Microbial lipid production using aqueous by-product streams from a lipid pyrolysis technology

by

Olga Georgiivna Mameeva

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

Master of Science

in

Bioresource and Food Engineering

Department of Agricultural, Food and Nutritional Science
University of Alberta

ABSTRACT

Currently, non-food carbon sources are making a valuable contribution to development of the next generation of hydrocarbon-based renewable fuels and value-added products. As non-food carbon feedstocks with low or negative value, yellow and brown greases, as well as biosolids, can potentially be processed through pyrolysis technologies to produce renewable fuels. Aqueous by-product streams are generated in these processes after hydrolysis, as well as after pyrolysis, and can potentially be used as a carbon and nutrient source to grow oleaginous microorganisms and to facilitate lipid production.

In this study, volatile fatty acids and other compounds were quantified in pyrolysis aqueous by-product streams. It was shown that volatile fatty acids, such as acetic, propionic and butyric acids, could be used as single or mixed carbon sources for fermentation of *Cryptococcus curvatus* and *Chlorella protothecoides.* Model media mimicked the volatile fatty acid levels in the original aqueous pyrolysis by-product stream, which needed to be diluted to enable growth and lipid accumulation in microbial cells. To avoid potential inhibition caused by low pH, neutralization of the original waste streams and model media was performed to allow high biomass and lipid production. The best growth and lipid productivity of *C. curvatus* along with *C. protothecoides* biomass were observed in three-times diluted model media. It was shown that volatile fatty acid consumption by *C. curvatus* and *C. protothecoides* is time- and concentration-dependent. Moreover, the lipid production of *C. curvatus* and *C. protothecoides* using acetic acid, propionic acid, and butyric acid as a single and mixed carbon source were comparable, in some cases, with

glucose. The fatty acid profiles of *C. curvatus* grown in the volatile fatty acids in the model media showed a higher amount of unsaturation in comparison to *C. protothecoides* fatty acid profiles.

The aqueous by-product streams after hydrolysis of 4 % biosolids, brown grease, and their mixtures, were characterized for their ability to promote *C. curvatus* and *C. protothecoides* growth and lipid accumulation. It was established that the aqueous by-product streams after hydrolysis of 4 % biosolids or a biosolids/brown grease mix cannot be used for fermentation and lipid production by oleaginous microorganisms unless supplemented with the essential minerals to stimulate oleaginous microorganisms growth as well as glycerol to increase the carbon to nitrogen ratio to force lipid production, respectively. In supplemented conditions, a significant rise in biomass production was detected compared to low amounts of total lipids production for both *C. curvatus* and *C. protothecoides*. Moreover, substantial changes in fatty acids profiles of yeast and microalgae were detected.

To summarize, the value-added application of the aqueous by-product streams after hydrolysis and pyrolysis of different lipid-containing feedstocks as a carbon and nutrient source to grow and produce lipids by oleaginous microorganisms is a promising approach. Both oleaginous microorganisms, *C. curvatus* and *C. protothecoides*, are suitable for high-concentration volatile fatty acids fermentations as well as for lipid production under the lipid-stimulated conditions.

PREFACE

This thesis is an original work by Olga Georgiivna Mameeva. No parts of this thesis have been previously published. All experimental procedures were designed and discussed with the assistance of Dr. David Bressler and Dr. Michael Chae. I was directly responsible for experiments performed. Dr. Chae assisted with revisions and editing. Amino acid analysis was performed with support from Jingui Lan (M.Sc.) in the Biorefining Conversions and Fermentation Laboratory. Gas chromatography coupled with thermal conductivity detection analysis of the water content was performed at the Department of Agricultural, Food and Nutritional Science Chromatography Facility, University of Alberta, with assistance from Lisa Nikolai (M.Sc.). Total organic carbon and total nitrogen were analyzed at the Natural Resources Analytical Laboratory, University of Alberta. The essential minerals were analyzed at the Canadian Center for Isotopic Microanalysis, University of Alberta.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my supervisor Dr. David Bressler for his guidance and valuable suggestions, constant encouragement and patience, and support during my program. I am grateful for all lessons that I have had, and for all conclusions that I made. I sincerely thank Dr. Michael Chae for his valuable suggestions and support, for his advice and help during my studying, and for his contribution to manuscript editing.

I would like to thank Dr. Michael Gänzle and Dr. David Stuart, my supervisory committee members, for their helpful feedback and advice on my research.

I want to thank my colleagues of the Biorefining Conversions and Fermentation Laboratory, Dr. Mehdi Omidghane, Lin Xia, and Chengyong Zhu, for providing the aqueous by-product stream samples used for this research. I would like to thank Jingui Lan for his assistance and advice. The technical assistance with samples characterization provided by Lisa Nikolai from the Department of Agricultural, Food and Nutritional Science Chromatography Facility, University of Alberta, and Maxxam Analytics, Edmonton, is gratefully acknowledged.

I want to express my gratitude to FORGE Hydrocarbons Corporation for the Mitacs learning opportunity. The results included within this research are focused on studying fermentability of oleaginous microorganisms to create valuable biorefining strategies. This research does not reflect the current FORGE Hydrocarbons Corporation technological process.

I would like to thank Mrs. Kate Hawrysh and the University of Alberta. I am greatly appreciative of being a recipient of the Babich-Babichuk Graduate Student Award. I am grateful to the Biorefining Conversions Network, FORGE Hydrocarbons Corporation, Mitacs, BioFuelNet Canada, Government of Alberta, the City of Edmonton, EPCOR, Alberta Livestock and Meat Agency Ltd., Natural Sciences and Engineering Research Council of Canada (NSERC), and the University of Alberta for project support.

I want to thank my labmates, present and former, and my friends for their support and encouragement.

To my family: because of your love and support, I am receiving a precious gift – education. You always believe in me, thank you!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
APT	All Purpose Tween medium
ASTM	American Society for Testing and Materials
BAR	Biofuels Annual Report
BDL	Below detection limit
BM	Biosolids Management
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
$CaCO_3$	Calcium carbonate
CBA	Canadian Bioenergy Association
CBR	CanBio report
CoA	Coenzyme A
FAS	Fatty acid synthetase
g	Gram
GC	Gas Chromatography
GC-FID	Gas chromatography coupled with Flame ionization detector
GC-TCD	Gas chromatography coupled with Thermal conductivity detection
h	Hours
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
HPLC-FD	High performance liquid chromatography coupled with Fluorescent Detector
HPLC-RID	High performance liquid chromatography coupled with Refractive Index Detector
IEA	International Energy Agency
L	Liters
min	Minutes
mg	Milligram
mL	Milliliters
N	Normality
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

NaOH	Sodium hydroxide
NRC	National Research Council
NRES	Non-renewable and Renewable Energy Sources
OD	Optical density
pH	Potentiometric hydrogen ion concentration
RFA	Renewable Fuels Association
RHB	Renewable Hydrocarbons Biofuel
rpm	Revolutions per minute
UNEP	United Nations Environment Programme
UNFCCC	United Nations Framework Convention on Climate Change
v/v	Volume per unit volume percentage
wt	Weight
w/w	Weight per unit weight percentage
WEO	World Energy Outlook
WWI	WorldWatch Institute
YPD	Yeast extract peptone dextrose media
$Y_{(b/s)}$	Yield (biomass/substrate)
$Y_{(l/s)}$	Yield (lipid/substrate)
µL	Microliter

