactose [304, 305]. Disaccharides are not commonly metabolized by microalgae; only sucrose is used by some species [306] although algal consumption of this disaccharide might result from sucrose hydrolysis in the acidic pH of the growth media [307].

The typical glucose use for growing heterotrophic algal cultures represents around 60-75% of the total cost of the culture media [26, 44]. An effective strategy to lower the cost of culturing *C. protothecoides* is the utilization of inexpensive substrates that contain suitable carbon sources [73-76, 78, 308]. The utilization of abundant industrial byproduct streams that have limited

[c] Parts of this chapter have been previously published.
Espinosa-Gonzalez I., A. Parashar, D.C. Bressler, Heterotrophic growth and lipid accumulation of *Chlorella protothecoides* in whey permeate, a dairy by-product stream, for biofuel production. Bioresource Technology, 2013. 155:p.170-176.

applications represents an ideal opportunity for the heterotrophic cultivation of microalgae in a cost-effective manner.

One such byproduct stream is whey permeate (WP) from the dairy industry. Whey permeate is obtained by ultrafiltration and removal of protein from whey that is generated during cheese manufacturing and represents about 85% of the total milk used in the process [309]. Whey permeate is composed mostly of lactose along with salts and non-protein nitrogen [310]. Considering the large amounts of whey permeate generated, developing methods to valorize this byproduct stream would be of key interest to the dairy sector. Thus far, the use of whey permeate as a direct source of lactose has been ignored due to the extensive processing required, which includes demineralization and dewatering [310].

Whey derivatives have been used to grow microalgae to evaluate the potential for such organisms to decrease organic matter and deplete nutrients from dairy and other industrial effluents [311, 312]. More recently, a dual approach for treating industrial waste streams was proposed that used algae as a biofilter, but also used the resulting algal biomass for biofuel production [313]. However, the direct use of WP, a consistent and readily available feedstock from the dairy industry, for controlled heterotrophic microalgal cultivation has not been reported.

C. protothecoides has been previously cultivated through heterotrophy by using different strategies such as batch and fed-batch [244, 314]. The fed-batch strategy is commonly used to increase the amount of cells in a bioreactor by constantly supplying the limiting nutrient(s) to the fermentation, thus allowing the microorganisms to extend their log phase. It is also useful for cultivations in which higher concentrations of a substrate negatively influence cell growth, as in the case of *C. protothecoides* grown in pure glucose [314].

The aim of this study was to evaluate the use of whey permeate as the main carbon source for the heterotrophic growth and lipid accumulation of the microalgae *C. protothecoides*. The potential application of pre-hydrolyzed whey permeate as feedstock for batch and high cell density fed-batch microalgal cultures was investigated along with the direct use of non-hydrolyzed whey permeate by simultaneous saccharification and fermentation using immobilized hydrolysing enzymes.

2. Materials and methods

2.1. Materials

Chlorella protothecoides (UTEX 256) was used in this study. Storage conditions, media preparation, and inoculation procedures were as described in section 2.1 of chapter II. Whey permeate was procured from a large dairy producer (liquid form; pH 5.5) and contained the following nutrients: lactose (182 g/L), phosphorous (0.8% dry weight basis), magnesium (0.2% dry weight basis), and calcium (0.8% dry weight basis). Whey permeate was used 'as is' or hydrolyzed as described below.

2.2. Methods

2.2.1. Whey permeate hydrolysis

Whey permeate was neutralized using 2 M KOH and hydrolyzed at 30°C for 24 h in shake flask at 200 rpm using 65 U of Lactozyme 3000 (Sigma-Aldrich, St. Louis, MO) per g lactose quantified in whey permeate. Hydrolyzed whey permeate (HWP) with a final composition of 95 g/L glucose and 85 g/L galactose was filtered (0.22 μm) and stored at 2°C.

2.2.2. Analytical methods

Lactose, glucose, and galactose content was determined using an Agilent 1200 series High Performance Liquid Chromatography (HPLC) instrument (Agilent, Santa Clara, CA), with a refractive index detector, and a Bio-Rad HP87H column (Bio-Rad Laboratories, Hercules, CA) under conditions previously described (section 2.2.7.2 of chapter II). Algal growth and lipid accumulation were determined by measuring OD_{600nm} and fluorescence using Nile Red as previously explained (sections 2.2.3.2 and 2.2.3.3 of chapter II). Total fatty acid composition in freeze-dried algal biomass was determined as previously described (section 2.2.7.4 of chapter II). Total nitrogen and total carbon were measured using the Dumas Combustion Method at the Natural Resources Analytical Laboratory, University of Alberta.

2.2.3. Batch fermentations

C. protothecoides was cultured in 250 mL Erlenmeyer flasks with 100 mL base mineral media [242]. The amount of glucose, galactose, lactose, WP, or HWP used as main carbon sources was standardized from 0.33 C mol/L to 1 C mol/L, which corresponds to 10 g/L and 30 g/L monomeric sugar, respectively. To trigger lipid accumulation, a low nitrogen concentration was used in the growth media: $NaNO_3$ (2.5 g/L) for preliminary experiments and yeast extract for all other experiments [244]. Yeast extract varied from 1 to 1.2 g/L ($7.8E^{-3}$ mol/L to $1E^{-2}$ mol/L; based on results from preliminary experiments) to establish carbon to nitrogen ratios of 50:1 to 100:1 [136]. The pH of the cultures was monitored and adjusted to 6.4 with 2M KOH. Flasks were inoculated with 5% (v/v) starter inoculum obtained from a shake flask culture in late exponential phase [136]. Cultures were grown in the dark at 25°C, 150 rpm with 24 h sampling intervals for a total of 168 or 216 h. Each experimental unit was monitored for contamination by

microscopic examination and streaking on Luria Bertani (LB) agar plates with subsequent incubation. All experiments were done in triplicate.

2.2.4. Fed-batch fermentations

2.2.4.1. Small scale

Small scale fermentations were performed in 250 mL Erlenmeyer flasks. The carbon sources used for fed-batch fermentations were either HWP or a control mixture of glucose and galactose prepared at the same concentration as the HWP. Initial C mole from sugar was standardized to 0.33 C mol/L (10 g/L of each monomeric sugar) and N was initially supplied through addition of 2 g/L yeast extract. Inoculation, incubation, pH control, and contamination analysis were done as indicated in section 2.2.3 of this chapter. Subsequent feeding (described in the results and discussion section) of HWP or the control mixture along with yeast extract (0.5 g/L) was done as required. Triplicate cultures were monitored for a total of 240 h.

2.2.4.2. Scale up

Scale-up of fed-batch fermentations (5 L bioreactors , Infors, Einsbach, Germany) were done with either HWP or the glucose control, which was prepared at the same sugar concentration as HWP. The initial fermentation volume was 3 L with a C mole amount from sugar doubled from the small scale experiments (HWP: 10 g/L glucose, 10g/L galactose; glucose control: 20 g/L glucose) and an initial yeast extract concentration of 4 g/L. Inoculation, pH control, and contamination checks were done as indicted in section 2.2.3 of this chapter. Subsequent feeding of HWP or glucose along with yeast extract (2 g/L) was performed as required. During fermentation, aeration rate and stirrer speed were varied between 1-2 vvm and

100-200 rpm, respectively, to keep air saturation over 20% [244]. When necessary, pure O_2 was mixed with air to maintain saturation. Duplicate cultures were monitored for a total of 240 h.

2.2.5. β-galactosidase immobilization

The enzyme β-galactosidase from *Aspergillus oryzae* (Sigma-Aldrich, St. Louis, MO) was immobilized by encapsulation using polyvinyl alcohol (PVA) and polyethylene glycol (PEG) according to the manufacturer's instructions (Lentikat's, Prague, Czech Republic). Briefly, PVA 10% (w/w) and PEG 6% (w/w) were solubilized in water by boiling, and the solution was cooled to 35°C before adding 5% (w/w) of a β-galactosidase enzyme solution (0.1 mg/L). Immobilized enzyme beads were produced using a LentiPrinter device (Lentikat's, Prague, Czech Republic), dried, and subsequently rehydrated with 0.1M Na_2SO_4 for 2 h before transferring into 0.1M potassium phosphate buffer with 2 mM $MgCl_2$, pH 6.5 for storage at 4°C. Enzymatic activity of the immobilized enzyme was measured according to manufacturer's instructions.

2.2.6. Simultaneous saccharification and fermentation (SSF)

C. protothecoides was cultured in 250 mL Erlenmeyer flasks with 100 mL media containing 25 mL of 4X concentrated mineral base [242], 0.25 g/L yeast extract, non-hydrolyzed whey permeate, and autoclaved double distilled water (MilliQ, Millipore, Billerica, MA). Whey permeate was filtered (0.22 μm) and added based on C mole from sugar, which was calculated from the lactose content. At inoculation, 3 U of encapsulated β-galactosidase activity were added to the flasks and yeast extract (2 g/L) was supplemented as required. Inoculation, incubation, pH control, and contamination checks were done as indicted in section 2.2.3 of this chapter. Triplicate cultures were monitored for a total of 216 h.

2.2.7. Calculations

Statistical analysis of variance for biomass, lipid production as well as fatty acid composition was done using one or two way- ANOVA with mean comparison by Tukey test (GradPad software, La Jolla, CA).

Yields (dry weight basis) were calculated at harvesting time as follows:

$$\text{Biomass yield } (Y_{b/s}) = \text{Biomass (g)/Utilized substrate (initial – residual) (g)}$$

$$\text{Lipid yield } (Y_{l/s}) = \text{Lipid (g)/Utilized substrate (initial – residual) (g)}$$

3. Results and Discussion

3.1. Growth of *C. protothecoides* in whey permeate (WP)

The ability of *C. protothecoides* to utilize lactose in WP or glucose and galactose in HWP was evaluated in batch cultures (Figure 31). Pure lactose, glucose, and galactose were included as controls, and $NaNO_3$ was used as the inorganic nitrogen source. *C. protothecoides* was unable to utilize lactose from WP or pure lactose as a carbon source, but was able to grow well in HWP through assimilation of glucose and galactose. The negligible consumption of lactose (from WP or in its pure form) was expected as this was previously reported for other *Chlorella* [306, 307]. However, the lactose hydrolysates (HWP), acted as a favourable substrate for algal growth (biomass production of 2.8 ± 0.4 g/L). With the glucose and galactose controls, lower biomass productions of 1.5 ± 0.1 and 1.7 ± 0.4 g/L, respectively, were observed. This indicates that the waste stream WP can be valorized by adding a simple hydrolysis step, which may be a better alternative to its current application in energy-intensive lactose recovery methods.

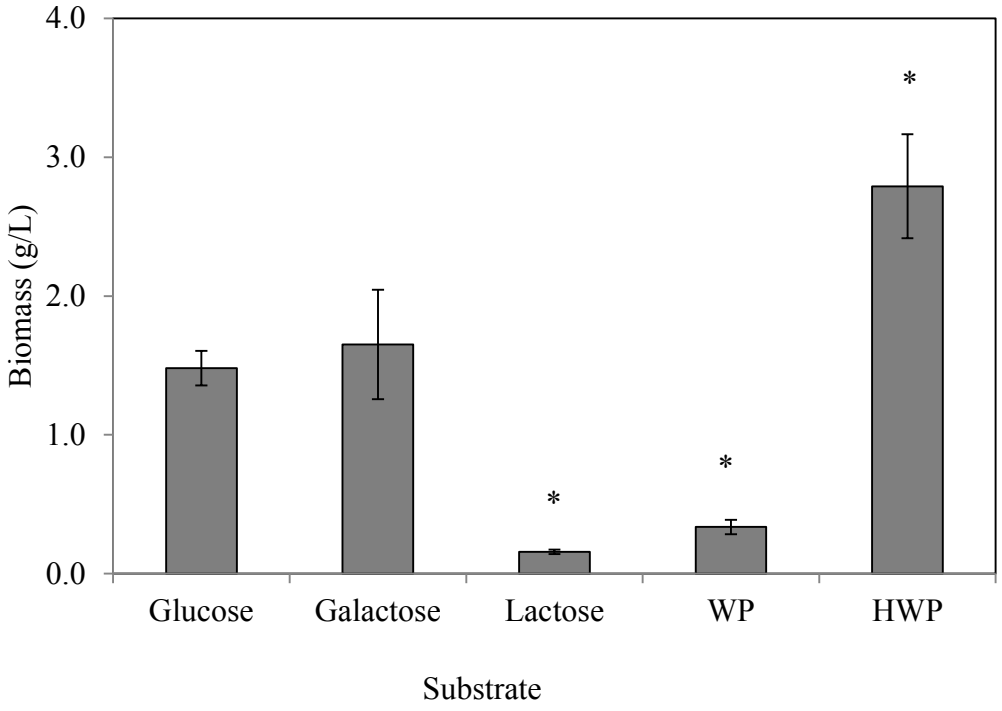


Figure 31. Growth of *C. protothecoides* in batch cultures for 168 h using different substrates as main carbon sources at a final concentration of 15 g/L monomeric sugar (0.5 C mol/L) and 2.5 g/L $NaNO_3$. Results express the means of replicate experiments (n=3).

3.2. Biomass production and lipid accumulation in batch cultures

To evaluate biomass production and lipid accumulation of *C. protothecoides* grown in HWP, batch cultures were grown using 10 g/L total monomeric sugar (0.33 C mol/L) and yeast extract as the main nitrogen source (1 g/L = $7.8E^{-3}$ N mol/L) resulting in a C to N ratio of 50:1. No contamination was observed in cultures during the entire growth period and harvesting. Glucose and galactose were used individually and in a mixture as controls. *C. protothecoides* was able to assimilate glucose and galactose simultaneously with a slight preference for glucose (Figure 32). The substrate utilization rate was higher for all three control mixtures compared to HWP (Figure 32). A biomass of 4.3 ± 0.6 g/L with a lipid content of 24.6 ± 1.5% was obtained when grown using HWP. Lipid content in control cultures, except for galactose, was not

significantly different (α=0.05) from the HWP culture (Figure 34). To promote higher lipid accumulation, cultures were then grown using 30 g/L total monomeric sugar and reduced yeast extract for a C to N ratio of 100:1 [275]. Higher concentrations of glucose have been previously shown to cause substrate inhibition in batch cultures [244, 314]. At a substrate concentration of 30 g/L, slower growth rates and residual sugars were observed for all controls relative to that of HWP (Figure 33). Higher biomass was obtained using HWP (9.1 ± 0.2 g/L) compared to the controls: glucose (5.9 ± 0.2 g/L), galactose (6.2 ± 0.2 g/L), and glucose and galactose mixture (5.9 ± 0.1 g/L). The biomass produced in the three controls was not significantly different (α= 0.05) from each other. Increased lipid accumulation of 42.0 ± 4.5% was also observed using HWP at 30 g/L compared to 10 g/L, which was not significantly different (α=0.05) from the other culture conditions (Figure 34).

Biomass and lipid production yields (Table 13) suggested comparable substrate utilization efficiencies for all the groups. In cultures at 30 g/L, the shorter lag phase and faster substrate utilization observed with HWP suggests its beneficial role in promoting algal growth, which likely is due to other nutrients present in the whey permeate, including nitrogen and minerals. Cultures at 10 g/L were set for an initial assessment of algal performance; the increase in substrate concentration to 30 g/L was also accompanied with a decrease in yeast extract, which was likely the reason for the higher lipid content in all cultures.

Table 13. Biomass ($Y_{(b/s)}$) and lipid ($Y_{(l/s)}$) yields of *C. protothecoides* grown in 10 and 30 g/L monomeric sugar, and 1 and 1.2 g/L yeast extract, respectively. Hydrolyzed whey permeate, HWP; Glucose and galactose mixture, glu+gal; Glucose, glu; Galactose, gal.