CHAPTER 1. INTRODUCTION

1.1. Project background

Minimizing waste generation and creating valuable revenue from its reapplication are significant priorities in any industry. Animal fats, cooking, trap grease – yellow and brown greases, as well as biosolids are considered as low-value or negative-value feedstocks, which can be utilized in the production of the renewable fuels (Wood, Layzell, 2003; Pandey, *et al.*, 2011). The biorefining of waste biomass, especially greases and biosolids, to produce renewable fuels and added-value products needs research and development. Utilization of waste streams and industrial by-products could be considered as waste minimization and value-added product generation approaches (Kargbo, 2010; Abou-Shanab *et al.,* 2013; Hadiyanto & Nur, 2014; Kouhia *et al.,* 2015). Waste streams enriched with lipids could serve as a feedstock for a drop-in fuel technology to produce biofuels. Drop-in fuels are fully compatible with fossil derived fuels and produced by thermochemical or biological processing of hydrocarbons from biomass (Karatzos *et al.,* 2014; Xu *et al.,* 2016). Animals fats (Asomaning *et al.,* 2014a), tall oils (Jenab *et al.,* 2014), greases (yellow and brown), and cold pressed camelina oil (Asomaning *et al.,* 2014a; Asomaning *et al.,* 2014b); oleic, stearic, and abietic acids (Maher, Bressler, 2007; Maher *et al.,* 2008; Asomaning *et al.,* 2014a; Asomaning *et al.,* 2014b; Asomaning *et al.,* 2014c; Jenab *et al.,* 2014); and yeast and algae biomass slurries (Espinosa-Gonzalez *et al.,* 2014a; Espinosa-Gonzalez *et al.,* 2014b; Reddy *et al.,* 2014; Dong *et al.,* 2016; Naghdi *et al.,* 2016; Ochsenreither *et al.,* 2016) were successfully studied and used as lipid feedstocks for biofuel production.

Lipid pyrolysis to produce renewable fuels was intensively studied and developed in the Biorefining Conversions and Fermentation Laboratory, University of Alberta, including the patenting (US8067653B2.230.) of a novel process (Maher, Bressler, 2007; Bressler, 2011; Maher *et al.,* 2008; Asomaning *et al.,* 2014a; Asomaning *et al.,* 2014b; Asomaning *et al.,* 2014c; Jenab *et al.,* 2014). Fatty acids (organic fraction) and an aqueous by-product stream containing glycerol were yielded after hydrolysis. The recovered fatty acids were then pyrolyzed in a second reactor to produce various forms of fuels. Fatty acid pyrolysis resulted in the production of the deoxygenated hydrocarbons as a liquid product, gas, solid products, and an aqueous by-product stream.

Previously published studies in our laboratory showed that depending on the feedstock used, the aqueous by-product stream accounted for 2.6 – 4 % of the product yields when camelina, brown and yellow greases, or beef tallow were used in lipid pyrolysis (Asomaning *et al.,* 2014a; Asomaning *et al.,* 2014b). Our laboratory and many other studies reported the presence of substantial amounts of volatile fatty acids in the aqueous by-product streams after hydrothermal liquefaction, fast pyrolysis and catalytic fast pyrolysis applied in biofuel production (Garcia-Perez et al., 2010; Lian *et al.,* 2012; Paasikallio *et al.,* 2014; Vitasari *et al.,* 2015; Black *et al.,* 2016).

The potential for oleaginous microorganisms to produce microbial oil has been well documented (Ratledge, 1974; Ratledge, Wynn, 2002; Miao & Wu, 2004; Liang *et al.,* 2009; Papanikolaou, Aggelis, 2011; Cerón-García *et al.*, 2013). Numerous studies have shown the application of some volatile fatty acids in

oleaginous microorganism fermentation (Chang *et al.,* 2010; Fei *et al.,* 2011; Fontanille *et al.,* 2012; Christophe *et al.,* 2012; Wen Q. *et al.*, 2013). A few studies demonstrated the utilization of the volatile fatty acids and their mixtures for microbial growth and indicated the concentrations and ratios used to increase lipid accumulation (Fei *et al.,* 2011; Fontanille *et al.,* 2012; Lian *et al.,* 2012; Liu *et al.,* 2016).

There has been no published report on the processing of the aqueous by-product streams received after hydrolysis of brown grease, 4 % biosolids, or their mixtures, after pyrolysis of lipid-containing feedstocks, or their characterization and application. Based on the uniqueness of the composition of the aqueous by-product stream received from lipid pyrolysis, it is very important to determine the compounds that could serve as a carbon source for the fermentation of oleaginous microorganisms and lipid accumulation. In this research study, the volatile fatty acids in the aqueous by-product streams were applied as a carbon source for the cultivation of oleaginous microorganisms: yeast *Cryptococcus curvatus* and microalgae *Chlorella protothecoides*.

Research hypothesis: The aqueous by-product streams that are generated from lipid pyrolysis can be used as substrates for the cultivation of oleaginous yeast and microalgae to accumulate lipids.

1.2. Objectives

The overall objective of this thesis project is to study and apply the aqueous by-product streams after hydrolysis and pyrolysis of different waste lipid-containing

feedstocks as a carbon and nutrient source to grow oleaginous microorganisms and to facilitate lipid production.

1.2.1. Short term objectives

1. To identify and quantify the chemical compounds in by-product streams that can be used as a nutrient source for fermentation of *C. curvatus* and *C. protothecoides.*
2. To evaluate the ability of *C. curvatus* and *C. protothecoides* to grow and tolerate acetic, propionic and butyric acids as a single and mixed carbon source.
3. To evaluate the ability of *C. curvatus* and *C. protothecoides* to produce lipids using acetic, propionic and butyric acids as a single and mixed carbon source.
4. To evaluate the feasibility of using the aqueous by-product streams after hydrolysis of 4 % biosolids, brown grease and their mixtures, as feedstocks for *C. curvatus* and *C. protothecoides* growth and lipid accumulation.

1.2.2. Long term objectives

1. Sustainable renewable fuel production by using lipids from oleaginous yeast and microalgae biomass slurries grown on by-product streams from lipid pyrolysis.
2. Using residual streams generated during the lipid pyrolysis process for development of other value-added products and recycling options.

CHAPTER 2. LITERATURE REVIEW

2.1. Lipids from oleaginous microorganisms as third generation renewable fuels

Fossil fuels are the derivatives of plants and animals formed over 350 millions years ago to 65 millions of years ago. Fossil fuels are the primary non-renewable energy source used today (NRES, 2017). It is a known fact that using fossil fuels leads to dangerous air pollution and gases emissions. Air pollution affects the environment as well as human health (Cohen, 1990). In addition, the main factors affecting global renewable fuel development are energy security and climate change (WEO, 2016). Reducing overall greenhouse gas emissions is the key function of the renewable fuels/biofuels that can help alleviate climate change (Hassan & Kalam, 2013; RHB, 2016). In addition, biofuel production stimulates growth of the agricultural sector, sustains the use of farmland and uses non-agricultural lands to promote agricultural development (Ho *et al.,* 2014).

There are four main generations or categories of biofuels. The first-generation biofuels are converted directly from food crops, animal fats, or vegetable oil into biodiesel or bioethanol. First-generation biofuels have some advantages. The most important one is feedstock availability; food crops can grow in many regions, and animal fats and vegetable oils are widely available. Moreover, the infrastructure is already accessible for planting, harvesting, processing, and relatively simple conversion (Kagan, 2010; Gupta, 2016). The production of first-generation biofuels does raise some concerns regarding its sustainability because they are derived from food crops for human consumption and have indirect land-use implications (Holt-

Gimenez, 2008; Naik *et al.,* 2009). In addition, geographic limitations are present. The feedstock costs for biofuels production are generally high as there is competition for the feedstock with the food market. The first-generation biofuels also suffer from feedstock commodity price volatility (Appadoo, 2011).

The second-generation biofuels are derived from non-food crops that are often cultivated on marginal lands, forestry resources, residues (*e.g.* residues of the lignocellulosic biomass), dedicated energy crops, waste vegetable oils, and urban waste (Naik *et al.,* 2009; Kagan, 2010). Thermochemical (gasification, pyrolysis, and torrefaction) and biochemical (fermentation, *etc.*) conversion technologies are used to produce second-generation biofuels. The non-food biomass is less expensive than the first-generation biomass and more available (Kagan, 2010; Appadoo, 2011). Biorefineries and integrated approaches are also envisioned as potential providers of all possible fuels, chemicals and materials from biomass (Kagan, 2010). Potential benefits of second-generation biofuels include the use of waste materials as low-value sources and, at the same time, the possibility of substantial greenhouse gases emissions reduction (Pearson & Dale, 2013). Several disadvantages of second-generation biofuels are present. For second-generation biofuel production, policies and market development as well as cost and technology development are currently determined as the most important barriers (Naik *et al.,* 2009; IEA, 2017). In addition, as mentioned in the disadvantages of the first-generation biofuels, second generation have some similar deficiencies in regards to water and fertilizer requirements. This includes that many of the feedstocks tested thus far have been rather high-

maintenance (Kagan, 2010) and geographically, grasses growth and application depends on the climate (Naik *et al.,* 2009).

Third-generation biofuels are focused on algal biomass feedstocks as an alternative to first- and second- generation biomass feedstocks (Barclay *et al.,* 2010; Lam & Lee, 2012; Behera *et al.,* 2014). This group also includes other oil-accumulating microorganisms such as yeast and fungi (Ratledge & Wynn, 2002; Ratledge, 2004), and bacteria (Bröker *et al.,* 2010). Oleaginous microorganisms as non-edible lipid sources can be considered for the biofuel production (Ratledge, 1974; Lam & Lee, 2012; Behera *et al.,* 2014). Lipids are stored inside of the yeast and algae cells as lipid droplets and lipid bodies, respectively (Ratledge, 1991; Guo *et al.,* 2009; Fei *et al.,* 2011; Murphy, 2012). Currently, up to 30 species of yeast and algae (microalgae included) are employed for renewable fuel production (Wang *et al.,* 2013). The yeasts *Cryptococcus curvatus* (Ryu *et al.,* 2013; Gong *et al.,* 2015) and *Yarrowia lipolytica* (Beopoulos *et al.,* 2008; Fontanille *et al.,* 2012; Xue *et al.,* 2013; Zhang *et al.,* 2013), the microalgae *Chlorella protothecoides* (Miao, Wu, 2004; Chen, Walker, 2011; Brennan, Owende, 2010; Wen *et al.,* 2013), and *Scenedesmus obtusiusculus* (Toledo-Cervantes *et al.,* 2013), as well as cyanobacteria *Synechocystis* species (Quintana *et al.,* 2011), and fungi *Aspergillus niger* and *Mucor circinelloides* have all been studied as biofuel sources (Ratledge, 2004).

The benefits of third-generation biofuels include the ability of the oleaginous organisms to fix carbon dioxide, to grow with high biomass productivity, and the use of waste feedstocks to produce biofuels (Mata *et al.,* 2010; Pittman *et al.,* 2011; Suganya *et al.,* 2014). Nevertheless, technology to produce third-generation biofuels

needs to be developed to eliminate the high capital cost of the downstream processing (Naik et al., 2009; Brennan & Owende, 2010). As well, some unresolved issues with the lipid extraction from oleaginous biomass and fatty acid composition exist (Brennan & Owende, 2010; Anthony *et al.,* 2012).

A relatively new fourth-generation grouping of biofuels is focused on bioenergy production as well as capturing and storing carbon dioxide using genetically modified bacteria (Howard *et al.,* 2013), yeast (Guadalupe-Medina *et al.*, 2013) and algae (Jones *et al.,* 2016). The fourth-generation biofuels can be made using marginal land, do not need biomass destruction, and geographic limitations are not a huge obstacle. The technology of producing fourth-generation biofuels can be used anywhere where water and carbon dioxide present. In the model, developed by Joule Biotechnology, waste carbon dioxide, sunlight, and engineered microorganisms integrate into a "solar converter" to create renewable fuel (Kagan, 2010). As a new technology, the approach to apply fourth-generation biofuels needs sufficient development and has high initial capital costs (Callebaut, 2010).

2.1.1. Feedstocks for biofuels

Biomass feedstocks for biofuel production are very diverse. The International Energy Agency (2017) defines biomass as “any organic, i.e. decomposable, matter available on a renewable basis” and is "derived from plants or animals" (IEA, 2017). The majority of biomass feedstocks used today are forestry and agricultural biomasses (Brown, 2003; Gupta & Tuohy, 2013; Khosla, 2017). The

continuously increasing amounts of urban waste including municipal solids waste, both residential and commercial, including primary and secondary industrial waste as well as food processing residues has led to a great solution: the possibility of using these waste biomasses as feedstocks for renewable fuels production (Brown, 2003; IEA, 2017; Khosla, 2017).

Value creation and environmental benefits of waste reuse are benefits of comprehensive utilization of the waste feedstocks (Dalrymple, *et al.,* 2013). Waste feedstocks are often relatively poor feedstocks with few options for utilization (Zhu, Chertow, 2016). The composition of the waste feedstocks are varied and depend on many factors such as initial feedstock content and the impact of the selected treatment process. In addition, the most important issue is the removal of high levels of contamination and associated handling hazards (Turovsky, Mathai, 2006). The opportunity for waste feedstocks is to be re-introduced into subsequent industrial processes as a feedstock, eliminating the cost of it waste disposal, possible emissions or environmental hazards.

2.1.1.1. Biomass feedstock availability in Canada

Canada has vast biological resources (Wood & Layzell, 2003). Availability of agricultural and forest biomass feedstocks make them a promising resource to provide renewable energy and products (CBA, 2004; BAR, 2013). Canada's biomass supply feedstock chain includes forestry, agricultural as well as municipal waste feedstocks (Wood & Layzell, 2003). Canada has an abundance of forest

resources. The country has 10 % of the world's forest resources of which 245 million hectares is considered productive forest. Canada's agricultural sources include about 68 million hectares of farmland of which 36.4 million hectares is cropland (CBA, 2004).

Forestry feedstocks can be used for traditional wood products or as an energy resource. Forestry biomass includes timber productive forest biomass, harvest waste residues, unused mill waste, woody residues from natural disturbances such as fires and infestations, and feedstocks derived from silviculture. Silviculture refers to growth of woody crop species with short-rotation harvested cycle (*e.g.* poplar, maple, eucalyptus, black locust, sycamore, and sweet gum) (NRC, 2000).

Agriculture feedstocks for bioindustrial applications (*i.e.* virgin and waste biomass) include bioenergy crops (*e.g.* canola, soy, and corn) as well as perennial grasses (*e.g.* switchgrass, napiergrass, rye, tall fescue, and timothy), legumes (*e.g.* alfalfa, clover or crown vetch), crop residues such as straw or stover residues, and livestock manure. Agricultural crop species grown in Canada belong to starch crops like corn, wheat, barley, rye, oats, as well as seed oil crops such as canola or soybeans and finally pulse crops such as peas and lentils (Wood & Layzell, 2003; CBA, 2004).

The available waste feedstock accounts for 46 % of biomass left after forestry harvest and 39 % of biomass left after harvesting in agriculture (Wood & Layzell, 2003). There are significant residual or waste biomass streams coming from municipalities, with around 387,166 ton/year of biosolids residues produced in Canada (Wood & Layzell, 2003). In general, Canada has major potential to utilize

its biomass forest and agricultural feedstocks resources as well as municipal waste feedstocks, including waste greases and biosolids, to provide biofuels.