	10 g/L C monomer- 1 g/L yeast						**30 g/L C monomer-1.2 g/L yeast**					
	$Y_{(b/s)}$			$Y_{(l/s)}$			$Y_{(b/s)}$			$Y_{(l/s)}$		
HWP	0.41	±	0.06	0.10	±	0.01	0.35	±	0.01	0.15	±	0.01
glu+gal	0.47	±	0.01	0.13	±	0.02	0.33	±	0.01	0.15	±	0.00
glu	0.49	±	0.06	0.15	±	0.01	0.36	±	0.02	0.15	±	0.01
gal	0.46	±	0.02	0.13	±	0.01	0.36	±	0.01	0.13	±	0.02

b/s = g biomass dry weight basis /g substrate; l/s= g lipid dry weight basis /g substrate

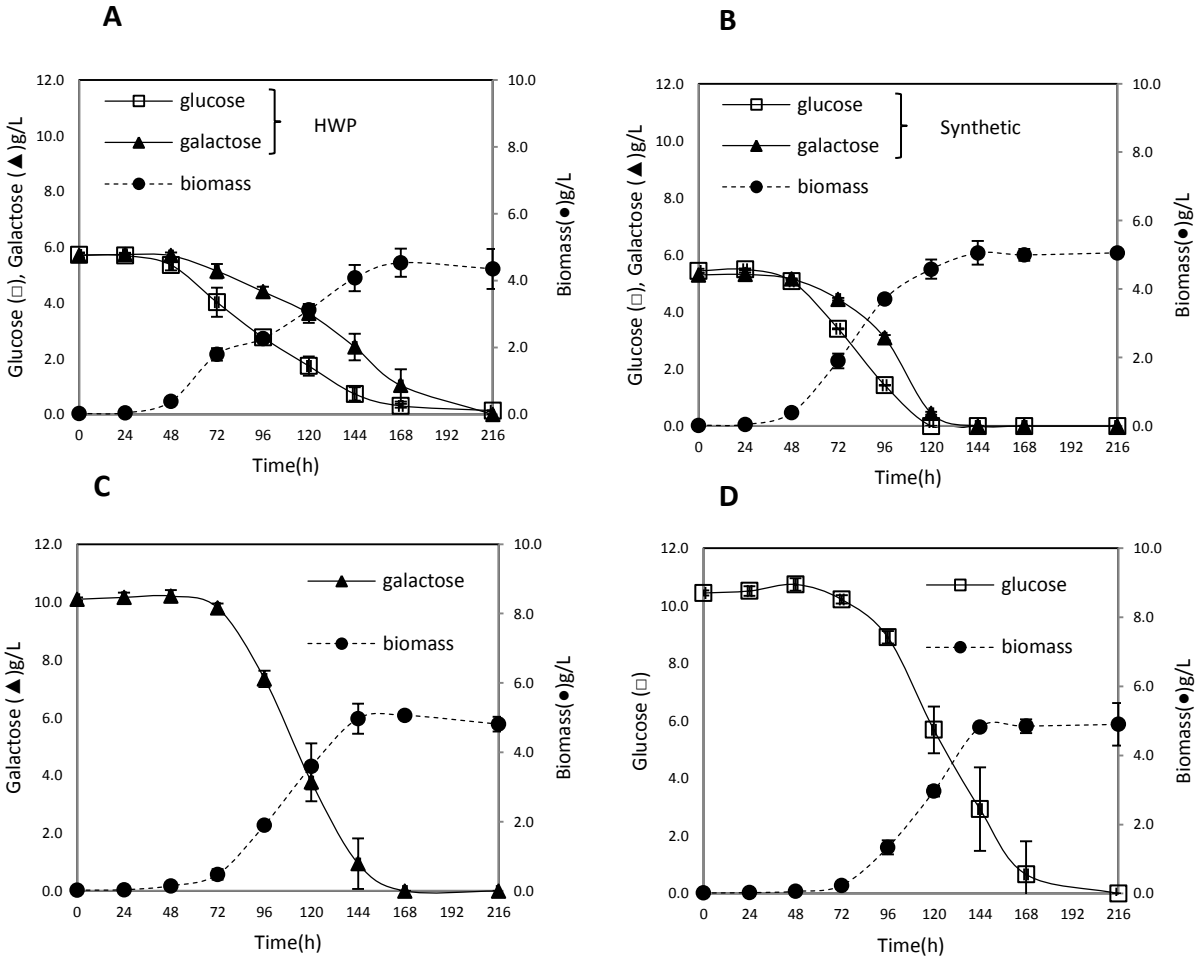


Figure 32. Sugar consumption and growth of *C. protothecoides* in batch cultures grown for 216 h using different substrates as main carbon sources at a final concentration of 10 g/L monomeric sugar (0.33 C mol/L) and 1 g/L yeast extract ($7.8E^{-3}$ N mol/L) with a C:N ratio of 50:1. A) HWP; B) Glucose and galactose mixture; C) Glucose; D) Galactose. Results express the means of replicate experiments (n=3).

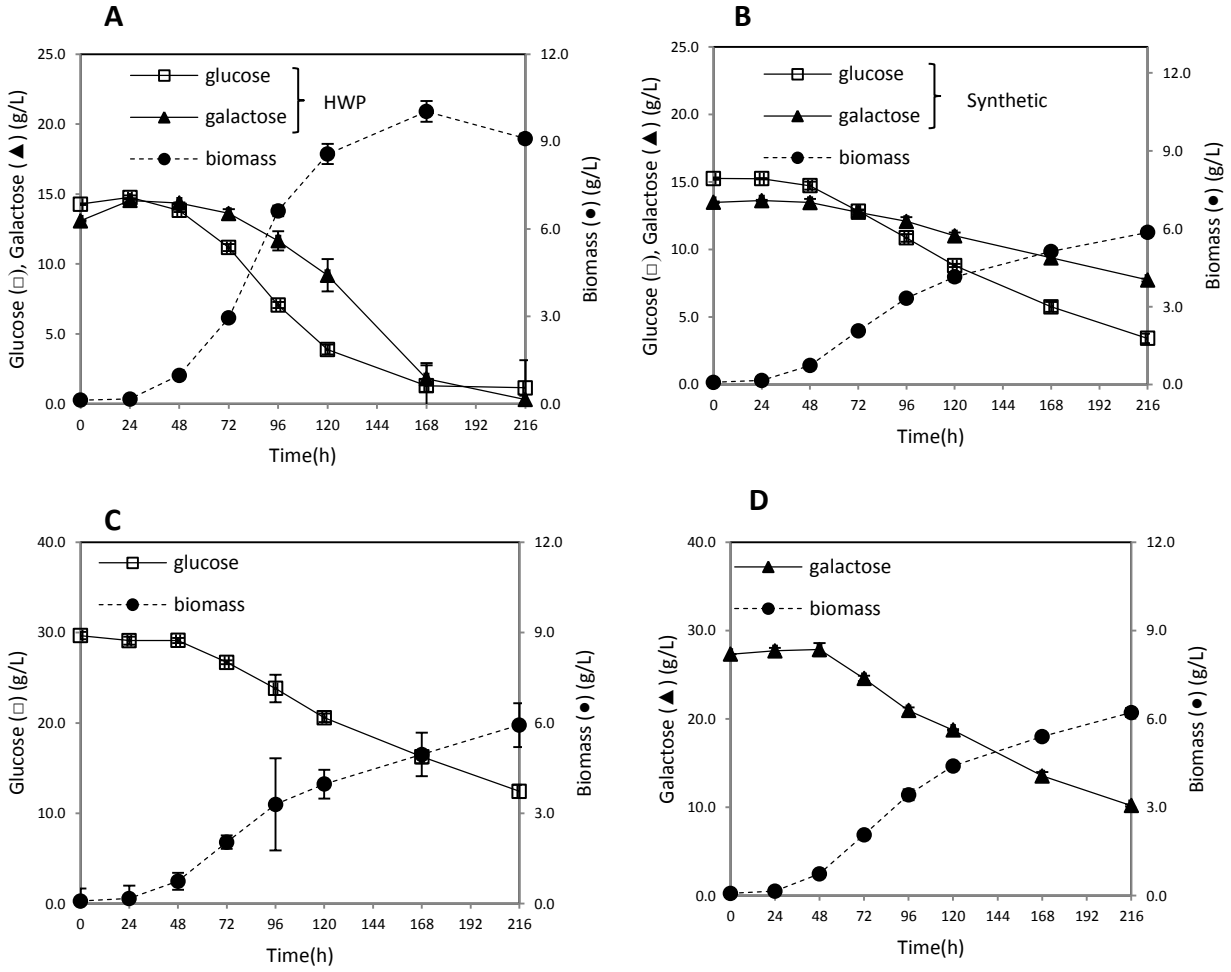


Figure 33. Sugar consumption and growth of *C. protothecoides* in batch cultures for 240 h using different substrates as main carbon sources at a final concentration of 30 g/L monomeric sugar (1 C mol/L) and 1.2 g/L yeast extract ($1E^{-2}$ mol/L) with a C:N ratios of 100:1. A) HWP; B) Glucose and galactose mixture; C) Glucose; D) Galactose. Results express the means of replicate experiments (n=3).

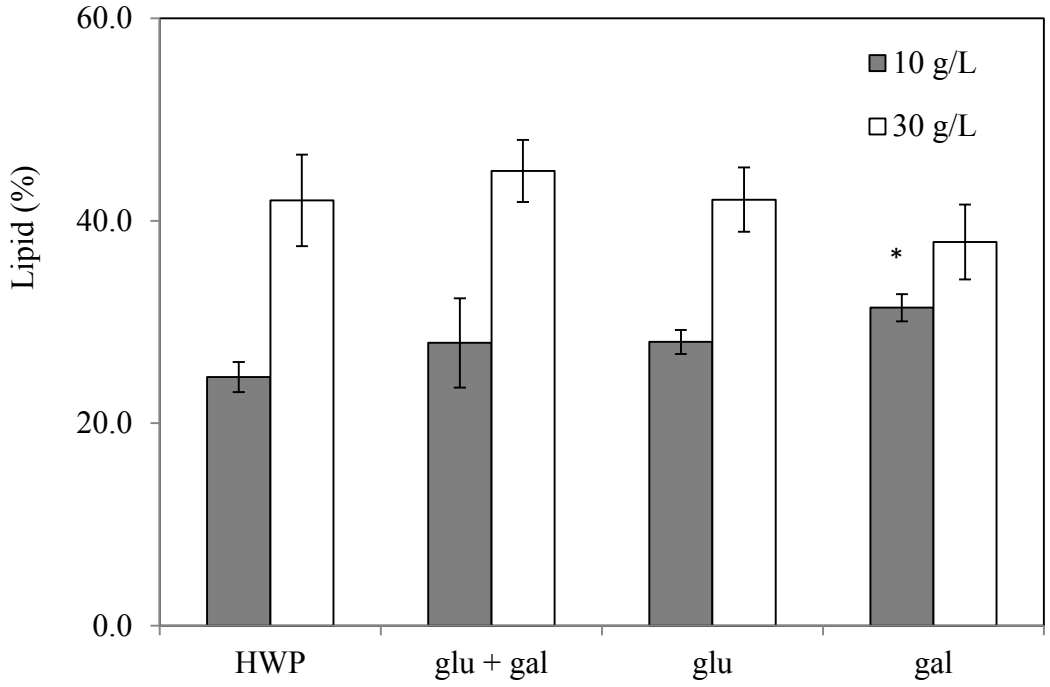


Figure 34. Lipid content of *C. protothecoides* at 216 h grown with different substrates at final concentrations of 10 and 30 g/L monomeric sugar and 1 or 1.2 g/L yeast extract, respectively. Hydrolyzed whey permeate,HWP; Glucose and galactose mixture, glu + gal; Glucose, glu; galactose, gal. Samples displaying significantly (α=0.05) different lipid compared to HWP are indicated with an asterisk (*). Results express the means of replicate experiments (n=3).

3.3. Fatty acid composition

The fatty acid composition of *C. protothecoides* grown using various feedstocks was analyzed to reveal any significant differences. The amount of fatty acids in the freeze-dried algal biomass was normalized to determine their composition relative to the total. The fatty acid profile of *C. protothecoides* grown in HWP revealed oleic (54.5%), linoleic (21.8%), and stearic (13.5%) acids, along with lower amounts of palmitic, myristic, linolenic, palmitoleic, and arachidic acids (Figure 35). The fatty acid composition of *C. protothecoides* grown in HWP was both qualitatively and quantitatively invariant compared to the other cultures grown in glucose, galactose or a mixture of both (α=0.05). It has been previously reported that the composition of

algal lipids can be altered by changing various physical conditions during culturing, including the feedstock [78, 297]. From these results, whey permeate may be an attractive alternative feedstock that produces a lipid profile identical to that obtained with a pure glucose based system.

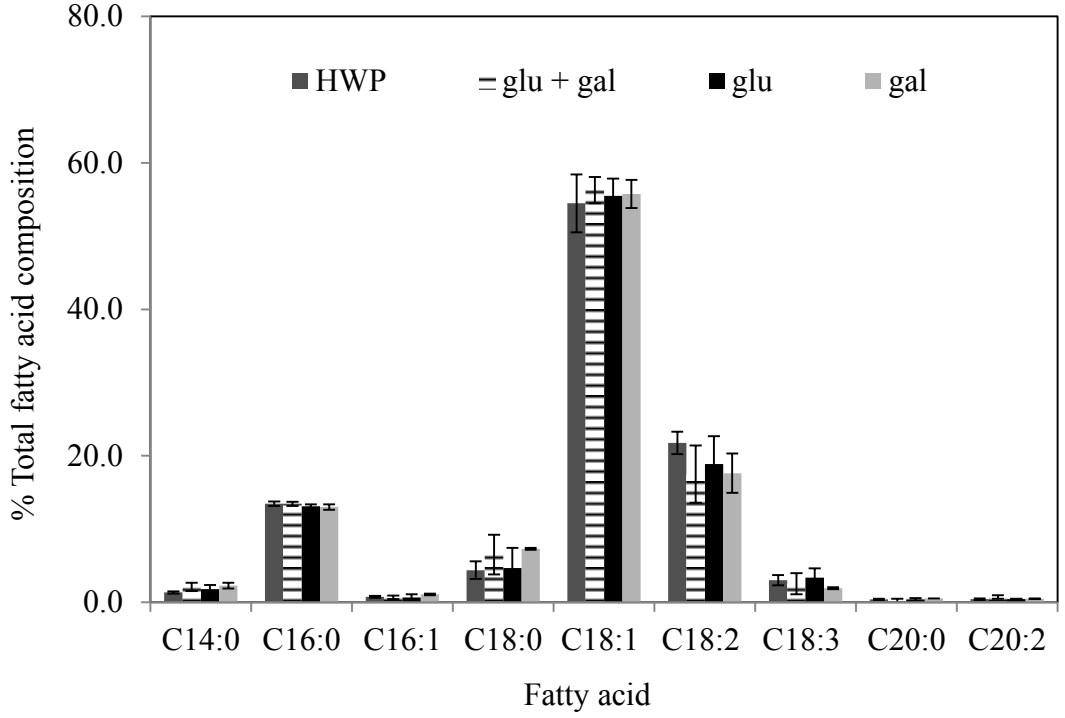


Figure 2. Fatty acid profile of *C. protothecoides* grown using different substrates containing 30 g/L monomeric sugar and supplementation of 1.2 g/L yeast extract. Results express the means of replicate experiments (n=3).

3.4. Fed-batch culture and scale up

To further increase biomass and lipid production, fed-batch cultures were established using HWP or a control mixture of glucose and galactose. After depletion of the initial carbon (72 h), HWP (5.2 g/L glucose, 4.6 g/L galactose) and yeast extract (0.5 g/L) were added. Subsequent additions of HWP (2.3 g/L glucose, 2.0 g/L galactose) with 0.5 g/L yeast extract occurred at 96, 120, and 168 h for a total of 32.3 g/L glucose and galactose from HWP; a total of 34.9 g/L of

monomeric sugar was used in the control. No contamination was observed in cultures during the entire growth process. Biomass after 240 h of culturing was 8.0 ± 0.3 g/L for HWP and 10.9 ± 0.4 g/L for the control (Figure 36A, B) and the percentage of glucose and galactose consumed was 91.1 and 69.2%, respectively, for HWP compared to 96.3 and 81.4%, respectively, for the control. Galactose was not efficiently consumed compared to glucose for HWP and the control, which was in agreement with previous reports [307]. HWP had a higher residual galactose than the control, which might be due to the presence of an external factor in HWP that limits galactose consumption or the formation of aggregates that decrease its utilization. The fed-batch approach using shake flasks did not promote better growth than batch cultures at 30 g/L when HWP was used as the substrate even though improved biomass accumulation was observed in the control.