2.1.2.2. Feedstocks currently used in commercial biofuel production

The transformation of biomass feedstocks to bio-energy and bio-products has been developing in Canada for more than 25 years. Numerous applications of the biomass feedstock are found to produce biofuels, biogas, renewable chemicals and materials (Bradburn, 2014).

The most intensively produced biofuels are bioethanol and biodiesel. In general, bioethanol can be produced from sugar, starch, and cellulose. The most popular first-generation bioethanol feedstocks are sugarcane (from Brazil), corn (from USA) (Rutz & Janssen, 2007), corn and grain (from Canada) (Bradburn, 2014), sugar beet (from Europe), grain and maize (from Europe and USA) (Rutz & Janssen, 2007). USA and Brazil are by far the biggest producers of bioethanol worldwide (WWI, 2011). In 2016, the United States produced 57.6 % of world output while Brazil produced 27.4 % and Canada produced 1.6 % (RFA, 2017). Capacity to produce first-generation bioethanol made from corn and grain in Canada reached 1,826 million liters from 14 plants in 2013. For example, Suncor produced 413 million liters of bioethanol in 2012 (retrieved from http://sustainability.suncor.com).

Production of the second-generation bioethanol from lignocellulosic feedstocks is now successfully transitioning from the research and development stage

to commercial development. Second-generation, cellulosic bioethanol, is already applicable at large-scale. The estimated price of bioethanol production from cellulosic feedstock is in for Raizens (Brazil) at $ 2.17 per gallon (Van der Hoeven, 2016). Currently, 14 cellulosic bioethanol production facilities including pilot plants (*e.g*. Lignol Innovations, Vancouver, British Columbia), commercial demonstration plants (*e.g.* Iogen Corporation, Ottawa, Ontario) and start-ups (*e.g.* Prairie Green Renewable Energy, Saskatoon, Saskatchewan and Varennes, Quebec) are now under operation in Canada (Bradburn, 2014; BAR, 2016). The total capacity to produce bioethanol made in Canada by 2016 was 1.75 billion liters where up to 19 % accounted for second-generation bioethanol (BAR, 2016).

The variety of feedstocks for biodiesel production is determined by climate, the geographical position of the country producer and the feedstock's agricultural availability. Up to 85 % of biodiesel worldwide is made from rapeseed (Europe), soybean (USA and Brasil), sunflower seed (Europe), palm oil (Indonesia and Malaysia), and jatropha (India) (Mittelbach, Remschmidt, 2004; Rutz & Janssen, 2007). The lipid resources that can be used as biodiesel feedstock are seeds oils, palm/fruit oils, algae/microalgae oils and waste oils. In Canada, canola is the primary used feedstock (Alberta and Saskatchewan), followed by recycled cooking oils, animal fats and greases (Ontario and Quebec) (CBR, 2014). The capacity to produce biodiesel in 2017 was estimated in the Global Information Agricultural Network Biofuels report at 550 million liters, compared to the 400 million liters produced in 2016 in Canada (BAR, 2016).

2.1.2. Waste feedstocks for biodiesel production

Forestry, livestock, farming, domestic solid and liquid waste including waste grease and oil from restaurants and food production facilities, and vegetable oil refinery waste are potential sources of feedstocks for biodiesel production (Chen *et al.,* 2009; Dizge, *et al.,* 2009). Waste valorization approach was applied for a wide range of waste feedstocks, such as sewage sludge (Kargbo, 2010), paper industry effluents (Kouhia *et al.,* 2015), palm oil mill effluent (Hadiyanto & Nur, 2014), piggery wastewater (Abou-Shanab *et al.,* 2013), and distillery wastewater (Gonzalez-Garcia *et al.,* 2013). Yellow and brown greases, animal fats, waste fish oil, spent coffee grounds, cold pressed camelina oil, and citrus waste are some resources that have been reported as valuable feedstocks enriched by lipids for biodiesel production (Kulkarni, Dalai, 2006; Didge *et al.,* 2009; Chen *et al.,* 2009; Du *et al.,* 2011; Chi *et al.,* 2011; Ryu *et al.,* 2013; Asomaning *et al.,* 2014a; Asomaning *et al.,* 2014b).

2.1.2.1. Yellow and brown greases

Currently, the small amount of biodiesel produced in Canada is mostly made from waste products (*e.g.* restaurant oils and greases, and animal renderings) (Bradburn, 2014). Yellow grease is typically generated from used cooking oil or vegetable oil with less than 15 % a free fatty acid level. In yellow grease, solids are filtered out, and moisture content is adjusted to meet industry specification standards by heating spent cooking oil (Stiefelmeyer, *et al.* (2006). Based on the population in Alberta, an estimated 18 million liters per year of yellow waste grease are produced (Bradburn, 2014). Two potato chip production plants in southern Alberta produced

one million liters (McCain Foods, Coaldale) and 0.43 million liters (Hostess Potato Chips, Taber) of grease per year in Alberta (Kulkarni & Dalai, 2006).

In brown grease, the free fatty acid level exceeds 20 %. This grease can be cleaned from restaurant grease traps but mainly represents trap and sewage grease that cannot be used for animal feed as is highly oxidized yellow grease (Pandey *et al.,* 2011).

The use of yellow and brown greases for biodiesel production as well as other waste oil can help to solve the problem of their disposal (Kemp, 2006). Application of different innovative strategies on the range of fat-containing feedstocks (*i.e.* brown grease and yellow grease) to produce green renewable fuel and the possibility to reduce greenhouse gas emissions is the best solution for their utilization (Kemp, 2006; UNFCCC, 2006; Pandey *et al.,* 2011).

2.1.2.2. Biosolids

Biosolids are the by-product in liquid, semi-solid or solid state generated after wastewater treatment and enriched by organics, nutrients, chemical elements, and water (UNEP, 2017; Evanylo, retrieved on 2017). In Canada, more than 660,000 metric tons (dry basis) of urban biosolids are produced each year, based on the assessment of the Canadian Council of Ministers of the Environment (CCME, 2014). The annual amount of biosolids managed by the City of Edmonton by the end of 2015 was 33,500 dry tons. The accumulated amount of biosolids at the Clover Bar lagoons at the end of 2010 was in the range of 18,000 – 200,000 dry tons (CCME,

2014). Currently, 50 % of the processing costs of wastewater treatment facilities are spent on the management fees for biosolids maintenance (Wheeler, 2007; Mangory *et al.,* retrieved on 2017).

Because of different wastewater treatments, the composition of biosolids varies substantially based on the municipality producing those biosolids. Common wastewater treatment processes to get land applicable biosolids include thickening, different general and advanced stabilization methods, and physical and chemical processes to enhance biosolids handling (Evanylo, 2017). They can be applied in the thickened, dewatered and dry form. For example, the dried biosolids can be used as a rich nutrient fertilizer (Lu *et al.,* 2012; BM, 2016). Benefits of this traditional method of biosolids application include assistance in the improvement of soil structure, reducing landfill disposal and groundwater protection (Evanylo, retrieved on 2017). The biosolids "exceptional quality" are a very important parameter of their land applicability; pollutant concentration limits and loading rates, and pathogen reduction should be met (Turovsky, Mathai, 2006, p. 5). Nevertheless, the drawbacks of the land biosolids application are the concerns about environmental impact because of incorrect application, accumulation of metals, or because of the public dislike of existing odors.