To overcome limitations associated with fed-batch cultures in shake flasks, the process was scaled up to 5 L bioreactors, in which HWP or glucose (control) was used. Experiments in shake flask (batch 30 g/L) showed that there is no significant difference in growth, lipid accumulation and fatty acid profile with the use of glucose/galactose mixture compared to glucose alone. Based on these results, the glucose/galactose mixture was not included for experiments using the 5 L bioreactor. Additionally, use of glucose alone would allow comparison of results to glucose benchmark fermentations reported in the literature. HWP or glucose was fed into the cultures as early as 48 h to avoid total depletion of the carbon substrate, and yeast extract was added only at 48 h at a concentration of 2 g/L. Feeding of HWP was done at 48, 72, 84, 108, 120, 144, 156, 168, and 192 h for a total volume of 1.1 L HWP (105.9 g glucose, 93.9 g galactose). Feeding of pure glucose was done at 48, 72, 78, 84, 108, 120, 144, 156, 168, 192, and 216 h for a total of 250 g of glucose. No contamination was observed in cultures during the entire growth period

until harvest. There was a greater than two fold increase in biomass relative to the shake flasks: 17.2 ±1.3 g/L for HWP and 23.1 ± 0.7 g/L for the control (Figure 36C, D). This increase was proportional to the substrate added. Lipid accumulation was less for HWP (20.5 ± 0.4%) compared to the glucose control (47.0 ± 3.7%) likely due to the continued supply of nitrogen from HWP feed during fermentation. Using HWP, the fed-batch strategy may be more beneficial for producing biomass rather than increasing lipid content of *C. protothecoides* since nitrogen depletion is essential for promoting lipid accumulation [23, 165, 315]. However, optimization of lipid production using HWP may also be achieved by lowering the amount of yeast extract added to the culture. The differences in the trend of growth promoted by HPW between batch and fed-batch cultures could be due to the accumulation of an inhibitory compound(s) from the WP added by the constant feedings. The carbon source concentrations used in batch and fed-batch fermentations (10-30 g/L) have been previously reported to prevent substrate (glucose) inhibition [244, 314].

The growth rate of the microalgae in this study is not as high as some of those reported in the literature since other variables such as temperature, micronutrients, salinity, and pH influence growth [275, 316-319]. It is important to note that some reported high cell density cultures of *C. protothecoides* have been cultivated using undefined "*Chlorella* growth factors" and hormones, which enhance growth rates but negatively impact the environment and cost-effectiveness [244]. Furthermore, our results suggest that the C: N ratio is critical for lipid accumulation, which is also influenced by the variables described above [275, 316-319].

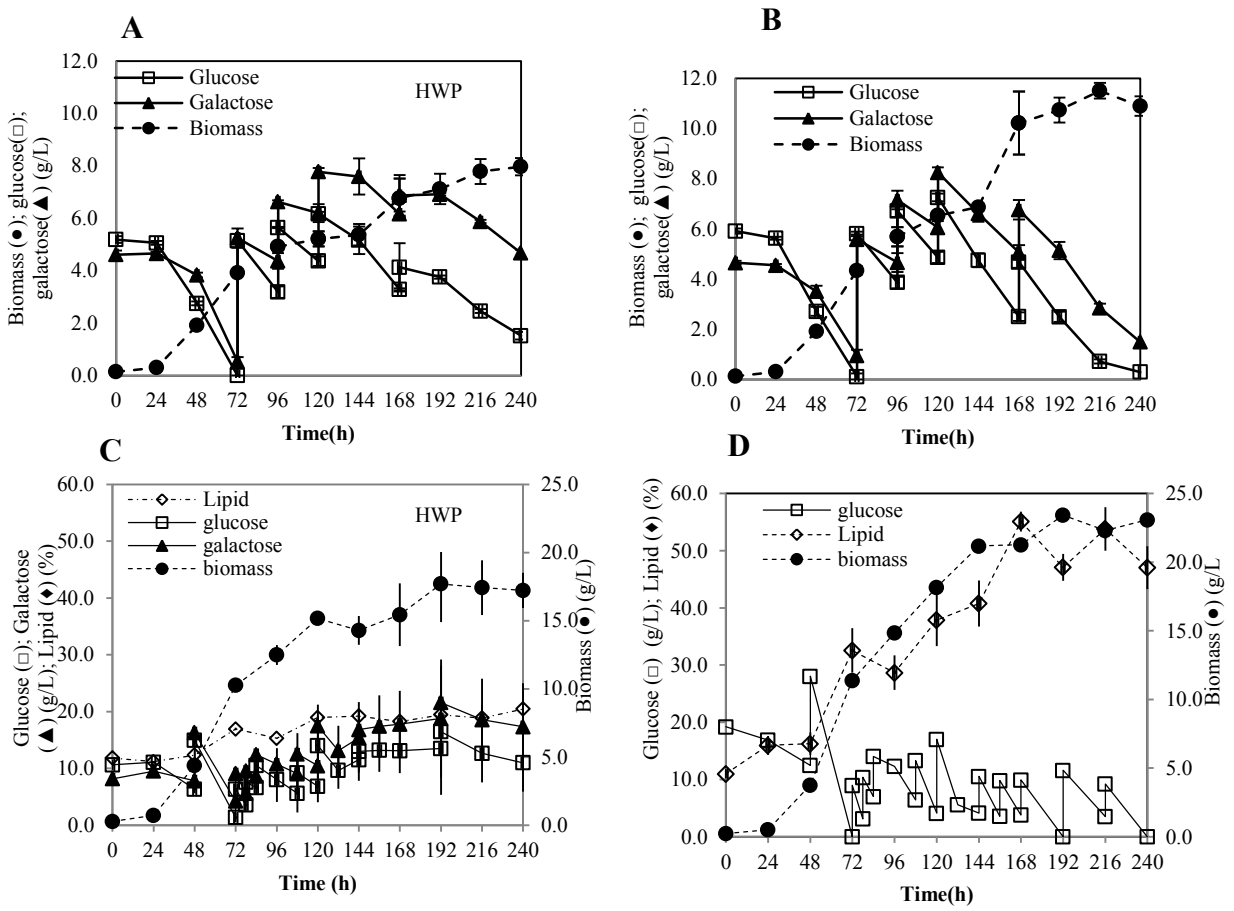


Figure 36. Biomass production and substrate consumption of *C. protothecoides* in fed-batch cultures grown for 240 h in 100 mL shake flasks and a 5 L reactor with different substrates. A) Shake flask, HWP (initially 5.19 g/L glucose, 4.61 g/L galactose, 0.5 g/L yeast extract); B) Shake flask, glucose and galactose mixture (equivalent to HWP); C) 5 L bioreactor, HWP (10 g/L glucose, 10 g/L galactose, 4 g/L yeast extract); D) 5 L bioreactor, glucose. Results express the means of replicate experiments (n=3).

3.5. SSF using non-hydrolyzed WP

An SSF strategy was employed to grow *C. protothecoides* in order to investigate the potential for the direct use of WP, eliminating pre-hydrolysis and using immobilized enzymes. This strategy involved enzymatic saccharification of lactose in the WP into glucose and galactose monomers, and parallel fermentation of these monomers by the growing algal culture. Based on

previous experiments (data not shown), β-galactosidase from *A. oryzae* was chosen for optimal release of sugars during algal growth. Enzymatic activity in the original re-suspended enzyme preparation was 5 U/mg. Considering the importance of reusability for the cost effectiveness of the process, enzyme immobilization (5% w/w) was achieved using PVA and PEG. The enzymatic activity of the immobilized beads was determined to be 3 U/mg solid beads. A series of experiments done with non-hydrolyzed whey permeate (20-40 g/L lactose) and immobilized enzymes showed that an initial lactose concentration of 25 g/L with yeast extract (0.25 g/L) added at 0, 48, 72, and 96 h resulted in significant biomass and lipid accumulation (data not shown). Complete saccharification of lactose was achieved and the monomers were consumed as expected, resulting in a biomass of 7.3 ±1.3 g/L and lipid accumulation of 49.9 ± 3.3% on a dry weight basis (Figure 37). No contamination was observed in cultures.

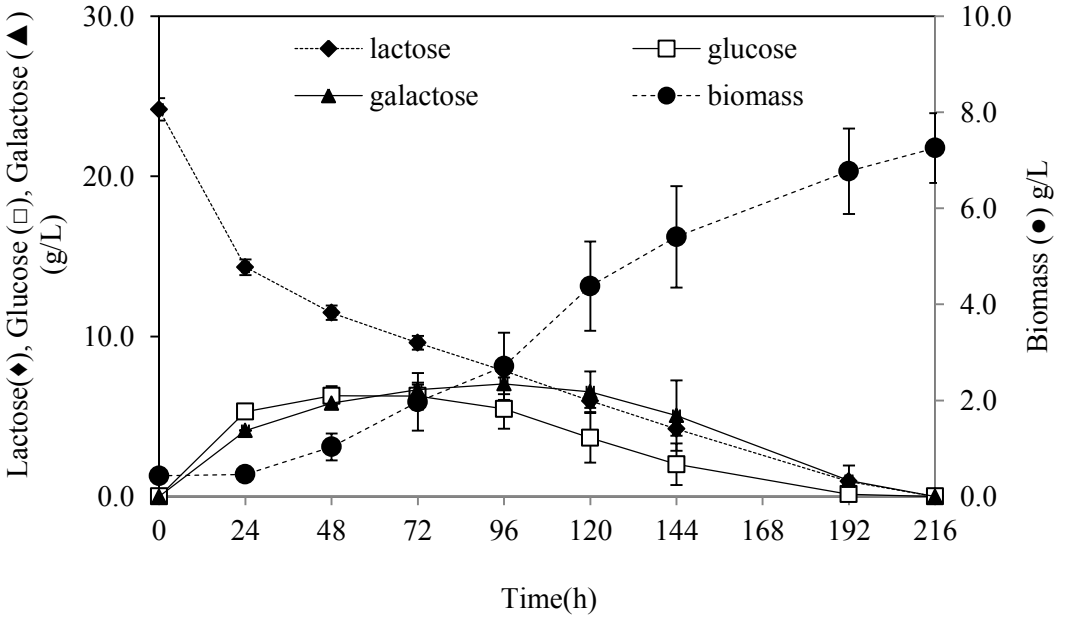


Figure 37. Biomass production and substrate consumption of *C. protothecoides* in SSF mode grown for 216 h in 100 mL shake flasks with non-hydrolyzed WP (final 25 g/L lactose), yeast extract (0.25 g/L initial, 2g/L feed), and 3 U of encapsulated β-galactosidase activity. Results express the means of replicate experiments (n=3).

Although whey or whey derivatives have been used for the production of industrially important products [311], its global abundance indicates that there is scope for alternative strategies as the one proposed in this work. The world trade of whey and whey products for 2009 was estimated at 1.2 million tons, of which, the major product was whey powder [320]. In the USA, 190,000 tons of whey protein was produced in 2009, which corresponds to an estimated 1.5 million tons of available lactose, mostly in the form of whey permeate [321]. The versatility of *C. protothecoides* to undergo heterotrophic growth and accumulate lipids by using different complex feedstocks has been previously demonstrated, however in all cases, the feedstock used was not a waste stream and was more expensive than whey permeate. Biomass concentration and lipid content in batch cultures using pre-hydrolyzed sweet sorghum (3.3 g/L, 52.3%)[78], cassava starch (4.3 g/L, 50.2%)[74] and corn powder (4.7g/L, 39.9%)[75] are comparable to those obtained in the present study (9.1 g/L, 42%: Figure 33 and Figure 34). In fed-batch cultures, biomass concentration and lipid content using pre-hydrolyzed sugar cane (121.3 g/L, 45%)[74], cassava starch (53.6 g/L, 53%)[75], and molasses (97.1 g/L, 57%)[76], are higher than those obtained in the present study (17.2 g/L, 20.5%). It should be noted that the feeding amounts used for the fed-batch cultures reported in this thesis were not optimized. No algorithms or models were applied to calculate the amount of substrates or rate of addition. Instead, empirical calculations based on consumption of substrate at regular intervals were used to maintain the reactor at the pre-established range of substrate concentration. Further optimization can be conducted in this area to obtain improvements in the overall fed-batch performance.

There are not many reports regarding the use of SSF for microalgal growth though a high biomass concentration (49.3 g/L) and lipid content (54.5%) were reported using SSF of cassava starch; however, this technique required continuous addition of enzyme [75]. In this study,

application of SSF resulted in 7.3 g/L biomass with 49.9% lipids with no residual lactose, glucose, or galactose and thus a maximal feedstock utilization. Moreover, this system offers the advantage of the potential reusability of the immobilized enzyme.

4. Conclusion

Whey permeate was found to be a promising waste stream that can be integrated in the heterotrophic cultivation of *C. protothecoides*. Batch and fed-batch strategies using HWP were implemented for the growth and lipid accumulation of *C. protothecoides*. The lipid accumulation and the fatty acid profile of the algal biomass grown with HWP as feedstock were comparable to those obtained using pure glucose in batch cultures at 30 g/L. The fed-batch strategy using HWP did not favour either biomass or lipid accumulation to the same extent as the glucose control. Further investigation is required towards optimization of this strategy to improve biomass production and lipid accumulation. It was also demonstrated that WP can be directly used for algal cultivation in a SSF strategy with immobilized enzymes that hydrolyzed unusable lactose into its constituent monomers (glucose and galactose) as they were consumed by the microalgae. The results from this study suggest that WP can be readily integrated into algal cultivation technologies, especially for biofuel production. Furthermore, this work has demonstrated a value added alternative for the utilization of a dairy byproduct stream that is produced in significantly large quantities, but has limited applications.

V. General discussion and conclusions

1. Final discussion

The production and processing of microbial lipids are two of the most critical aspects that need to be addressed with different strategies in order to advance the field of renewable fuels. A previously patented LTH technology produces renewable hydrocarbons through hydrolysis of lipids followed by thermal cracking of fatty acids. The research in this thesis was devoted to study the use of high-lipid content biomass as a feedstock for the LTH process. In addition, several waste streams, including those generated during LTH bioprocessing and other industries were also investigated as nutrient sources for the growth and lipid accumulation of algal and yeast oleaginous microorganisms.

The undertaking of this research required the production of high lipid content microbial biomasses. Two different model organisms, the microalgae *C. protothecoides* and the yeast *C. curvatus*, were chosen to evaluate the use of oleaginous microbial biomass for the LTH conversion. These strains are among the most studied high-lipid content organisms in the available literature. However, it should be noted that the selection of the most appropriate microorganism for biofuel production depends on the specific application or conversion intended. For instance, the selection criteria must prioritize the desired characteristics, the most important of which are generally the ability of the microorganism to: 1) grow quickly (high biomass productivity), 2) produce valuable byproducts, and 3) produce the lipids required (qualitatively and quantitatively) [322]. Another characteristic for strain selection is oil productivity per area, which is relevant when the surface utilized for cultivation has to be maximized [23]. The two microorganisms selected in this thesis are benchmark strains that accumulate high lipid amounts. Genetically modified microorganisms were not considered in this

thesis as their industrial applications are limited due to present and/or future restrictions or regulations [115].

Different microbial cultivation scales (microplate, flask, 5L and 10 L reactors) and various strategies (batch, fed-batch, and SSF) were established in this work depending on the purpose of the study. The large amounts of algae and yeast required for the experiments described in chapters II were produced using fed-batch cultivation in 5L and 10L bioreactors, which is a technique that is successful in increasing cell density of cultures. Glucose, which is the preferred carbon source for both microorganisms, was used as feedstock for these bioreactor fermentations. However, other carbon sources were also studied including pure and crude glycerol (chapter III), and HWP (chapter IV).

The performance (growth, lipid accumulation, and yields) of the two oleaginous microorganisms in the experiments conducted in this thesis is summarized in Table 14. The lipid contents of the microalgae *C. protothecoides* and the yeast *C. curvatus* ranged between 21 to 50% and from 21 to 59% (dry basis), respectively (Table 14). These values confirm the previously reported lipid accumulation capacity of these two organisms [30,75, 244, 245] and correspond to the expected accumulation amounts (at least 20%, but up to 70%) [51] of most oleaginous microorganisms. Since the calculated lipid productivities for both microorganisms (Table 14) are all below the maximum theoretical conversion (0.22 for glucose to oil [27]), the culture conditions may be further modified to decrease lipid free biomass and to increase oil accumulation. The total lipid content of *C. protothecoides* and *C. curvatus* grown in glycerol were lower than their respective cultures in glucose. Since this is not a desired attribute for our purposes, further optimization studies are required.

It should be noted that the cultivation of both oleaginous organisms (in all chapters) was focused on the production of large amounts of lipids. Thus, some of the potential high value-added metabolites, such as light-induced pigments of microalgae, enzymes, or polysaccharides, were disregarded even though their simultaneous exploitation might increase profitability compared to the exclusive production of lipids [27]. Microbial cultures were intentionally directed towards lipid production, as this was the desired trait for use in the LTH applications.

The overall fatty acid distribution in *C. protothecoides* and *C. curvatus* grown in different conditions was composed of more unsaturated (oleic, linoleic, and linolenic acids) than saturated (palmitic and stearic) acids. The relative amounts of each fatty acid in both microbial oil profiles displayed the same trend of change when glycerol was used instead of glucose (palmitic, linolenic, and linoleic acids increased and a decrease in stearic and oleic acids; Figure 38).

In chapter II, slurries of the oleaginous microorganisms *C. protothecoides* and *C. curvatus* were hydrolyzed under subcritical conditions as established for the initial stage of the LTH conversion. The microbial cultures were minimally processed (centrifugation was used at laboratory scale) to obtain slurries with a moisture content close to 70% (sections 2.2.1 and 3.2.2.2., chapter II), sufficient for conducting hydrolysis reactions. The significance of this observation is related to the minimization of dewatering of microbial biomass, which is required for the production of typical biodiesel [244]. Ideally, higher cell density cultures could be directly hydrolyzed without further separation, although other cell recovery methods (i.e. sedimentation or filtration) can be also used as alternatives to centrifugation in an industrial setting.

Table 14. Performance of oleaginous microorganisms in this research.

	Substrate	Initial substrate (g/L)	Cultivation mode	*Y (b/s)*	*Y (l/s)*	*mBP* (g/L.h)	Biomass g/L	Lipid (% dwb)	T (h)
C. protothecoides	Glucose		5L reactor (fed-batch)	0.25	0.12	0.22	23	47	240
	Glucose		10L reactor (fed-batch)	0.27	0.11	0.33	32	40	268
	HWP		5L reactor (fed-batch)	0.21	0.05	0.20	17	21	240
	HWP	10 (m)	Flask (batch)	0.41	0.10	0.06	4	25	216
	HWP	30 (m)	Flask (batch)	0.35	0.15	0.12	9	42	216
	WP	25	Flask (SSF)	0.30	0.15	0.04	7	50	216
	Glycerol	20	Flask (batch)	0.49	0.16	0.03	3	32	264
	Glycerol + H	20	Flask (batch)	0.49	0.19	0.05	6	39	264
C. curvatus	Glucose		5L reactor (fed-batch)	0.22	0.12	0.38	30	53	192
	Glucose	30 (m)	Flask (batch)	0.35	0.21	0.20	10	59	72
	Glycerol	20	Flask (batch)	0.52	0.13	0.07	7	21	168
	Glycerol + H	20	Flask (batch)	0.48	0.10	0.09	10	21	168

m, monomer; H, hydrolysate; Biomass yield ($Y_{b/s}$) = Biomass (g)/Utilized substrate (initial – residual) (g); Lipid yield ($Y_{l/s}$) = Lipid (g)/Utilized substrate (initial – residual) (g); Maximum biomass productivity (*mBP*; selecting 2 points between exponential phase) = (Δ Biomass) /(Δ Time).

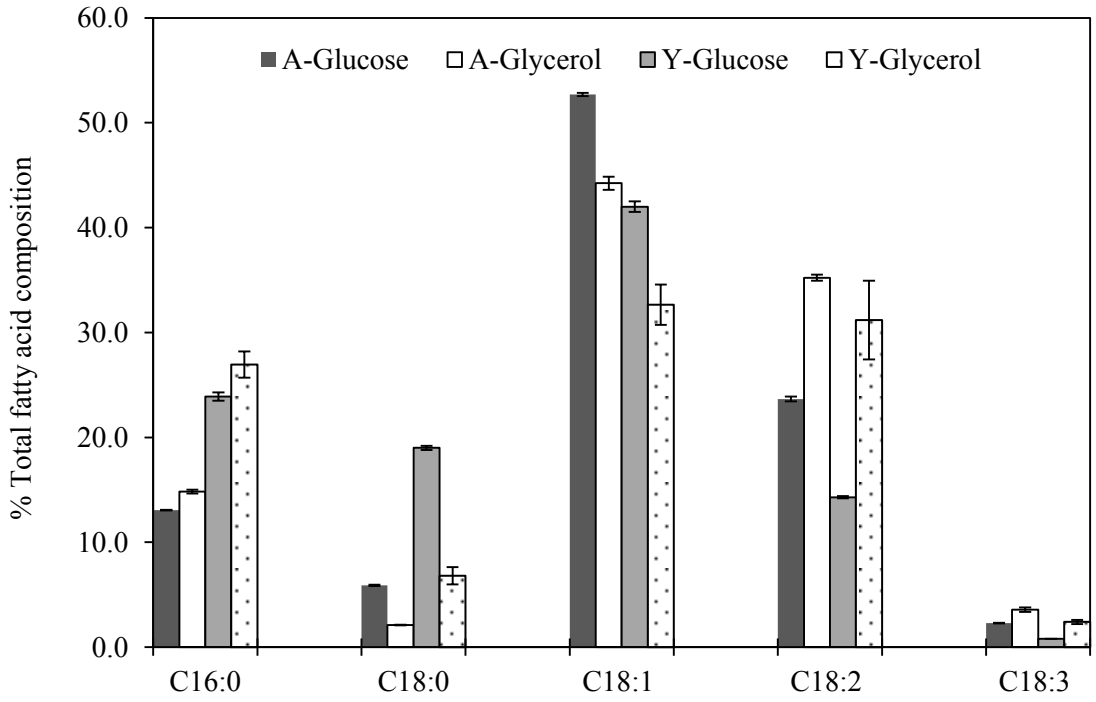


Figure 38. Fatty acid profile in oleaginous organisms. A-Algae (*C. protothecoides)* grown in glucose (dark bars) or glycerol (white bars), Y-yeast *C. curvatus* grown in glucose (grey bars) or glycerol (dotted bars).

Free fatty acids were recovered from hydrolysis products using a one-step hexane extraction. The use of hexane in this step might raise some concerns since industrial processes are expected to minimize environmental footprints by reducing or substituting hazardous toxic compounds (such as hexane) involved in manufacturing [323]. In this process, however, hexane utilization can be optimized through its efficient recirculation, which would minimize the amount of hexane waste.

Using hydrolysis as a preprocessing technique facilitated cell disruption (for lipid release) and lipid hydrolysis in a single step. These findings support earlier work of hydrothermal liquefaction of algal biomass [216, 222-227] and add an evaluation of the use of yeast and algal

oleaginous microorganisms for lipid hydrolysis, which has not been previously described. The fatty acids recovered from the hydrothermal treatment of algal biomass were compatible as drop in feedstocks for the second stage (pyrolysis) of LTH (as demonstrated in section 3.5, Chapter II). Thus, the technical feasibility of using oleaginous biomasses as lipid sources to produce biofuels was demonstrated in this thesis. These findings contribute to the area of developing alternative methods for production of biofuels using microbial biomass, which has received limited attention in the available literature.

Other alternative lipid extraction methods applied to biomass (particularly algae) commonly produce spent biomass that can be directly used as feed supplements or can serve as feedstock for biochemical (anaerobic digestion) [188] or thermochemical (pyrolysis) [205] processes. Conversely, the hydrothermal pretreatment of microbial biomass, which degraded proteins, lipids, and carbohydrates, used in this work generated an insoluble residue and an aqueous byproduct stream; the latter was valorized in chapter III.

In chapters III and IV, alternative nutrients from industrial waste streams were integrated in the culture of two oleaginous microorganisms (algae and yeast). Aqueous streams of hydrothermally processed oleaginous biomasses (chapter II) were used for nutrient supplementation of microalgal and yeast cultures. Recycling of aqueous streams from hydrothermal liquefaction of algae in algal cultivation confirms previous findings [125-131,133,135]. This study is unique in a sense that it as it examines heterotrophic systems for lipid accumulation purposes, which has not been previously reported. Recycling of nutrients proposed in this thesis, with regards to both approach and scope, is also novel for oleaginous yeast. Oleaginous yeasts, in comparison with algae, have not been typically seen as microorganisms that could tolerate potential toxic compounds from industrial streams. This study

proves that the oleaginous yeast *C. curvatus* in batch cultures not only survived, but actually thrived when exposed to recycled nutrients contained in aqueous hydrothermal streams derived from *C. curvatus* or *C. protothecoides* bioprocessing (section 3.4.2.2 Chapter III). Batch cultures of algae and yeast supplemented with certain byproduct microbial hydrolysates displayed: 1) an improvement in microbial growth; 2) favorable lipid accumulation; and 3) minimal changes in microbial fatty acid profiles (sections 3.4.2.1. to 3.4.2.4, Chapter III).

In chapter III, it was demonstrated that crude glycerol (from the aqueous byproduct streams of hydrothermally processed model fats and oils) could serve as the sole carbon source for both microalgae and yeast. The use of crude glycerol in algal cultivation was beneficial for growth and lipid accumulation compared to the use of pure glycerol (section 3.2.1., Chapter III). This observation confirms algal utilization of non-pure glycerol [252]. Yeast, on the other hand, grew and accumulated lipids in crude glycerol to the same extent as pure glycerol (section 3.2.2., Chapter III). Nevertheless, our results show that the byproduct aqueous streams from hydrolysis of fats and oils can be used to provide a carbon source to growth media used for microbial cultivation.

In chapter IV, whey permeate (WP), a dairy industry waste stream that contains lactose, was used for producing high lipid content *C. protothecoides* in three different cultivation modes. The use of WP for the growth and lipid accumulation of the yeast *C. curvatus* was not investigated as this process has been previously reported [324]. Use of an SSF strategy allowed the simultaneous hydrolysis of lactose and microbial consumption of the resulting glucose and galactose to promote growth and lipid accumulation (section 3.5, chapter IV). Previously hydrolyzed WP was also used for the batch and fed-batch cultivations of *C. protothecoides*. Although the fed-batch strategy underperformed compared to glucose controls (section 3.4, chapter IV), batch cultures

with 30 g/L sugar concentration had an improved biomass production with similar lipid accumulation, when compared to glucose cultures (section 3.2, Chapter IV). Overall, these results demonstrated a value added alternative for the utilization of an industrial byproduct stream with limited applications. At the same time, the feasibility of producing microbial oils using a cheap carbon source was improved.

The research aims initially proposed in this thesis were achieved. LTH pre-processing was shown to release fatty acids from biomasses of oleaginous microorganisms, which could be subsequently recovered via a simple hexane extraction and subjected to pyrolysis to produce a clean biofuel. Furthermore, byproduct streams from LTH processing (fats, oils, and oleaginous microorganism biomass) and the dairy sector were integrated into recycling programs to cultivate oleaginous microorganisms that could be used for biofuels production. The data obtained in this thesis can be applied to hypothetical microbial production and processing scenarios as explained in Figure 39.

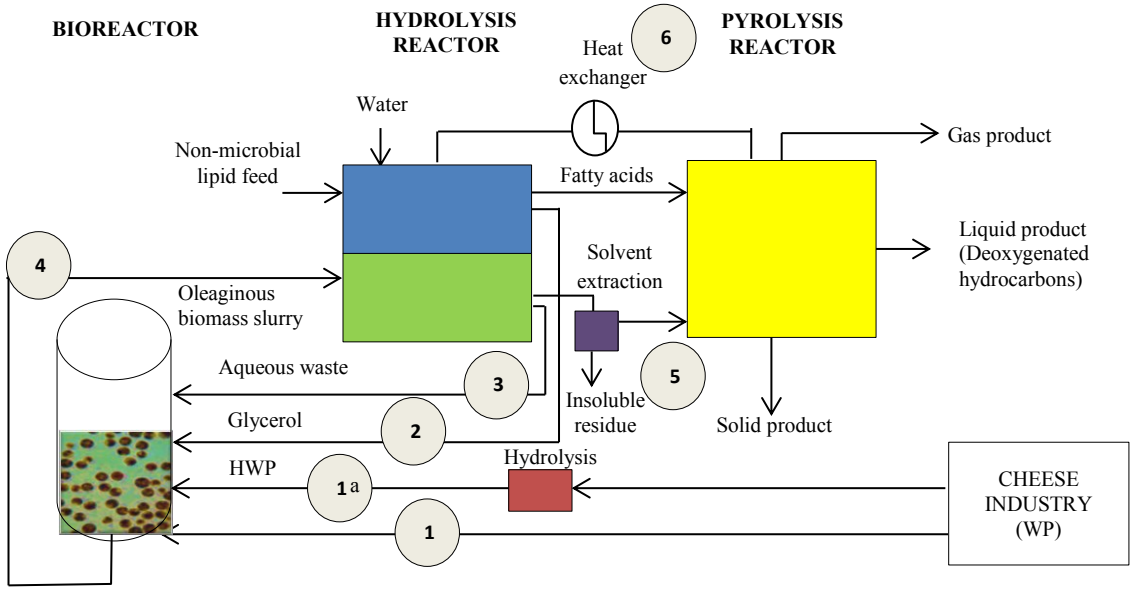


Figure 3. Integration of the various strategies developed in this thesis.

Microbial production can be established by optimizing use of different combinations of various nutrients derived from waste streams from: 1) the cheese industry (WP; implementing SSF with immobilized enzymes for microalgae), 1a) the cheese industry (WP) after lactose hydrolysis (HWP; only for microalgae), 2) LTH preprocessing of various fats and oils (a crude glycerol source), and 3) LTH preprocessing of oleaginous microbial biomass. Resulting microbial slurries of high cell density can be introduced to the hydrolysis reactor (4). After solvent extraction, microbial fatty acids (5) can enter in the pyrolysis reactor to produce hydrocarbons. A heat exchanger between pyrolysis and hydrolysis (6) supplies energy for the hydrothermal treatment.

2. Recommendations for future research

2.1. Integrate and optimize the use of waste streams for the production of oleaginous microorganisms

Although alternative carbon and nutrient sources were individually evaluated for cultivation of oleaginous microorganisms, the use of more than one waste stream was not explored. Further studies might include the optimization of oleaginous microorganism cultivation in which waste streams from various sources (whey permeate, crude glycerol, and microbial hydrolysates) are used. In doing this, it may be possible to cultivate oleaginous organisms using only recycled nutrient streams, which would be a huge financial benefit to the biofuels sector. Optimized cultures might also be scaled up to produce sufficient amounts of biomass for large scale LTH preprocessing.

2.2. Establish alternative culture strategies to reduce energy requirements

Since the use of typical bioreactors for high density cultivation of oleaginous microorganisms represent an energy-intensive practice, a cost analysis may be conducted to evaluate if the price of the final biofuel is greater than its production costs. Other cultivation modes or bioreactor configurations could also be explored (i.e. airlift or bubble columns reactors).

2.3. Co-culture of algae or yeast with other species

Since the effects of co-culturing algae and other microorganisms for lipid accumulation have not been widely explored, further research may include the study of the interactions between oleaginous microorganisms. Co-cultures might be established using different algal strains or algal strains with yeast or bacteria.