Biosolids, as a municipal waste feedstock, can be successfully used for biofuel production. Integration of the biosolids from municipal wastewater processing facilities to produce biofuels has been examined through different processes (Moller, 2007; CCME, 2014; Muller *et al*., 2014). They include sludge to biogas an anaerobic digestion (Ryckebosch, 2011; Cao, Pawłowski, 2012), microbial

syngas fermentation or the Fischer-Tropsch process (Dry, 2004; Latif *et al.,* 2014), sewage sludge pyrolysis (Fonts *et al.,* 2012), catalytic pyrolysis (Heo *et al.,* 2011), and fast pyrolysis (Park *et al.,* 2010). Several studies have demonstrated that wastewater treatment products typically represented by waste activated sludge can be directly used as a growth substrate by oleaginous microorganisms for further biofuel synthesis (Pittman, 2011; Murphy, 2012; Dalrymple *et al.,* 2013). Waste activated sludge was used to grow the oleaginous yeast *Cryptococcus curvatus* to achieve lipid accumulation of up to 23 % of dry weight of biomass (Seo *et al.,* 2013). The yeasts *C. curvatus* and *Rhodotorula glutinis* were tested for their ability to grow and accumulate lipids in municipal primary wastewater. It was shown that lipids could be accumulated up to 28.6 ± 2.2 % and 19.6 ± 0.2 % of the dry weight for *C. curvatus* and *Rhodotorula glutinis*, respectively (Chi *et al.,* 2011). Some microalgae species of *Chlorella* and *Scenedesmus* have been shown to use a sewage-based wastewater for biodiesel production and, additionally, in many cases, they can facilitate removal of ammonia, nitrate and total phosphorus (Wen *et al.,* 2013; Stemmler *et al.,* 2016). The potential of the oleaginous microorganisms to use by-products of wastewater treatments as a substrate to grow and to enhance further the functional capacity for production of lipids provide substantial benefits for sustainable biofuels production.

2.1.3. Algae as a source of renewable fuels

Third-generation feedstock, algae, is considered the most promising feedstock for the development of the next generation of biofuel and value-added products from non-food carbon sources (Lam & Lee, 2012; Behera *et al.,* 2014; Suganya *et al.,* 2016). Algae can grow in ocean water, non-potable water, and in different feedstocks that are unsuitable for agriculture usage and variable wastewater (Mata *et al.,* 2010). These advantages led to widespread use of algae (both microalgae and macroalgae) for biofuel production. Based on cell composition, algae can be used to produce a variety of biofuels including bioethanol, biodiesel, bio-oil, biohydrogen, and syngas (Behera *et al.*, 2014; Suganya *et al.,* 2016). Both macroalgae and microalgae can be used to produce biofuels.

Macroalgae are enriched in carbohydrates, which after further pre-treatment and subsequent enzymatic hydrolysis, can be used for bioethanol production. Different red, brown and green macroalgae species were tested for bioethanol production. Some macroalgae cells show high levels of carbohydrates (per dry weight): *Caulerpa laetevirens* at 56.25 %, *Codium decorticatum* at 50.63 %, *Cladophora fascicularis* at 49.50 % (Kaliaperumal *et al.*, 2002). Laminarin and mannitol represent fermentable sugars in *Saccharina latissima* macroalgae (Adams *et al*., 2009). As shown by Borines *et al., Sargassum spp.* biomass is a raw source of fermentable sugars (Borines *et al.*, 2013). It was determined that the α-cellulose, hemicellulose, holocellulose, and mannitol are the main structural components of *Sargassum spp.* After acid pre-treatment and then enzymatic hydrolysis with cellulase and cellobiase, the fermentable sugars, mainly glucose and cellobiose, are

released. Then, depending on the different fermentation conditions applied, the percentage of the ethanol converted by *Saccharomyces cerevisiae* was between 65 – 89 %.

Microalgae enriched with lipids (> 20 % per cell dry weight) are also considered as feedstock for biodiesel production (Behera *et al.*, 2014). In general, depending on cultivation conditions, microalgae can accumulate from 20 % to 50 % lipids, while some microalgae accumulated lipids up to 80 % of their dry weight (Bellou *et al.,* 2014). As reported, *Botryococcus spp.* accumulated up to 75 % by weight of dry biomass (Mata *et al.,* 2010), *Chlorella zofingiensis* at 65.1 % (Feng *et al.,* 2012), and *C. protothecoides* at 50 % (Cerón-García *et al.*, 2013). In addition, the fatty acids composition of the microalgae oil is affected by feedstock used for growth (Hu *et al.,* 2008; Mata *et al.,* 2010). Microalgae can be one of the options to produce biofuels from oily biomass (Suganya *et al.*, 2016).

For biodiesel production, the algal lipids must be extracted from the cells. Enzyme-catalysed (Robles-Medina *et al.,* 2013) and non-catalyzed transesterification under supercritical ethanol conditions (temperature of 245 - 270 ^{o}C and pressure of 6.5 – 20 MPa) (Demirbas, 2003; Reddy *et al.*, 2014) or a lipid pyrolysis technology (Bressler, 2011) can be used. In addition, conventional, flash and fast pyrolysis processes were applied to receive sufficient amounts of bio-oil from algae. The macroalgae *Ulva lactuce* was tested for bio-oil production by fast pyrolysis and showed 78 % yield (Trinh et al., 2012). The yield of bio-oil obtained for *Saccharina japonica* at 450 °C was 47 % (Choia *et al.,* 2014). As was shown by Miao and Wu in 2004, fast pyrolysis can be a useful tool to get 57.9 % yield of bio-oil using

heterotrophically grown *Chlorella protothecoides* as a feedstock (Miao & Wu, 2004). Microalgae *Botryococcus braunii, Chlorella vulgaris* and *Dunaliella tertiolecta* were used for catalytic conversion at low temperatures. The results showed that oil recovery rates for *B. braunii* and *D. tetriolecta* were 64 % and 42 %, respectively (Tsukahara & Sawayama, 2005).

2.1.4. Yeast as a source of renewable fuels

Yeast is a unique source of biofuel as it can produce and serve as a source of both bioethanol and biodiesel. The ability of yeast to ferment hexose sugar feedstocks to ethanol and other chemical compounds has been described previously (Sanchez & Cardona, 2008). The ability of yeast *S. cerevisiae* and *Schizosaccharomyces pombe* to produce bioethanol was studied using different feedstocks like sugars (molasses), starch, lignocellulosic substrates, wastes, under various cultivation and operation conditions (Jouzani & Taherzadeh, 2015). Moreover, as a convenient model for genetic modifications and metabolic pathways, yeast have been engineered to ferment pentose sugars (Kim *et al.,* 2013) and various lignocellulosic substrates (Delgenes *et al.,* 1996; Matsushika *et al.,* 2009; Olofsson *et al.,* 2011) to increase ethanol yield. Alternatively, oleaginous yeast is a valuable feedstock to produce biodiesel due to their excellent ability to accumulate lipids. The genera *Cryptococcus, Yarrowia, Lypomyces, Rhodotorula* and *Candida* have found a wide application as biodiesel feedstocks. The yeast *Yarrowia lipolytica*, *Candida curvata*, *Rhodotorula glutinis, Lipomyces starkeyi,* and *Cryptococcus albidus* can accumulate 50 – 70 % of intracellular lipids per cell dry weight (Ratledge & Wynn,

2002; Ratledge, 2004; Meng, 2009; Fei *et al.,* 2011; Fontanille *et al.,* 2012; Christophe *et al.,* 2012; Ryu *et al.,* 2013; Gong *et al.,* 2015).