2.4. Novel strategies to control contamination

The control of contamination in microbial cultivation represents a challenge for large-scale production. A novel strategy that can be studied to combat contamination in algal cultures is the use of allelophatic compounds. Some of these naturally occurring chemicals that affect contaminant organisms can be explored by triggering natural algal excretion or by applying them as supplements during cultivation [107]. The potential of this approach could be assessed in algae produced under autotrophic, mixotrophic, and heterotrophic conditions. The same principle can be studied for specific compounds (such as bacteriocins) excreted by oil-accumulating yeasts that target prevalent contaminating organisms.

2.5. Evaluate and assess aqueous stream toxicity

Limited information is available on the characterization and composition of potentially toxic organic compounds in wastewaters from hydrothermal processing of microbial biomass. An

alternative technique for analysis and characterization of aqueous products from hydrothermal treatment of algal biomass was recently published [325]. In this report, Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR MS) with both positive- and negative-ion electrospray ionization was utilized to identify almost three thousands peaks in the algal aqueous phase analyzed (positive and negative modes) [325]. These data were organized in 3D mass spectral images to group components by carbon number and aromatic nature. However, this method can provide more detailed information about the specific composition of these mixtures [325]. A detailed characterization of these streams may help evaluate the toxicity and the environmental impact of microbial hydrothermal hydrolysates [131].

2.6. Recycling of microbial spent culture media

This thesis addressed the recycling of nutrients from different waste streams in microbial cultivation. However, the culture media that remains after the separation of the microbial slurry at the end of the fermentation may also contain various unused nutrients. Therefore, research for recycling microbial spent culture media is another approach that can contribute to efficiently utilize resources.

2.7. Investigate properties and potential applications of the insoluble residue

Complete characterization of insoluble residues derived from hydrothermal treatment of oleaginous biomasses is needed to find potential value-added applications. Solid residues of defatted algae have been used for anaerobic digestion (biogas), animal feed, fermentation, and hydrothermal gasification [122]. However, considering the nature of the insoluble residue after hydrothermal treatment (i.e. high in minerals but also containing N and P), this residue may be used as fertilizer. For instance, the agronomic potential of the biochar generated through slow pyrolysis (500°C for 20 min) of the marine microalgae *Tetraselmis chui* has been reported [326].

To confirm this possible application, proximate, ultimate, detailed ash composition, trace elements analysis, intrusion studies (porosity and density), and field experimental data are required.

2.8. Conduct a Life Cycle Assessment (LCA) and economic analysis of the process

A detailed LCA of the LTH technology using oleaginous organisms, such as algae and yeast, would provide an accurate estimation of the viability and environmental sustainability of such methods. An economic evaluation would also establish the economic feasibility of each step of the LTH process as well as the overall costs of inputs and processing.

References

1. Hill, J., *et al*., Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proceedings of the National Academy of Sciences of the United States of America, 2006. 103(30): p. 11206-11210.

2. Lynd, L.R., C.E. Wyman, and T.U. Gerngross, Biocommodity engineering. Biotechnology Progress, 1999. 15(5): p. 777-793.

3. Demirbas, M.F., Biorefineries for biofuel upgrading: A critical review. Applied Energy, 2009. 86: p. S151-S161.

4. Demirbas, A., Combustion of biomass. Energy Sources Part a-Recovery Utilization and Environmental Effects, 2007. 29(6): p. 549-561.

5. Taylor, G., Biofuels and the biorefinery concept. Energy Policy, 2008. 36(12): p. 4406-4409.

6. Demirbas, A., Biorefineries: Current activities and future developments. Energy Conversion and Management, 2009. 50(11): p. 2782-2801.

7. FitzPatrick, M., *et al*., A biorefinery processing perspective: Treatment of lignocellulosic materials for the production of value-added products. Bioresource Technology, 2010. 101(23): p. 8915-8922.

8. Fernando, S., *et al*., Biorefineries: Current status, challenges, and future direction. Energy & Fuels, 2006. 20(4): p. 1727-1737.

9. Octave, S. and D. Thomas, Biorefinery: Toward an industrial metabolism. Biochimie, 2009. 91(6): p. 659-664.

10. Kamm, B. and M. Kamm, Principles of biorefineries. Applied Microbiology and Biotechnology, 2004. 64(2): p. 137-145.

11. Yang, S.T. and M.R. Yu, Integrated biorefinery for sustainable production of fuels, chemicals and polymers, in Bioprocessing Technologies in Biorefinery for Sustainable Production of Fuels, Chemicals, and Polymers, S.T. Yang, H.A. ElEnshasy, and N. Thongchul, Editors. 2013, John Wiley & Sons: Chichester. p. 1-26.

12. Tilman, D., P.B. Reich, and J.M.H. Knops, Biodiversity and ecosystem stability in a decade-long grassland experiment. Nature, 2006. 441(7093): p. 629-632.

13. Subhadra, B. and G. Grinson, Algal biorefinery-based industry: an approach to address fuel and food insecurity for a carbon-smart world. Journal of the Science of Food and Agriculture, 2011. 91(1): p. 2-13.

14. Sun, Y. and J.Y. Cheng, Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology, 2002. 83(1): p. 1-11.

15. Galbe, M. and G. Zacchi, A review of the production of ethanol from softwood. Applied Microbiology and Biotechnology, 2002. 59(6): p. 618-628.

16. Pan, X.J., *et al*., Strategies to enhance the enzymatic hydrolysis of pretreated softwood with high residual lignin content. Applied Biochemistry and Biotechnology, 2005. 121: p. 1069-1079.

17. Jorgensen, H., J.B. Kristensen, and C. Felby, Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. Biofuels Bioproducts & Biorefining-Biofpr, 2007. 1(2): p. 119-134.

18. Wyman, C.E., What is (and is not) vital to advancing cellulosic ethanol. Trends in Biotechnology, 2007. 25(4): p. 153-157.

19. Menon, V. and M. Rao, Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. Progress in Energy and Combustion Science, 2012. 38(4): p. 522-550.

20. Bautista, L.F., G. Vicente, and V. Garre, Biodiesel from microbial oil, in Advances in Biodiesel Production: Processes and Technologies, R. Luque and J.A. Melero, Editors. 2012, Woodhead Publ Ltd: Cambridge. p. 179-203.

21. Chisti, Y., Biodiesel from microalgae. Biotechnology Advances, 2007. 25(3): p. 294.

22. Chisti, Y., Response to Reijnders: Do biofuels from microalgae beat biofuels from terrestrial plants? Trends in Biotechnology, 2008. 26(7): p. 351-352.

23. Rodolfi, L., *et al*., Microalgae for Oil: Strain Selection, Induction of Lipid Synthesis and Outdoor Mass Cultivation in a Low-Cost Photobioreactor. Biotechnology and bioengineering, 2009. 102(1): p. 100.

24. Stephens, E., *et al*., Future prospects of microalgal biofuel production systems. Trends in plant science, 2010. 15(10): p. 554.

25. Xavier Malcata, F., Microalgae and biofuels: A promising partnership? Trends in biotechnology, 2011. 29(11): p. 542.

26. Menetrez, M.Y., An Overview of Algae Biofuel Production and Potential Environmental Impact. Environmental science & technology, 2012. 46(13): p. 7073.

27. Ratledge, C. and Z. Cohen, Microbial and algal oils: Do they have a future for biodiesel or as commodity oils? Lipid Technology, 2008. 20(7): p. 155.

28. Ageitos, J.M., *et al*., Oily yeasts as oleaginous cell factories. Applied Microbiology and Biotechnology, 2011. 90(4): p. 1219.

29. Meesters, P., G.N.M. Huijberts, and G. Eggink, High cell density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. Applied Microbiology and Biotechnology, 1996. 45(5): p. 575.

30. Thiru, M., S. Sankh, and V. Rangaswamy, Process for biodiesel production from *Cryptococcus curvatus*. Bioresource technology, 2011. 102(22): p. 10436.

31. Gualtieri, P. and L. Barsanti, Algae [electronic resource] : anatomy, biochemistry, and biotechnology by Paolo Gualtieri and Laura Barsanti. 2006: Boca Raton : Taylor & Francis, 2006.

32. Rajkumar, R. and Z. Yaakob, The biology of microalgae, in Biotechnological Applications of Microalgae, F. Bux, Editor. 2013, CRC Press. p. 7-16.

33. Cardozo, K.H.M., *et al.*, Metabolites from algae with economical impact. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology, 2007. 146(1-2): p. 60-78.

34. Chisti, Y., Technical Means for Algae Production in Microalgal biotechnology: Potential and production, C. Posten and C. Walter, Editors. 2012, Walter de Gruyter: Berlin.

35. Carvalho, A.P., L.A. Meireles, and F.X. Malcata, Microalgal reactors: A review of enclosed system designs and performances. Biotechnology Progress, 2006. 22(6): p. 1490-1506.

36. Ugwu, C.U., H. Aoyagi, and H. Uchiyama, Photobioreactors for mass cultivation of algae. Bioresource Technology, 2008. 99(10): p. 4021-4028.

37. Behrens, P.W., Photobioreactor and fermentors: the light and the dark sides of the growing algae, in Algal Culturing Techniques, R.A. Andersen, Editor. 2005, Elsevier Academic Press: New York, USA. p. 189-204.

38. Apt, K.E. and P.W. Behrens, Commercial developments in microalgal biotechnology. Journal of Phycology, 1999. 35(2): p. 215-226.

39. Chen, F., High cell density culture of microalgae in heterotrophic growth. Trends in Biotechnology, 1996. 14(11): p. 421-426.

40. Yang, C., Q. Hua, and K. Shimizu, Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. Biochemical Engineering Journal, 2000. 6(2): p. 87-102.

41. Perez-Garcia, O., *et al*., Heterotrophic cultures of microalgae: Metabolism and potential products. Water research, 2011. 45(1): p. 11.

42. Liu, J., Z. Sun, and F. Chen, Heterotrophic Production of Algal Oils. Biofuels from Algae, 2014: p. 111-142.

43. Sansawa, H. and H. Endo, Production of intracellular phytochemicals in *Chlorella* under heterotrophic conditions. Journal of Bioscience and Bioengineering, 2004. 98(6): p. 437-444.

44. Li, X., H. Xu, and Q. Wu, Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotropic Cultivation in bioreactors. Biotechnology and bioengineering, 2007. 98(4): p. 764.

45. Vazhappilly, R. and F. Chen, Eicosapentaenoic acid and docosahexaenoic acid production potential of microalgae and their heterotrophic growth. Journal of the American Oil Chemists Society, 1998. 75(3): p. 393-397.

46. Barclay, W.R., K.M. Meager, and J.R. Abril, Heterotrophic production of long-chain Omega-3-Fatty-Acids utilizing algae and algae-like microorganisms. Journal of Applied Phycology, 1994. 6(2): p. 123-129.

47. Lee, Y.-K., Algal Nutrition, Heterotrophic Carbon Nutrition, in Handbook of Microalgal Culture: Biotechnology and Applied Phycology, A. Richmond, Editor. 2004, Blckwell Publishing. p. 116-124.

48. Lee, Y.K., *et al*., Mixotrophic growth of *Chlorella sorokiniana* in outdoor enclosed photobioreactor. Journal of Applied Phycology, 1996. 8(2): p. 163-169.

49. Haass, D. and W. Tanner, Regulation of hexose transport in *Chlorella vulgaris* - Characteristics of induction and turnover. Plant Physiology, 1974. 53(1): p. 14-20.

50. Neilson, A.H. and R.A. Lewin, The uptake and utilization of organic carbon by algae an essay in comparative biochemistry. Phycologia, 1974. 13(3): p. 227-264.

51. Ratledge, C. and J.P. Wynn, The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Advances in Applied Microbiology, 2002. 51(51): p. 1-51.

52. Khozin-Goldberg, I., U. Iskandarov, and Z. Cohen, LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. Applied Microbiology and Biotechnology, 2011. 91(4): p. 905-915.

53. Harwood, J.L. and I.A. Guschina, The versatility of algae and their lipid metabolism. Biochimie, 2009. 91(6): p. 679-684.

54. Harwood, J.L. and A.L. Jones, Lipid-metabolism in algae. Advances in Botanical Research Incorporating Advances in Plant Pathology, 1989. 16: p. 1-53.

55. Thompson, G.A., Lipids and membrane function in green algae. Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism, 1996. 1302(1): p. 17-45.

56. Guschina, I.A. and J.L. Harwood, Lipids and lipid metabolism in eukaryotic algae. Progress in Lipid Research, 2006. 45(2): p. 160-186.

57. Wen, Z.Y. and F. Chen, Heterotrophic production of eicosapentaenoic acid by microalgae. Biotechnology Advances, 2003. 21(4): p. 273-294.

58. Adl, S.M., *et al*., The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. Journal of Eukaryotic Microbiology, 2005. 52(5): p. 399-451.

59. Keeling, P.J., The endosymbiotic origin, diversification and fate of plastids. Royal Society Philosophical Transactions Biological Sciences, 2010. 365(1541): p. 729-748.

60. Borza, T., C.E. Popescu, and R.W. Lee, Multiple metabolic roles for the nonphotosynthetic plastid of the green alga Prototheca wickerhamii. Eukaryotic Cell, 2005. 4(2): p. 253-261.

61. Kobayashi, T., I. Tanabe, and A. Obayashi, On the Properties of the Starch Granules from Unicellular Green Algae. Agricultural and Biological Chemistry, 1974. 38(5): p. 941-946.

62. Suzuki, E. and R. Suzuki, Variation of Storage Polysaccharides in Phototrophic Microorganisms. Journal of Applied Glycoscience, 2013. 60(1): p. 21-27.

63. Domozych, D.S., Algal Cell Walls, in eLS. 2001, John Wiley & Sons, Ltd.

64. Corre, G., *et al*., Influence of cell wall composition on the resistance of two *Chlorella* species (chlorophyta) to detergents. Journal of Phycology, 1996. 32(4): p. 584-590.

65. Tomaselli, L., The Microalgal Cell, in Handbook of Microalgal Culture: Biotechnology and Applied Phycology, A. Richmond, Editor. 2004, Blackwell Science. p. 3-20.

66. Griffiths, D.J., Microalgal Cell Cycles. Cell Biology Research Progress. 2010, New York: Novinka/Nova Science Publishers.

67. Friedl, T., N. Rybalka, and A. Kryvenda, Phylogeny and systematics of microalgae: An overview, in Microalgal Biotechnology: Potential and Production, C. Walter and C. Posten, Editors. 2012, Berlin: Walter de Gruyter, 2012.

68. Shihira, I. and R.W. Krauss, *Chlorella*. Physiology and taxonomy of forty-one isolates. 1965: Maryland: University of Maryland, College Park.

69. Campenni, L., *et al*., Carotenoid and lipid production by the autotrophic microalga *Chlorella protothecoides* under nutritional, salinity, and luminosity stress conditions. Applied Microbiology and Biotechnology, 2013. 97(3): p. 1383-1393.

70. Wei, D., *et al*., Enhanced production of lutein in heterotrophic *Chlorella protothecoides* by oxidative stress. Science in China Series C-Life Sciences, 2008. 51(12): p. 1088-1093.

71. Running, J.A., D.K. Severson, and K.J. Schneider, Extracellular production of L-ascorbic acid by *Chlorella protothecoides*, Prototheca species, and mutants of P-moriformis during aerobic culturing at low pH. Journal of Industrial Microbiology & Biotechnology, 2002. 29(2): p. 93-98.

72. Brooks, G., *et al*.,. Food product, useful as e.g. brownie, cookie, cake, crackers, pizza crust, breadstick and biscuit, formed by baking mixture of microalgal (*Chlorella protothecoides*) biomass, edible liquid and edible ingredient, 2010. Patent US2010297296-A1.

73. Cheng, Y., *et al*., Alga-Based Biodiesel Production and Optimization Using Sugar Cane as the Feedstock. Energy & Fuels, 2009. 23(8): p. 4166.

74. Lu, Y., *et al*., Biodiesel production from algal oil using cassava (*Manihot esculenta Crantz*) as feedstock. Journal of Applied Phycology, 2010. 22(5): p. 573.