Biodiesel properties depend on the composition of raw materials used for yeast cultivation (Ramos *et al.,* 2009). Carbon sources for direct production of oleaginous yeast biomass included glucose and other sugars, sugar substitutes, volatile fatty acids, glycerol, starch, cellulosic hydrolysates, industrial, commercial, and food wastes (Ochsenreither *et al.,* 2016). *Lipomyces tetrasporus* and *Lipomyces lipofer* can grow and accumulate lipids 54.8 to 62.2 % (w/w) using L-arabinose, glucose, and xylose (Dien *et al.,* 2016). With glucose used as the only carbon source, *Rhodotorula glutinis* accumulated 66 % (w/w) lipids, while *Lipomyces starkei* and *Trichosporon pullulans* amassed 63 % and 65 % lipids, respectively (Ratledge, Wynn, 2002). Acetic, propionic and butyric acids were used as the carbon sources, and growth and lipid accumulation by *Yarrowia lipolitica* were measured (Fontanille *et al.,* 2012). The highest lipid content was detected as 30.7 %, 25.7 % and 25.0 % (w/w), for acetic, propionic and butyric acids, respectively (Fontanille *et al.,* 2012). *Candida albidus* were cultivated in mix fermentation conditions (acetic, propionic and butyric acids with the ratio 8:1:1), and the highest lipid content detected was 27.8 % (w/w) (Fei *et al.,* 2011). *Cryptococcus curvatus* strains grown on glycerol accumulated 25 - 69 % (w/w) lipids (Meesters *et al.,* 1996; Iassonova *et al.,* 2008). The heterologous alpha-amylase and glucoamylase enzymes were expressed in *Y. lipolitica* to enable the use of starch as a carbon source, resulting in 21 - 27 % lipid production (Ledesma-Amaro *et al.,* 2015). Another approach applied by Gong *et al.* in 2003, used corn stover as a feedstock for lipid production by *C. curvatus* (Gong *et*

al., 2013). The cellulose conversion and release of sugars with simultaneous lipid accumulation effectively led to 55 % (w/w) of lipids produced by *C. curvatus*. Yeast can also grow and accumulate lipids in various industrial, commercial, and food wastes feedstocks. For example, oleaginous yeast *C. curvatus* can grow using waste activated sludge as a substrate and can accumulate up to 23 % (w/w) lipids (Seo *et al.,* 2013).

The approach to use yeast whole biomass to reduce costs of lipids for production of biofuels has actively developed over the last decade (Dong *et al.,* 2016; Ochsenreither *et al.,* 2016). In addition, direct hydrothermal liquefaction with the presence of catalyst, sodium carbonate, was reported as a method to produce renewable diesel using *C. curvatus* biomass (Jena *et al.,* 2015). From our laboratory, Espinosa-Gonzales *et al.* in 2014 investigated microbial slurry of *C. curvatus* as a lipid feedstock to produce renewable hydrocarbons through two-step thermal conversion technology (Espinosa-Gonzales *et al.*, 2014a; Espinosa-Gonzalez, 2014b).

2.2. Oleaginous microorganisms as microbial oil producers

The total lipid content of the cells of oleaginous microorganism can be described based on their chemical nature, subcellular distribution, and function. Additionally, lipids can be divided into "bounded" and "un-bounded" forms depending on their extraction mechanisms (Rattray *et al.*, 1975). The lipids that can be present in the cell are triacylglycerols, sterols, glycerophospholipids,

sphingolipids, and glycolipids, which differ based on their chemical nature and biological significance (Ratledge, Wynn, 2002).

Yeast *Cryptococcus curvatus* and microalgae *Chlorella protothecoides* are effective microbial oil producers (more information see in Chapters 2.1.3. and 2.1.4.). Single cell oils are considered a valuable feedstock source for biofuels and value-added chemicals because of the specific ability of oleaginous microorganisms to accumulate different types of neutral lipids in the form of a lipid droplet/lipid bodies. In particular, neutral lipids as triacylglycerols, sterol esters, polyhydroxyalkanoates, and wax esters, lipid molecules with high energy density are best for biofuel production by oleaginous microorganisms (Garay *et al.*, 2014). In general, oleaginous yeast and microalgae lipids are formulated by esters of glycerol and C14 – C22 saturated or unsaturated fatty acids. Triacylglycerols are the most common storage lipids in these organisms (Borowitzka, 2010; Ochsenreither *et al.,* 2016).

The composition and concentration of produced fatty acids varies based on many different factors including strain specificity. The composition of single cell oils produced by oleaginous microorganisms represents the perfect material for biodiesel production (Papanikolaou, Aggelis, 2011). Thus, in the oleaginous yeast, triacylglycerols and sterol esters, in bacteria, mainly polyhydroxyalkanoates, also triacylglycerols and wax esters, are the main components of single cell oil. It is well known that biodiesel properties highly depend on the level of unsaturation of fatty acid methyl esters. A high degree of unsaturation leads to production of biodiesel with a better cloud point and a lower cetane index. Schenk *et al.* in 2008 stated that a

low oxidative potential to avoid long-term instability of biodiesel can be achieved with the "ideal mix" of fatty acids, which "has been suggested to be 16:1, 18:1 and 14:0 in the ratio 5:4:1" (Schenk *et al.,* 2008, p. 36).

2.2.1. Carbon to nitrogen ratio and lipid accumulation

Factors that affect lipid accumulation are macronutrients (*e.g.* carbon, nitrogen, and phosphorus), growth factors (*e.g.* inositol, pantothenic acid, Vitamin B_6, and biotin), micronutrients (*e.g.* trace metals), and environmental factors (*e.g.* temperature, photoperiod and light intensity, culture pH, oxygen concentration, oxidative stress, salinity, and culture mode) (Papanikolaou, Aggelis, 2011; Yilancioglu *et al.*, 2014; Miazek *et al.*, 2015). The molar ratio between carbon and nitrogen was considered a major factor in the induction of lipid accumulation in both oleaginous yeast and algae (Ratledge, Wynn, 2002; Fei *et al.,* 2011; Papanikolaou, Aggelis, 2011; Fontanille *et al.,* 2012; Braunwald *et al.* 2013). The other factors, which induced cell stress, also affect lipid accumulation. For example, as a result of oxidative stress caused by addition of hydrogen peroxide, the lipid production by green algae strain *Dunaliella salina* was increased up to 44 % (Yilancioglu *et al.*, 2014).

The most useful method to increase microbial lipid production is to keep the ratio of carbon to nitrogen greater than 20 (Ratledge, Wynn, 2002). It was observed that increasing amounts of carbon with a constant nitrogen level leads to extra lipid accumulation in yeast. To determine the lipid production by *Rhodotorula glutinis*, the carbon to nitrogen ratios of 20, 70, and 120 were tested (Braunwald *et al.* 2013).

It was shown that enhancing the carbon to nitrogen ratio by increasing the glucose content led to higher total lipid and fatty acid methyl ester yields. The highest levels of C18:1 and C18:2 were detected at carbon to nitrogen ratio at 20 to 1 (Braunwald *et al.* 2013). Carbon to nitrogen ratios from 8.6 – 200, which were manipulated by altering glycerol and yeast extract levels, were applied to grow *Candida freyschussii* ATCC 18737 (Raimondi *et al*., 2014). The conclusion of Raimondi *et al*. (2014) was that at the carbon to nitrogen ratios of 52 and 100 were the best for lipid production by *Candida freyschussii* ATCC 18737.

The influence of a high carbon to nitrogen ratio on lipid accumulation by algae cells was also observed (Brennan, Owende, 2010; Lam & Lee, 2012). Nitrogen depletion along with excess supplementation of a carbon source was studied using *Chlorella vulgaris* and *Chlorella protothecoides* to increase lipid production from 33 % and 38 % to 50 and 55.2 %, respectively (Hu *et al.,* 2008; Liang *et al.,* 2009; Cerón-García *et al.*, 2013).

Excess carbon supplementation along with nitrogen depletion shifted cells from biomass generation to lipid accumulation as a mechanism to utilise additional carbon in the form of neutral lipids (Ratledge, Wynn, 2002; Papanikolaou, Aggelis, 2011; Braunwald *et al.* 2013). During lipid accumulation by oleaginous microorganisms, the high carbon to nitrogen ratio plays a key role.