75. Lu, Y., Y. Ding, and Q. Wu, Simultaneous saccharification of cassava starch and fermentation of algae for biodiesel production. Journal of Applied Phycology, 2011. 23(1): p. 115.

76. Yan, D., *et al.*, Waste molasses alone displaces glucose-based medium for microalgal fermentation towards cost-saving biodiesel production. Bioresource technology, 2011. 102(11): p. 6487.

77. Cheng, Y., *et al*., Biodiesel production from Jerusalem artichoke (*Helianthus Tuberosus L.*) tuber by heterotrophic microalgae *Chlorella protothecoides*. Journal of Chemical Technology and Biotechnology, 2009. 84(5): p. 777.

78. Gao, C., *et al*., Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*. Applied Energy, 2010. 87(3): p. 756.

79. Johnson, E.A. and C. Echavarri-Erasun, Yeast Biotechnology, in The yeasts: a taxonomic study, C.P. Kurtzman, J.W. Fell, and T. Boekhout, Editors. 2011, Amsterdam ; Oxford : Elsevier, 2010. p. 21–44.

80. Johnson, E.A., Biotechnology of non-*Saccharomyces* yeasts-the basidiomycetes. Applied Microbiology and Biotechnology, 2013. 97(17): p. 7563-7577.

81. Flores, C.L., *et al*., Carbohydrate and energy-yielding metabolism in non-conventional yeasts. Fems Microbiology Reviews, 2000. 24(4): p. 507-529.

82. Johnson, E.A., Biotechnology of non-*Saccharomyces* yeasts-the ascomycetes. Applied Microbiology and Biotechnology, 2013. 97(2): p. 503-517.

83. Davies, R.J., Scale up of yeast oil technology, in Industrial Applications of Single Cell Oils, D.J. Kyle and C. Ratledge, Editors. 1992, American Oil Chemists' Society: Champaign, IL, USA. p. 196-218.

84. Hassan, M., *et al*., Production of single-cell oil from prickly-pear juice fermentation by *Cryptococcus curvatus* grown in batch culture. World Journal of Microbiology & Biotechnology, 1994. 10(5): p. 534-537.

85. Lages, F. and C. Lucas, Characterization of a glycerol H+ symport in the halotolerant yeast *Pichia sorbitophila*. Yeast, 1995. 11(2): p. 111-119.

86. Lages, F., M. Silva-Graca, and C. Lucas, Active glycerol uptake is a mechanism underlying halotolerance in yeasts: a study of 42 species. Microbiology-Uk, 1999. 145: p. 2577-2585.

87. Gancedo, C., J.M. Gancedo, and A. Sols, Glycerol metabolism in yeasts - Pathways of utilization and production. European Journal of Biochemistry, 1968. 5(2): p. 165-&.

88. May, J.W., J.H. Marshall, and J. Sloan, Glycerol utilization by *Schizosaccharomyces pombe* phosphorilation of dihydroxyacetone by a specific kinase as the 2nd step. Journal of General Microbiology, 1982. 128(AUG): p. 1763-1766.

89. Ratledge, C., Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. Biochimie, 2004. 86(11): p. 807-815.

90. Boekhout, T., *et al*., Discussion of Teleomorphic and Anamorphic Basidiomycetous Yeasts, in The yeasts: a taxonomic study, C.P. Kurtzman, J.W. Fell, and T. Boekhout, Editors. 2011, Amsterdam ; Oxford : Elsevier, 2010. p. 1339-1371.

91. Fonseca, Á., T. Boekhout, and J.W. Fell, *Cryptococcus vuillemin* (1901), in The Yeasts A taxonomic study, C. Kurtzman, J.W. Fell, and T. Boekhout, Editors. 2011, Elsevier: London. p. 1661-1737.

92. Lee, I., E.G. Hammond, and B.A. Glatz, Triacylglycerol Assembly from Lipid Substrates by *Apiotrichum curvatum*, in Industrial Applications of Single Cell Oils, D.J. Kyle and C. Ratledge, Editors. 1992, AOCS Publishing.

93. Iassonova, D.R., E.G. Hammond, and S.E. Beattie, Oxidative stability of polyunsaturated triacylglycerols encapsulated in oleaginous yeast. Journal of the American Oil Chemists Society, 2008. 85(8): p. 711-716.

94. Liang, Y.N., *et al*., Converting crude glycerol derived from yellow grease to lipids through yeast fermentation. Bioresource Technology, 2010. 101(19): p. 7581-7586.

95. Seo, Y.H., I.G. Lee, and J.I. Han, Cultivation and lipid production of yeast *Cryptococcus curvatus* using pretreated waste active sludge supernatant. Bioresource Technology, 2013. 135: p. 304-308.

96. Chi, Z.Y., *et al*., Oleaginous yeast *Cryptococcus curvatus* culture with dark fermentation hydrogen production effluent as feedstock for microbial lipid production. International Journal of Hydrogen Energy, 2011. 36(16): p. 9542-9550.

97. Ryu, B.G., *et al*., High-cell-density cultivation of oleaginous yeast *Cryptococcus curvatus* for biodiesel production using organic waste from the brewery industry. Bioresource Technology, 2013. 135: p. 357-364.

98. Gonzalez-Garcia, Y., *et al*., Lipids accumulation in *Rhodotorula glutinis* and *Cryptococcus curvatus* growing on distillery wastewater as culture medium. Environmental Progress & Sustainable Energy, 2013. 32(1): p. 69-74.

99. Yu, X.C., *et al*., Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. Bioresource Technology, 2011. 102(10): p. 6134-6140.

100. Zhang, G.C., *et al*., Microbial lipid production as biodiesel feedstock from N-acetylglucosamine by oleaginous microorganisms. Journal of Chemical Technology and Biotechnology, 2011. 86(5): p. 642-650.

101. Liang, Y.N., *et al*., Lipid production from sweet sorghum bagasse through yeast fermentation. Renewable Energy, 2012. 40(1): p. 130-136.

102. Leflaive, J. and L. Ten-Hage, Algal and cyanobacterial secondary metabolites in freshwaters: a comparison of allelopathic compounds and toxins. Freshwater Biology, 2007. 52(2): p. 199-214.

103. Case, R.J., M. Labbate, and S. Kjelleberg, AHL-driven quorum-sensing circuits: their frequency and function among the Proteobacteria. Isme Journal, 2008. 2(4): p. 345-349.

104. Zhang, Y., *et al*., The effect of bacterial contamination on the heterotrophic cultivation of *Chlorella pyrenoidosa* in wastewater from the production of soybean products. Water Research, 2012. 46(17): p. 5509-5516.

105. Angelis, S., *et al*., Co-Culture of Microalgae, Cyanobacteria, and Macromycetes for Exopolysaccharides Production: Process Preliminary Optimization and Partial Characterization. Applied Biochemistry and Biotechnology, 2012. 167(5): p. 1092-1106.

106. Hong, Y. and K. Xu, Co-existing growth relationships of a lipid-producing alga with three microalgae. Allelopathy Journal, 2013. 32(2): p. 301-314.

107. Bacellar Mendes, L.B. and A.B. Vermelho, Allelopathy as a potential strategy to improve microalgae cultivation. Biotechnology for Biofuels, 2013. 6.

108. Basanta, A., *et al*., Development of Bacteriocinogenic Strains of *Saccharomyces cerevisiae* Heterologously Expressing and Secreting the Leaderless Enterocin L50 Peptides L50A and L50B from *Enterococcus faecium* L50. Applied and Environmental Microbiology, 2009. 75(8): p. 2382-2392.

109. Courchesne, N.M.D., *et al*., Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches. Journal of Biotechnology, 2009. 141(1-2): p. 31-41.

110. Dunahay, T.G., *et al*., Manipulation of microalgal lipid production using genetic engineering. Applied Biochemistry and Biotechnology, 1996. 57-8: p. 223-231.

111. Radakovits, R., *et al*., Genetic Engineering of Algae for Enhanced Biofuel Production. Eukaryotic Cell, 2010. 9(4): p. 486-501.

112. Rosenberg, J.N., *et al*., A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. Current Opinion in Biotechnology, 2008. 19(5): p. 430-436.

113. Dunahay, T.G., E.E. Jarvis, and P.G. Roessler, Genetic transformation of the diatoms *Cyclotella cryptica* and *Navicula saprophila*. Journal of Phycology, 1995. 31(6): p. 1004-1012.

114. Radakovits, R., P.M. Eduafo, and M.C. Posewitz, Genetic engineering of fatty acid chain length in *Phaeodactylum tricornutum*. Metabolic Engineering, 2011. 13(1): p. 89-95.

115. Henley, W.J., *et al*., Initial risk assessment of genetically modified (GM) microalgae for commodity-scale biofuel cultivation. Algal Research-Biomass Biofuels and Bioproducts, 2013. 2(1): p. 66-77.

116. Lam, K.S. and J.W.D. Grootwassink, Enzymatic digestion of spent yeast-cells for nutrient recycling in inulinase production. Journal of Industrial Microbiology, 1990. 6(3): p. 207-210.

117. Hsiao, T.Y., C.E. Glatz, and B.A. Glatz, Broth recycle in yeast fermentation. Biotechnology and Bioengineering, 1994. 44(10): p. 1228-1234.

118. Yanagida, T. and Y. Matsumura, Proposal for Bioethanol Fermentation System with N, P, K Recycling by Wet Oxidation. Journal of the Japan Petroleum Institute, 2011. 54(1): p. 45-49.

119. Cai, T., S.Y. Park, and Y.B. Li, Nutrient recovery from wastewater streams by microalgae: Status and prospects. Renewable & Sustainable Energy Reviews, 2013. 19: p. 360-369.

120. Wijffels, R.H. and M.J. Barbosa, An Outlook on Microalgal Biofuels. Science, 2010. 329(5993): p. 796.

121. Slade, R. and A. Bauen, Micro-algae cultivation for biofuels: Cost, energy balance, environmental impacts and future prospects. Biomass & Bioenergy, 2013. 53: p. 29.

122. Rosch, C., J. Skarka, and N. Wegerer, Materials flow modeling of nutrient recycling in biodiesel production from microalgae. Bioresource Technology, 2012. 107: p. 191-199.

123. van Egmond, K., T. Bresser, and L. Bouwman, The European nitrogen case. Ambio, 2002. 31(2): p. 72.

124. Lardon, L., *et al.*, Life-Cycle Assessment of Biodiesel Production from Microalgae. Environmental science & technology, 2009. 43(17): p. 6475.

125. Zhou, Y., *et al*. Environment-Enhancing Energy: A Novel Wastewater Treatment System that Maximizes Algal Biofuel Production and Minimizes Greenhouse Gas Emissions. 2011. Alexandria Va, Water Environment Federation.

126. Minowa, T. and S. Sawayama, A novel microalgal system for energy production with nitrogen cycling. Fuel, 1999. 78(10): p. 1213.

127. Jena, U., *et al*., Evaluation of microalgae cultivation using recovered aqueous co-product from thermochemical liquefaction of algal biomass. Bioresource Technology, 2011. 102(3): p. 3380-3387.

128. Du, Z., *et al*., Cultivation of a microalga *Chlorella vulgaris* using recycled aqueous phase nutrients from hydrothermal carbonization process. Bioresource Technology, 2012. 126: p. 354-357.

129. Biller, P., *et al*., Nutrient recycling of aqueous phase for microalgae cultivation from the hydrothermal liquefaction process. Algal Research, 2012. 1(1): p. 70.

130. Alba, L.G., *et al*., Microalgae growth on the aqueous phase from Hydrothermal Liquefaction of the same microalgae. Chemical Engineering Journal, 2013. 228: p. 214.

131. Pham, M., *et al*., Chemical and Biological Characterization of Wastewater Generated from Hydrothermal Liquefaction of *Spirulina*. Environmental science & technology, 2013. 47(4): p. 2131.

132. Nelson, M., *et al*., Microbial utilization of aqueous co-products from hydrothermal liquefaction of microalgae *Nannochloropsis oculata*. Bioresource technology, 2013. 136: p. 522.

133. Zhou, Y., *et al*., A synergistic combination of algal wastewater treatment and hydrothermal biofuel production maximized by nutrient and carbon recycling. Energy & Environmental Science, 2013. 6(12): p. 3765-3779.

134. Alba, L.G., *et al*., Recycling Nutrients in Algae Biorefinery. Chemsuschem, 2013. 6(8): p. 1330.

135. Cherad, R., *et al*., Macroalgae Supercritical Water Gasification Combined with Nutrient Recycling for Microalgae Cultivation. Environmental Progress & Sustainable Energy, 2013. 32(4): p. 902-909.

136. De la Hoz, H., Optimization of Biomass and Lipid Production in Heterotrophic Microalgal Cultures, in Department of Chemical and Materials Engineering. 2012, University of Alberta.

137. Livansky, K., *et al*., Influence of the nutrient solution recycling on the productivity of *Scenedesmus obliquus*, utilization of nutrients and water in outdoor cultures. Archiv fuer Hydrobiologie Supplement, 1996. 114(0): p. 105-113.

138. Rodolfi, L., *et al*., Growth medium recycling in *Nannochloropsis* sp mass cultivation. Biomolecular Engineering, 2003. 20(4-6): p. 243-248.

139. Hadj-Romdhane, F., *et al*., The culture of *Chlorella vulgaris* in a recycled supernatant: Effects on biomass production and medium quality. Bioresource Technology, 2013. 132: p. 285-292.

140. Hadj-Romdhane, F., *et al*., Development and validation of a minimal growth medium for recycling *Chlorella vulgaris* culture. Bioresource Technology, 2012. 123: p. 366-374.

141. Alyabyev, A.J., *et al*., The effect of changes in salinity on the energy yielding processes of *Chlorella vulgaris* and *Dunaliella maritima* cells. Thermochimica Acta, 2006. 458(1-2): p. 65-70.

142. Gouveia, L., Microalgae as a Feedstock for Biofuels, 2011, Springer-Verlag Berlin: Berlin. p. 1-69.

143. Johnson, E.A. and C. Echavarri-Erasun, Yeast Biotechnology, in The yeasts: a taxonomic study, C.P. Kurtzman, J.W. Fell, and T. Boekhout, Editors. 2011, Amsterdam ; Oxford : Elsevier, 2010. p. 21-44.

144. Chandel, A.K., *et al*., Bioconversion of pentose sugars into ethanol: a review and future directions. Biotechnology and Molecular Biology Reviews, 2011. 6(1): p. 008-020.

145. Steen, E.J., *et al*., Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol. Microbial Cell Factories, 2008. 7.

146. Branduardi, P., *et al*., A novel pathway to produce butanol and isobutanol in *Saccharomyces cerevisiae*. Biotechnology for Biofuels, 2013. 6.

147. Savage, N., The ideal biofuel. Nature, 2011. 474(7352): p. S9-S11.

148. Ghirardi, M.L., *et al*., Microalgae: a green source of renewable H^{-2}. Trends in Biotechnology, 2000. 18(12): p. 506-511.

149. Levin, D.B., L. Pitt, and M. Love, Biohydrogen production: prospects and limitations to practical application. International Journal of Hydrogen Energy, 2004. 29(2): p. 173-185.

150. Sakurai, H., *et al*., Photobiological hydrogen production: Bioenergetics and challenges for its practical application. Journal of Photochemistry and Photobiology C-Photochemistry Reviews, 2013. 17: p. 1-25.

151. Yeh, T.M., *et al*., Hydrothermal catalytic production of fuels and chemicals from aquatic biomass. Journal of Chemical Technology and Biotechnology, 2013. 88(1): p. 13.

152. Cescut, J., *et al*., Optimizing pressurized liquid extraction of microbial lipids using the response surface method. Journal of Chromatography a, 2011. 1218(3): p. 373.

153. Uduman, N., *et al*., Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. Journal of Renewable & Sustainable Energy, 2010. 2(1): p. 012701.

154. Mata, T.M., A.A. Martins, and N.S. Caetano, Microalgae for biodiesel production and other applications: A review. Renewable & Sustainable Energy Reviews, 2010. 14(1): p. 217-232.

155. Halim, R., M.K. Danquah, and P.A. Webley, Extraction of oil from microalgae for biodiesel production: A review. Biotechnology Advances, 2012. 30(3): p. 709-732.