2.2.2. Volatile fatty acids

In the cell, *de novo* synthesis of lipids occurs using a variety of carbon sources. The oleaginous microorganisms utilize carbon compounds through a wide range of biochemical reactions and different metabolic pathways to produce acetyl-CoA, the first important intermediate metabolite of fatty acid synthesis (Garay *et al.,* 2014). Traditionally, studies assessing lipid accumulation in yeast implicated the use of glucose (Ratledge, Wynn, 2002; Liang *et al.,* 2009; Papanikolaou, Aggelis, 2011; Cerón-García *et al.*, 2013; Braunwald *et al.* 2013; Wen *et al.,* 2013; Pagnanelli *et al.,* 2014, *etc*.). More recently, it has been shown that glycerol could provide carbon for both microalgae and yeast growth (Liang et al., 2010; Chen, Walker, 2011; Cerón-García *et al.,* 2013; Espinosa-Gonzalez, 2014b; Raimondi *et al.,* 2014).

For yeast and algae fermentation, a variety of low- and negative- value carbon-containing feedstocks can be employed. It has been demonstrated that food wastes (Fei *et al.,* 2015), C5 and C6 sugars from waste cellulosic materials (Santamauro *et al.,* 2014), waste activated sludge (Liu *et al.,* 2016), effluent waste stock (Wen *et al.,* 2013), and the aqueous phase after pyrolysis (Lian *et al.,* 2012) can be used for lipid production for biofuels. It was shown that volatile fatty acids, such as acetic, propionic and butyric acids, found in the variety of the feedstocks, can be applied as source of carbon for fermentation of oleaginous microorganisms and lipid production (Chang *et al.,* 2010; Fei *et al.,* 2011; Fontanille *et al.,* 2012; Christophe *et al.,* 2012; Wen Q. *et al.,* 2013; Fei *et al.,* 2015). A mix of volatile fatty acids was evaluated by Fei*, et al.* in 2011, in which acetic, propionic and butyric acids were present in the media in a ratio of 8:1:1. The highest lipid content reached by *Candida albidus* was 27.8 % (Fei *et al.,* 2011). It was reported that *Cryptococcus curvatus* can use 5 g/L

acetic acid with a lipid yield up to 50 % (Christophe *et al.,* 2012). Fontanille *et al.* in 2012 determined the maximal *Yarrowia lipolytica* lipid content obtained was 40.7 % when cultivated on acetate, with a carbon to nitrogen ratio of 50 to 1 (Fontanille *et al.,* 2012). The highest lipid content of 73.4 % was reported for *C. curvatus* after cultivation in 30 g/L acetate in the media, pH 7.0 with a carbon to nitrogen ratio of 50 to 1 (Gong *et al.,* 2015). Wen *et al.,* 2013 evaluated growth of heterotrophic microalgae and consumption of volatile fatty acids in the hydrolyzate of waste activated sludge. It was shown that acetic acid and isovaleric acid are the major volatile fatty acids utilized for *Chlorella protothecoides* growth and lipid accumulation up to 21.5 %, and the hydrolysate needs to be supplemented with trace elements for a better biomass yield (Wen *et al.,* 2013). Fei *et al.* demonstrated 48.7 % lipid accumulation using *C. protothecoides* growing on volatile fatty acids composed of acetic acid, propionic acid and butyric acid in an 8:1:1 ratio (Fei *et al.,* 2015). It has been demonstrated that the volatile fatty acids can be applied for fermentation of the oleaginous microorganisms *C. curvatus* and *C. protothecoides.*

2.2.3. Biosynthesis of lipids in oleaginous microorganisms

Oleaginous microorganisms can effectively use volatile fatty acids for lipid formation. Glucose, acetate, propionate, and butyrate can all be converted to acetyl-CoA, and then, acetyl-CoA is turned into fatty acids in a process that will be described below. The fatty acid synthesis pathway employing acetate, butyrate, and propionate, in comparison with the glucose utilization pathway, are shown in Figure 2.1.

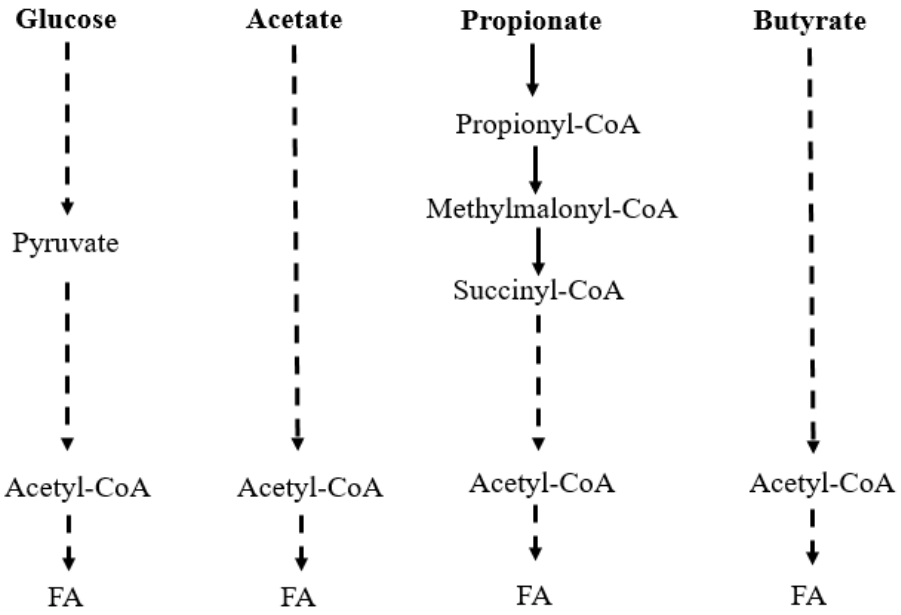

Figure 2.1. Fatty acid schematic synthesis pathways derived from glucose, acetate, butyrate and propionate (modified from Pronk *et al.,* 1994; Duncan *et al.,* 2002; Vital *et al.,* 2014).

Fatty acids need to be esterified/activated with coenzyme CoA to yield activated form of fatty acid - acetyl-CoA, where high energy thioester linkage is present (Pol *et al.,* 2014). Acetate is activated into acetyl-CoA through two reactions catalyzed by acyl-CoA synthetase (reaction 1 and 2), both of which are ATP-dependent (Ellis *et al.,* 2010; Pol *et al.,* 2014):

$$\text{Acetate} + \text{ATP} \longrightarrow \text{Acyl-AMP} + \text{PPi} \quad (1)$$

$$\text{Acyl-AMP} + \text{Coenzyme-A} \longrightarrow \text{Acetyl-CoA} + \text{AMP} \quad (2)$$

Similarly, the conversion of butyrate into two acetyl-CoA using butyryl-CoA:acetate-CoA transferase is possible through formation of butyryl-CoA in bacteria cells (Duncan *et al.,* 2002; Vital *et al.,* 2014). In yeast and microalgae cells, the butyrate undergoes ß-oxidation (reaction 3 (Maggio-Hall & Keller, 2004)) through formation acyl-CoA thioester and subsequent yielding acetyl-CoA (Feron *et al.,* 2005; Gabriel *et al.,* 2014; Baroukh *et al.,* 2017).

$$\text{Butyrate} + 2\ \text{ATP} + 2\ \text{Coenzyme-A} \longrightarrow 2\ \text{Acetyl-CoA} + 2\ \text{AMP} + 2\ \text{PPi} \quad (3)$$

The methylmalonyl-CoA pathway regulates fatty acid synthesis from propionate. Propionate is first activated into propionyl-CoA (reaction 4) and then converted into succinyl-CoA (reaction 5), the intermetabolite of the tricarboxylic acid cycle (TCA cycle) (Pronk *et al.,* 1994).

$$\text{Propionate} + \text{ATP} + \text{Coenzyme-A} \longrightarrow \text{Propionyl-CoA} + \text{AMP} + \text{PPi} \quad (4)$$

$$\text{Propionyl-CoA} \longrightarrow \text{(2S) methylmalonyl-CoA} \longleftrightarrow \text{(2R) methylmalonyl-CoA} \longrightarrow \text{Succinyl-CoA} \quad (5)$$

After formation of succinyl-CoA (reaction 6), an intermediate of the TCA cycle, the reactions of the cycle are triggered and leaded to formation of acetyl-CoA.