156. Schenk, P.M., *et al*., Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. Bioenergy Research, 2008. 1(1): p. 20-43.

157. Grima, E.M., *et al*., Recovery of microalgal biomass and metabolites: process options and economics. Biotechnology Advances, 2003. 20(7-8): p. 491-515.

158. Edzwald, J.K., Algae, bubbles, coagulants, and dissolved air flotation. Water Science and Technology, 1993. 27(10): p. 67-81.

159. Yoon, R.H. and G.H. Luttrell, The Effect of Bubble Size on Fine Particle Flotation. Mineral Processing & Extractive Metallurgy Review, 1989. 5(1-4): p. 101.

160. Chen, C.Y., *et al*., Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. Bioresource Technology, 2011. 102(1): p. 71-81.

161. Brennan, L. and P. Owende, Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable & Sustainable Energy Reviews, 2010. 14(2): p. 557-577.

162. Divakaran, R. and V.N.S. Pillai, Flocculation of algae using chitosan. Journal of Applied Phycology, 2002. 14(5): p. 419-422.

163. Heasman, M., *et al*., Development of extended shelf-life microalgae concentrate diets harvested by centrifugation for bivalve molluscs - a summary. Aquaculture Research, 2000. 31(8-9): p. 637-659.

164. Prakash, J., *et al*., Microalgal biomass drying by a simple solar device. International Journal of Solar Energy, 1997. 18(4).

165. Widjaja, A., C.-C. Chien, and Y.-H. Ju, Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. Journal of the Taiwan Institute of Chemical Engineers, 2009. 40(1): p. 13.

166. Lee, J.-Y., *et al*., Comparison of several methods for effective lipid extraction from microalgae. Bioresource technology, 2010. 101: p. S75.

167. Jacob, Z., Yeast Lipids - Extraction, Quality Analysis, and Acceptability. Critical reviews in biotechnology, 1992. 12(5-6): p. 463.

168. Vanthoor-Koopmans, M., *et al*., Biorefinery of microalgae for food and fuel. Bioresource Technology, 2013. 135: p. 142-149.

169. Robles Medina, A., *et al*., Downstream processing of algal polyunsaturated fatty acids. Biotechnology Advances, 1998. 16(3): p. 517-580.

170. Hejazi, M.A., E. Holwerda, and R.H. Wijffels, Milking microalga *Dunaliella salina* for beta-carotene production in two-phase bioreactors. Biotechnology and Bioengineering, 2004. 85(5): p. 475-481.

171. Molina Grima, E., *et al*., Comparison between extraction of lipids and fatty acids from microalgal biomass. Journal of the American Oil Chemists' Society, 1994. 71(9): p. 955-959.

172. Cooney, M., G. Young, and N. Nagle, Extraction of Bio-oils from Microalgae. Separation and Purification Reviews, 2009. 38(4): p. 291.

173. Lim, G.B., *et al*., Separation of astaxanthin from red yeast *Phaffia rhodozyma* by supercritical carbon dioxide extraction. Biochemical Engineering Journal, 2002. 11(2-3): p. 181-187.

174. Mendiola, J.A., *et al*., Screening of functional compounds in supercritical fluid extracts from *Spirulina platensis*. Food Chemistry, 2007. 102(4): p. 1357-1367.

175. Herrero, M., A. Cifuentes, and E. Ibanez, Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae - A review. Food Chemistry, 2006. 98(1): p. 136-148.

176. Macias-Sanchez, M.D., *et al*., Supercritical fluid extraction of carotenoids and chlorophyll a from *Synechococcus* sp. Journal of Supercritical Fluids, 2007. 39(3): p. 323-329.

177. Mercer, P. and R.E. Armenta, Developments in oil extraction from microalgae. European Journal of Lipid Science and Technology, 2011. 113(5): p. 539-547.

178. Quijano, G., A. Couvert, and A. Amrane, Ionic liquids: Applications and future trends in bioreactor technology. Bioresource Technology, 2010. 101(23): p. 8923-8930.

179. Teixeira, R.E., Energy-efficient extraction of fuel and chemical feedstocks from algae. Green Chemistry, 2012. 14(2): p. 419-427.

180. Kim, Y.-H., *et al*., Ionic liquid-mediated extraction of lipids from algal biomass. Bioresource Technology, 2012. 109: p. 312-315.

181. Choi, S.A., *et al*., Effects of ionic liquid mixtures on lipid extraction from *Chlorella vulgaris*. Renewable Energy, 2014. 65: p. 169-174.

182. Lovejoy, K.S., *et al*., Evaluation of ionic liquids on phototrophic microbes and their use in biofuel extraction and isolation. Journal of Applied Phycology, 2013. 25(4): p. 973-981.

183. Catchpole, O., *et al*., Extraction of lipids from fermentation biomass using near-critical dimethylether. Journal of Supercritical Fluids, 2010. 53(1-3): p. 34.

184. Hegel, P.E., *et al*., Influence of pretreatments for extraction of lipids from yeast by using supercritical carbon dioxide and ethanol as cosolvent. Journal of Supercritical Fluids, 2011. 58(1): p. 68.

185. Milanesio, J., *et al*., Extraction of lipids from *Yarrowia Lipolytica*. Journal of Chemical Technology and Biotechnology, 2013. 88(3): p. 378.

186. Jin, G., *et al*., Enzyme-assisted extraction of lipids directly from the culture of the oleaginous yeast *Rhodosporidium toruloides*. Bioresource technology, 2012. 111: p. 378.

187. Tsukahara, K. and S. Sawayama, Liquid fuel production using microalgae. Journal of the Japan Petroleum Institute, 2005. 48(5): p. 251-259.

188. Sialve, B., N. Bernet, and O. Bernard, Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnology Advances, 2009. 27(4): p. 409-416.

189. Hirano, A., *et al*., CO_2 fixation and ethanol production with microalgal photosynthesis and intracellular anaerobic fermentation. Energy, 1997. 22(2-3): p. 137-142.

190. Kim, N.J., *et al*., Ethanol production from marine algal hydrolysates using *Escherichia coli* KO11. Bioresource Technology, 2011. 102(16): p. 7466-7469.

191. Ueno, Y., N. Kurano, and S. Miyachi, Ethanol production by dark fermentation in the marine green alga, *Chlorococcum littorale*. Journal of Fermentation and Bioengineering, 1998. 86(1): p. 38-43.

192. Horn, S.J., I.M. Aasen, and K. Ostgaard, Ethanol production from seaweed extract. Journal of Industrial Microbiology & Biotechnology, 2000. 25(5): p. 249-254.

193. McKendry, P., Energy production from biomass (part 2): conversion technologies. Bioresource Technology, 2002. 83(1): p. 47-54.

194. Demirbas, A., Hydrogen from Mosses and Algae via Pyrolysis and Steam Gasification. Energy Sources Part a-Recovery Utilization and Environmental Effects, 2010. 32(2): p. 172-179.

195. Hirano, A., *et al*., Temperature effect on continuous gasification of microalgal biomass: theoretical yield of methanol production and its energy balance. Catalysis Today, 1998. 45(1-4): p. 399-404.

196. Hanaoka, T., S. Hiasa, and Y. Edashige, Syngas production by CO_2/O^{-2} gasification of aquatic biomass. Fuel Processing Technology, 2013. 116: p. 9-15.

197. Hanaoka, T., S. Hiasa, and Y. Edashige, Syngas production by gasification of aquatic biomass with CO_2/O^{-2} and simultaneous removal of H_2S and COS using char obtained in the gasification. Biomass & Bioenergy, 2013. 59: p. 448-457.

198. Chakinala, A.G., *et al*., Catalytic and Non-catalytic Supercritical Water Gasification of Microalgae and Glycerol. Industrial & Engineering Chemistry Research, 2010. 49(3): p. 1113-1122.

199. Chow, M.C., *et al*., Thermal Treatment of Algae for Production of Biofuel. Energy & Fuels, 2013. 27(4): p. 1926-1950.

200. Haiduc, A.G., *et al*., SunCHem: an integrated process for the hydrothermal production of methane from microalgae and CO_2 mitigation. Journal of Applied Phycology, 2009. 21(5): p. 529-541.

201. Stucki, S., *et al*., Catalytic gasification of algae in supercritical water for biofuel production and carbon capture. Energy & Environmental Science, 2009. 2(5): p. 535-541.

202. Goyal, H.B., D. Seal, and R.C. Saxena, Bio-fuels from thermochemical conversion of renewable resources: A review. Renewable & Sustainable Energy Reviews, 2008. 12(2): p. 504-517.

203. Goodacre, R. and D.B. Kell, Pyrolysis mass spectrometry and its applications in biotechnology. Current Opinion in Biotechnology, 1996. 7(1): p. 20-28.

204. Peng, W.M., Q.Y. Wu, and P.G. Tu, Effects of temperature and holding time on production of renewable fuels from pyrolysis of *Chlorella protothecoides*. Journal of Applied Phycology, 2000. 12(2): p. 147-152.

205. Peng, W.M., *et al*., Pyrolytic characteristics of microalgae as renewable energy source determined by thermogravimetric analysis. Bioresource Technology, 2001. 80(1): p. 1-7.

206. Zou, S.P., *et al*., Pyrolysis characteristics and kinetics of the marine microalgae *Dunaliella tertiolecta* using thermogravimetric analyzer. Bioresource Technology, 2010. 101(1): p. 359-365.

207. Babich, I.V., *et al*., Catalytic pyrolysis of microalgae to high-quality liquid bio-fuels. Biomass & Bioenergy, 2011. 35(7): p. 3199-3207.

208. Grierson, S., *et al*., Thermal characterisation of microalgae under slow pyrolysis conditions. Journal of Analytical and Applied Pyrolysis, 2009. 85(1-2): p. 118-123.

209. Campanella, A., *et al*., Thermolysis of microalgae and duckweed in a CO_2-swept fixed-bed reactor: Bio-oil yield and compositional effects. Bioresource Technology, 2012. 109: p. 154-162.

210. Miao, X.L., Q.Y. Wu, and C.Y. Yang, Fast pyrolysis of microalgae to produce renewable fuels. Journal of Analytical and Applied Pyrolysis, 2004. 71(2): p. 855-863.

211. Pan, P., *et al*., The direct pyrolysis and catalytic pyrolysis of *Nannochloropsis* sp residue for renewable bio-oils. Bioresource Technology, 2010. 101(12): p. 4593-4599.

212. Du, Z., *et al*., Hydrothermal pretreatment of microalgae for production of pyrolytic bio-oil with a low nitrogen content. Bioresource technology, 2012. 120: p. 13.

213. Mohan, D., C.U. Pittman, and P.H. Steele, Pyrolysis of wood/biomass for bio-oil: A critical review. Energy & Fuels, 2006. 20(3): p. 848-889.

214. Hu, Z.F., X.Q. Ma, and C.X. Chen, A study on experimental characteristic of microwave-assisted pyrolysis of microalgae. Bioresource Technology, 2012. 107: p. 487-493.

215. Duan, P. and P.E. Savage, Upgrading of crude algal bio-oil in supercritical water. Bioresource technology, 2011. 102(2): p. 1899.

216. Alba, L.G., *et al*., Hydrothermal Treatment (HIT) of Microalgae: Evaluation of the Process As Conversion Method in an Algae Biorefinery Concept. Energy & Fuels, 2012. 26(1): p. 642.

217. Demirbas, A., Combustion characteristics of different biomass fuels. Progress in Energy and Combustion Science, 2004. 30(2): p. 219-230.

218. Demirbas, A., Biomass resource facilities and biomass conversion processing for fuels and chemicals. Energy Conversion and Management, 2001. 42(11): p. 1357-1378.

219. Kadam, K.L., Environmental implications of power generation via coal-microalgae cofiring. Energy, 2002. 27(10): p. 905-922.

220. Balat, M., Mechanisms of thermochemical biomass conversion processes. Part 3: Reactions of liquefaction. Energy Sources Part a-Recovery Utilization and Environmental Effects, 2008. 30(7): p. 649-659.

221. Barreiro, D.L., *et al*., Hydrothermal liquefaction (HTL) of microalgae for biofuel production: State of the art review and future prospects. Biomass & Bioenergy, 2013. 53: p. 113.

222. Ross, A.B., *et al*., Hydrothermal processing of microalgae using alkali and organic acids. Fuel, 2010. 89(9): p. 2234.

223. Zhou, D., *et al*., Hydrothermal Liquefaction of Macroalgae *Enteromorpha prolifera* to Bio-oil. Energy & Fuels, 2010. 24: p. 4054.

224. Anastasakis, K. and A.B. Ross, Hydrothermal liquefaction of the brown macro-alga *Laminaria saccharina*: Effect of reaction conditions on product distribution and composition. Bioresource technology, 2011. 102(7): p. 4876.

225. Jena, U., K.C. Das, and J.R. Kastner, Effect of operating conditions of thermochemical liquefaction on biocrude production from *Spirulina platensis*. Bioresource technology, 2011. 102(10): p. 6221.

226. Valdez, P.J., J.G. Dickinson, and P.E. Savage, Characterization of Product Fractions from Hydrothermal Liquefaction of *Nannochloropsis* sp and the Influence of Solvents. Energy & Fuels, 2011. 25(7): p. 3235.

227. Vardon, D.R., *et al*., Thermochemical conversion of raw and defatted algal biomass via hydrothermal liquefaction and slow pyrolysis. Bioresource technology, 2012. 109: p. 178.

228. Levine, R.B., T. Pinnarat, and P.E. Savage, Biodiesel Production from Wet Algal Biomass through in Situ Lipid Hydrolysis and Supercritical Transesterification. Energy & Fuels, 2010. 24: p. 5235.

229. Bressler, D.C., Methods for producing fuels and solvents. 2011. Patent US8067653B2.

230. Asomaning, J., P. Mussone, and D.C. Bressler, Two-stage thermal conversion of inedible lipid feedstocks to renewable chemicals and fuels. 2014, Bioresource Technology. p. 55-62.

231. Maher, K., Pyrolysis of lipids for the production of renewable fuels and chemicals. 2007, University of Alberta.

232. Asomaning, J., Thermal cracking of lipids to produce renewable fuels and platform chemicals, in Department of Agricultural, Food and Nutritional Science. 2014, University of Alberta

233. Meng, X., *et al*., Biodiesel production from oleaginous microorganisms. Renewable Energy, 2009. 34(1): p. 1.

234. Galafassi, S., *et al*., Lipid production for second generation biodiesel by the oleaginous yeast *Rhodotorula graminis*. Bioresource technology, 2012. 111: p. 398.

235. Amaretti, A., *et al*., Production of single cell oils by the cold-adapted oleaginous yeast *Rhodotorula glacialis* AS 4.7: effects of the growth temperature and the C:N ratio. Ibic2010: 2nd International Conference on Industrial Biotechnology, 2010. 20: p. 109-114.

236. Ghulam, M., *et al*., Lipid production by *Chlorella vulgaris* after a shift from nutrient-rich to nitrogen starvation conditions. Bioresource Technology, 2012. 123: p. 279-283.

237. Illman, A.M., A.H. Scragg, and S.W. Shales, Increase in *Chlorella strains* calorific values when grown in low nitrogen medium. Enzyme and Microbial Technology, 2000. 27(8): p. 631-635.

238. Fjerbaek, L., K.V. Christensen, and B. Norddahl, A Review of the Current State of Biodiesel Production Using Enzymatic Transesterification. Biotechnology and bioengineering, 2009. 102(5): p. 1298.

239. Parawira, W., Biotechnological production of biodiesel fuel using biocatalysed transesterification: A review. Critical reviews in biotechnology, 2009. 29(2): p. 82.

240. Azocar, L., *et al*., Biotechnological processes for biodiesel production using alternative oils. Applied Microbiology and Biotechnology, 2010. 88(3): p. 621.

241. Toor, S.S., L. Rosendahl, and A. Rudolf, Hydrothermal liquefaction of biomass: A review of subcritical water technologies. Energy, 2011. 36(5): p. 2328-2342.

242. Siegler, H.D.l.H., *et al*., The dynamics of heterotrophic algal cultures. Bioresource technology, 2011. 102(10): p. 5764.

243. Hassan, M., *et al*., Lipid Production by an Unsaturated Fatty-Acid Auxotroph of the Oleaginous Yeast *Apiotrichum curvatum* Grown in Single-Stage Continuous-Culture. Applied Microbiology and Biotechnology, 1993. 40(4): p. 483.

244. Xiong, W., *et al*., High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. Applied Microbiology and Biotechnology, 2008. 78(1): p. 29.

245. Zhang, J., *et al*., Microbial lipid production by the oleaginous yeast *Cryptococcus curvatus* O3 grown in fed-batch culture. Biomass & Bioenergy, 2011. 35(5): p. 1906.

246. Maher, K.D., *et al*., Pyrolytic decarboxylation and cracking of stearic acid. Industrial & Engineering Chemistry Research, 2008. 47(15): p. 5328.

247. Asomaning, J., P. Mussone, and B. David, Thermal deoxygenation and pyrolysis of mono unsaturated fatty acids. 2013, Journal of Analytical Applied Pyrolysis, 2014. 105:p1-7.

248. De la Hoz Siegler, H., *et al*., Improving the reliability of fluorescence-based neutral lipid content measurements in microalgal cultures. Algal Research, 2012. 1(2): p. 176.

249. Shin, H.-Y., *et al*., Thermal stability of fatty acids in subcritical water. Journal of Analytical and Applied Pyrolysis, 2012. 98: p. 250.

250. Roussis, S.G., R. Cranford, and N. Sytkovetskiy, Thermal Treatment of Crude Algae Oils Prepared Under Hydrothermal Extraction Conditions. Energy & Fuels, 2012. 26(8): p. 5294.

251. Yang, J., *et al*., Life-cycle analysis on biodiesel production from microalgae: Water footprint and nutrients balance. Bioresource technology, 2011. 102(1): p. 159.

252. Chen, Y.-H. and T.H. Walker, Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel-derived crude glycerol. Biotechnology Letters, 2011. 33(10): p. 1973.

253. Tsigie, Y.A., *et al*., Maximizing biodiesel production from *Yarrowia lipolytica* Po1g biomass using subcritical water pretreatment. Bioresource Technology, 2012. 111: p. 201-207.

254. Miao, C., *et al*., Sequential hydrothermal fractionation of yeast *Cryptococcus curvatus* biomass. Bioresource technology, 2014. 164: p. 106-12.

255. Lamoolphak, W., *et al*., Hydrothermal decomposition of yeast cells for production of proteins and amino acids. Journal of hazardous materials, 2006. 137(3): p. 1643.

256. Hammerschmidt, A., *et al*., Conversion of yeast by hydrothermal treatment under reducing conditions. Fuel, 2011. 90(11): p. 3424.

257. Zheng, Y., *et al*., Oleaginous yeast *Cryptococcus curvatus* for biofuel production: Ammonia's effect. Biomass & Bioenergy, 2012. 37: p. 114.

258. Park, W.S., P.A. Murphy, and B.A. Glatz, Lipid metabolism and cell composition of theoleaginous yeast *Apiotrichum curvatum* grown at different carbon to nitrogen ratios. Canadian Journal of Microbiology, 1990. 36(5): p. 318-326.

259. Immer, J.G. and H.H. Lamb, Fed-Batch Catalytic Deoxygenation of Free Fatty Acids. Energy & Fuels, 2010. 24: p. 5291.

260. Prado, C.M.R. and N.R. Antoniosi Filho, Production and characterization of the biofuels obtained by thermal cracking and thermal catalytic cracking of vegetable oils. Journal of Analytical and Applied Pyrolysis, 2009. 86(2): p. 338.

261. Melero, J.A., *et al*., Production of Biofuels via the Catalytic Cracking of Mixtures of Crude Vegetable Oils and Nonedible Animal Fats with Vacuum Gas Oil. Energy & Fuels, 2010. 24: p. 707.

262. Idem, R.O., S.P.R. Katikaneni, and N.N. Bakhshi, Thermal cracking of Canola oil: Reaction products in the presence and absence of steam. Energy & Fuels, 1996. 10(6): p. 1150.

263. Adebanjo, A.O., A.K. Dalai, and N.N. Bakhshi, Production of diesel-like fuel and other value-added chemicals from pyrolysis of animal fat. Energy & Fuels, 2005. 19(4): p. 1735.

264. Kubatova, A., *et al*., New path in the thermal cracking of triacylglycerols (canola and soybean oil). Fuel, 2011. 90(8): p. 2598.

265. Koutinas, A.A. and S. Papanikolaou, Biodiesel production from microbial oil. Handbook of Biofuels Production: Processes and Technologies, 2011(15): p. 177.

266. Papanikolaou, S. and G. Aggelis, Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture. Bioresource technology, 2002. 82(1): p. 43.

267. Koutinas, A.A., *et al*., Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. Fuel, 2014. 116: p. 566.

268. Blaise, C., P. Vasseur, Algal microplate toxicity test, in: Small-Scale freshwater toxicity investigations, Vol.1 -Toxicity test methods, Blaise, C. and Ferard, JF, Editors. 2005. DOI: 10.1007/1-4020-3120-3_4. p. 137.

269. Papanikolaou, S., *et al*., Industrial derivative of tallow: a promising renewable substrate for microbial lipid, single-cell protein and lipase production by *Yarrowia lipolytica*. Electronic Journal of Biotechnology, 2007. 10(3): p. 425-435.

270. Bednarski, W., *et al*., Growth of 3 fungi on poultry fat or beef tallow. World Journal of Microbiology & Biotechnology, 1993. 9(6): p. 656-659.

271. Bednarski, W., *et al*., Biotechnological methods for the upgrading and modification of animal waste fats. Acta Biotechnologica, 1994. 14(4): p. 387-393.

272. Quitain, A.T., *et al*., Low-molecular-weight carboxylic acids produced from hydrothermal treatment of organic wastes. Journal of Hazardous Materials, 2002. 93(2): p. 209-220.

273. Wilson, C.A. and J.T. Novak, Hydrolysis of macromolecular components of primary and secondary wastewater sludge by thermal hydrolytic pretreatment. Water Research, 2009. 43(18): p. 4489-4498.

274.Mekonnen, T.H., *et al*., Subcritical hydrolysis and characterization of waste proteinaceous biomass for value added applications. Journal of Chemical Technology & Biotechnology. DOI: 10.1002/jctb.4333, 2014.

275. Heredia-Arroyo, T., W. Wei, and B. Hu, Oil Accumulation via Heterotrophic/Mixotrophic *Chlorella protothecoides*. Applied Biochemistry and Biotechnology, 2010. 162(7): p. 1978.

276. Ogbonna, J.C., H. Yoshizawa, and H. Tanaka, Treatment of high strength organic wastewater by a mixed culture of photosynthetic microorganisms. Journal of Applied Phycology, 2000. 12(3-5): p. 277.

277. Ullah, A., *et al*., Quantitative Analysis of the Modes of Growth Inhibition by Weak Organic Acids in *Saccharomyces cerevisiae*. Applied and Environmental Microbiology, 2012. 78(23): p. 8377.

278. Christophe, G., *et al*., Production of Oils from Acetic Acid by the Oleaginous Yeast *Cryptococcus curvatus*. Applied Biochemistry and Biotechnology, 2012. 167(5): p. 1270.

279. Abadie, F.,Utilization by yeasts of certain aminoacids as nitrogen source and as nitrogen and carbon source. Annales De L Institut Pasteur, 1967. 113(1): p. 81-&.

280. Schultz, A.S. and S. Pomper, Amino acids as nitrogen source for the growth of yeasts. Archives of Biochemistry, 1948. 19(2): p. 184-192.

281. Peterson, A.A., *et al*., Thermochemical biofuel production in hydrothermal media: A review of sub- and supercritical water technologies. Energy & Environmental Science, 2008. 1(1): p. 32.

282. Kang, K.Y. and B.S. Chun, Behavior of amino acid production from hydrothermal treatment of fish-derived wastes. Korean Journal of Chemical Engineering, 2004. 21(6): p. 1147.

283. Leuchtenberger, W., K. Huthmacher, and K. Drauz, Biotechnological production of amino acids and derivatives: current status and prospects. Applied Microbiology and Biotechnology, 2005. 69(1): p. 1-8.

284. Cho, B.H. and E. Komor, The amino acid transport systems of the autotrophically grown green alga *Chlorella*. Biochimica Et Biophysica Acta, 1985. 821(3): p. 384-392.

285. McAuley, P.J.,Uptake of amino acids by cultured and freshly isolated symbiotic *Chlorella*. New Phytologist, 1986. 104(3): p. 415-427.

286. Barrajon-Simancas, N., *et al*., Amino acid uptake by wild and commercial yeasts in single fermentations and co-fermentations. Food Chemistry, 2011. 127(2): p. 441-446.

287. Antia, N.J., P.J. Harrison, and L. Oliveira, The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology. Phycologia, 1991. 30(1): p. 1-89.

288. Ghasemi, Y., S. Rasoul-Amini, and E. Fotooh-Abadi, The biotransformation, biodegradation, and bioremediation of organic compounds by microalgae. Journal of Phycology, 2011. 47(5): p. 969-980.

289. Munoz, R., *et al*., Combined carbon and nitrogen removal from acetonitrile using algal-bacterial bioreactors. Applied Microbiology and Biotechnology, 2005. 67(5): p. 699-707.

290. Palmqvist, E., J.S. Almeida, and B. Hahn-Hagerdal, Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. Biotechnology and Bioengineering, 1999. 62(4): p. 447-454.

291. Palmqvist, E., *et al*., Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnology and Bioengineering, 1999. 63(1): p. 46-55.

292. Hu, C.M., *et al*., Effects of biomass hydrolysis by-products on oleaginous yeast *Rhodosporidium toruloides*. Bioresource Technology, 2009. 100(20): p. 4843-4847.

293. Palmqvist, E. and B. Hahn-Hagerdal, Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresource Technology, 2000. 74(1): p. 25-33.

294. Munoz, R. and B. Guieysse, Algal-bacterial processes for the treatment of hazardous contaminants: A review. Water Research, 2006. 40(15): p. 2799-2815.

295. Palmer, C.M., A composite rating of algae tolerating organic pollution. Journal of Phycology, 1969. 5(1): p. 78-&.

296. Ergashev, A.E. and S. Tajiev, Seasonal variations of phytoplankton in a series of waste treatment lagoons (Chimkent, Central Asia) 2. Distribution of phytoplankton numbers and biomass. Acta Hydrochimica Et Hydrobiologica, 1986. 14(6): p. 613-625.

297. Knothe, G., A technical evaluation of biodiesel from vegetable oils vs. algae. Will algae-derived biodiesel perform? Green Chemistry, 2011. 13(11): p. 3048.

298. Knothe, G., Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Processing Technology, 2005. 86(10): p. 1059-1070.

299. Knothe, G., "Designer" biodiesel: Optimizing fatty ester (composition to improve fuel properties. Energy & Fuels, 2008. 22(2): p. 1358-1364.

300. Sitepu, I.R., *et al*., Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeast species. Bioresource Technology, 2013. 144: p. 360-369.

301. Beopoulos, A., *et al*., *Yarrowia lipolytica* as a model for bio-oil production. Progress in lipid research, 2009. 48(6): p. 375.

302. Droop, M.R., Heterotrophy of carbon. Stewart, W. D. (Ed.). Botanical Monographs, Vol. 10. Algal Physiology and Biochemistry. Blackwell Scientific Publications: London, England (Dist. in U.S. by University of California Press: Berkeley, Calif.). Isbn 0-520-02410-9, 1974: p. (1975)-(1975).

303. Bumbak, F., *et al*., Best practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations. Applied Microbiology and Biotechnology, 2011. 91(1): p. 31.

304. Griffiths, D.J., C.L. Thresher, and H.E. Street, The heterotrophic nutrition of *Chlorella vulgaris* (Brannon No. 1 Strain). Ann Bot, 1960. 24(93): p. 1-11.

305. Rodrigue.M, Utilization of Sugars by *Chlorella* Under various Conditions. Journal of general microbiology, 1966. 43(1): p. 139.

306. Miller, J.S. and M.M. Allen, Carbon utilization patterns in heterotrophic blue algae *Chlorogloea fritschii*. Archiv Fur Mikrobiologie, 1972. 86(1): p. 1-&.

307. Schmidt, R.A., M.G. Wiebe, and N.T. Eriksen, Heterotrophic high cell-density fed-batch cultures of the phycocyanin-producing red alga *Galdieria sulphuraria*. Biotechnology and Bioengineering, 2005. 90(1): p. 77-84.

308. Sun, X., *et al*., Microalgal cultivation in wastewater from the fermentation effluent in Riboflavin (B2) manufacturing for biodiesel production. Bioresource technology, 2013. 143(0): p. 499.

309. Panesar, P.S. and J.F. Kennedy, Biotechnological approaches for the value addition of whey. Critical reviews in biotechnology, 2012. 32(4): p. 327.

310. Jelen, P., Dried Whey, Whey Proteins, Lactose and Lactose Derivative Products. 2009: p. 267.

311. Durmus, M., O. Tasli, and A. Kadioglu, The effect of whey on the growth of some algae. Fresenius Environmental Bulletin, 1999. 8(5-6): p. 296.

312. Woertz, I., *et al*., Algae Grown on Dairy and Municipal Wastewater for Simultaneous Nutrient Removal and Lipid Production for Biofuel Feedstock. Journal of Environmental Engineering-Asce, 2009. 135(11): p. 1115.

313. Kothari, R., *et al*., Experimental study for growth potential of unicellular alga *Chlorella pyrenoidosa* on dairy waste water: An integrated approach for treatment and biofuel production. Bioresource technology, 2012. 116: p. 466.

314. Shi, X.M., *et al*., Production of biomass and lutein by *Chlorella protothecoides* at various glucose concentrations in heterotrophic cultures. Process Biochemistry, 1999. 34(4): p. 341.

315. Jakobsen, A.N., et al., Accumulation of docosahexaenoic acid-rich lipid in *Thraustochytrid aurantiochytrium* sp strain T66: effects of N and P starvation and O(2) limitation. Applied Microbiology and Biotechnology, 2008. 80(2): p. 297.

316. Takagi, M., Karseno, and T. Yoshida, Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. Journal of Bioscience and Bioengineering, 2006. 101(3): p. 223.

317. Chen, M., *et al*., Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresource technology, 2011. 102(2): p. 1649.

318. Bartley, M., *et al*., pH effects on growth and lipid accumulation of the biofuel microalgae *Nannochloropsis salina* and invading organisms. Journal of Applied Phycology, 2013: p. 1.

319. Han, F., *et al*., Changes of biomass, lipid content and fatty acids composition under a light–dark cyclic culture of *Chlorella pyrenoidosa* in response to different temperature. Bioresource technology, 2013. 132(0): p. 182.

320. International Dairy Federation, The world dairy situation, in Bulletin no. 446/2010. 2010: Brussels, Belgium.

321. Mawson, A.J., Bioconversions for Whey Utilization and Waste Abatement. Bioresource technology, 1994. 47(3): p. 195.

322. Griffiths, M.J. and S.T.L. Harrison, Lipid productivity as a key characteristic for choosing algal species for biodiesel production. Journal of Applied Phycology, 2009. 21(5): p. 493-507.

323. Pollet, P., *et al*., Solvents for sustainable chemical processes. Green Chemistry, 2014. 16(3): p. 1034-1055.

324. Ykema, A., *et al*., Optimization of lipid production in the oleaginous yeast *Apiotrichum curvatum* in whey permeate. Applied Microbiology and Biotechnology, 1988. 29(2-3): p. 211-218.

325. Sudasinghe, N., *et al*., High resolution FT-ICR mass spectral analysis of bio-oil and residual water soluble organics produced by hydrothermal liquefaction of the marine microalga *Nannochloropsis salina*. Fuel, 2014. 119: p. 47-56.

326. Grierson, S., V. Strezov, and P. Shah, Properties of oil and char derived from slow pyrolysis of *Tetraselmis chui.* Bioresource technology, 2011. 102(17): p. 8232.