Lipid accumulation occurs through similar mechanisms in oleaginous yeast and heterotrophically grown microalgae. Lipid accumulation in yeast and microalgae cells is a complex process including the production of acetyl-CoA and NADH, fatty acid chain biosynthesis, generation of neutral and polar lipids due to allocation of acyl moieties, and lipid droplet formation (Garay *et al.,* 2014). In addition, acetyl-CoA, propionyl-CoA and butyryl-CoA can be used as a substrate for condensation with malonyl-CoA. Acetyl-CoA can be easily replaced by propionyl-CoA due to their high affinity to NADPH dehydrogenase, when the butyryl-CoA has very small degree of incorporation into fatty acids (Bressler & Wakil, 1961). Biosynthesis of lipids in oleaginous microorganisms is forced by under-regulation of the AMP deaminase activity due to nutrient limitation, usually nitrogen (Ratledge, 2004; Jin *et al.,* 2015). AMP breakdown leads to citrate accumulation through an interrupted TCA cycle (Pol *et al.,* 2014).

The first step involved in *de novo* lipid accumulation is the production of 1 mol of the key intermediate acetyl-CoA (along with oxaloacetate) from excess citrate derived from TCA cycle, and the production of 2 mol NADPH. To form malonyl-CoA, acetyl-CoA is carboxylated by acetyl-CoA carboxylase (ACC 1) (reaction 6) (Ratledge, 2004).

$$HCO_3^- + ATP + \text{acetyl-CoA} \xrightarrow[\text{ACC 1}]{} ADP + PPi + \text{malonyl-CoA} \qquad (6)$$

This reaction is an ATP-dependent carboxylation with biotin as the enzyme prosthetic group (Ratledge, 2004). Then, seven units of activated malonate (malonyl-CoA) are added to acetyl-CoA, adding 2-carbon units to the growing fatty acyl chain until palmitoyl-CoA is formed (catalyzed by fatty acyl synthase (FAS)). Type I fatty acyl synthase (FAS I) in yeast cells facilitates elongation of the fatty acyl chain. The fatty acyl chain elongation occurs via plastidial FAS II or cytosolic FAS I during heterotrophic growth in algae cells. Palmitate is a precursor for different fatty acids formed through subsequent elongation or/and desaturation reactions (Ratledge, 2004; Jacquier *et al.,* 2011; Garay *et al.,* 2014; Pol *et al.,* 2014; Jin *et al.,* 2015).

As a third step, the generation of neutral and polar lipids occurs due to acylation by acyltransferases of glycerol 3-phosphate by fatty acyl-CoA to yield phosphatidic acid. The phosphate group of the phosphatidic acid is removed via phosphatidic acid phosphatase forming 1,2-diacylglycerol and, then, the acyl transferase transfers the third acyl group from an acyl-CoA molecule to generate a triacylglycerol (Ratledge, 2004; Pol *et al.,* 2014; Jin *et al.,* 2015).

Lipid droplets formation is the final step of lipid accumulation by oleaginous microorganisms. According to Pol *et al.* (2014), lipid droplets are "*the intracellular organelle specialized in assembling, storing, and then supplying lipids*" (Pol *et al.,* 2014, p. 635). Lipid droplets consist of randomly packed lipids, with the centre consisting mainly of triacylglycerols, with some diacylglycerols, monoacylglycerols and sterol esters, surrounded by monolayer of phospholipids with perilipin family proteins contained within (Ratledge, 2004).

There are several models describing lipid droplet formation. The first two, the lensing model and bicell formation model, involve accumulation of triacylglycerides between two endoplasmic reticulum membranes, with the phospholipid monolayer surrounding the lipids originating from the outer membrane or both leaflets of the endoplasmic reticulum membrane, respectively. The third model, the vesicles model, describes the possibility of forming secretory vesicles directly from the endoplasmic reticulum (Guo *et al.*, 2009).

Recent functional assays determined solid association of lipid droplets with the endoplasmic reticulum membrane (Jacquier *et al.,* 2011, Fei *et al.,* 2011). It was shown that lipid droplets do not constitute an independent cytosolic organelle, but rather are functionally linked to the endoplasmic reticulum membrane (Jacquier *et al.,* 2011). The seipin homolog Fld1p facilitates trafficking of lipids and proteins between endoplasmic reticulum and lipid droplets, and regulates the fusion process (Fei *et al.,* 2011). However, the mechanisms involved in the maintenance of the discrete size of lipid droplets, their fusion and ripening are actively studying (Wang, 2015).

2.3. Aqueous by-product streams generated from conversion technologies

Currently, lignocellulose derivatives and waste greases, as well as oil-rich algae and yeast biomass are the preferred feedstocks for thermochemical conversion to produce liquid fuels (Brown, 2003; Lee & Lavoie, 2013; Elliot *et al.,* 2015; Xu *et al.,* 2016). Feedstock is subjected to high temperature, either with or without the

action of the different catalysts or chemicals, to transform biomass into the desired product, liquid biofuel. Lipid conversion routes include transesterification, hydrotreating, metal-catalyzed deoxygenation, acid-catalyzed cracking, and pyrolysis (Crocker, 2010). The products generated include fatty acid alkyl esters, n-alkanes, n-alkenes, aromatics and other chemical compounds (Brown, 2003).

Hydrothermal liquefaction, catalytic pyrolysis, and fast pyrolysis are common, well developed, and extensively applied technologies (Crocker, 2010; Elliot *et al.,* 2015; Xu *et al.,* 2016). Lipid pyrolysis can produce drop-in fuels, without catalyst application, that are functionally equivalent to petroleum fuels (Maher, Bressler, 2007; Maher *et al.,* 2008; Asomaning *et al.,* 2014a; Asomaning *et al.,* 2014b; Bressler, 2011; Espinosa-Gonzalez, 2014a; Espinosa-Gonzalez, 2014b, Jenab *et al.,* 2014). These technologies led to the production of aqueous by-product streams that can potentially be used for subsequent biofuel generation (Echim *et al.,* 2009; Paasikallio *et al.,* 2014; Espinosa-Gonzalez *et al.,* 2014a), value-added applications (Tsuoko *et al.,* 2015), and recycling options (Espinosa-Gonzalez, 2014b), depending on their composition.

2.3.1. Hydrothermal liquefaction

Hydrothermal liquefaction is the biomass-to-biocrude pyrolysis in water using temperatures from 280 – 370 °C and pressures between 4-24 MPa (Crocker, 2010). Lignocellulosic biomass, as well as macroalgae and microalgae biomasses, are usually applied as feedstocks for hydrothermal liquefaction, with biocrude yields of 35 %, 27 %, and 38 - 64 %, respectively (Elliot *et al.,* 2015). The biocrude

recovered is more deoxygenated compared to the bio-oil produced through fast pyrolysis. Because of the wide range of compounds contained within, biocrude needs to be upgraded before it can be combusted for energy production and used as biofuel (Jena *et al.,* 2015). During the hydrothermal liquefaction of organic compounds, up to 40 % are distributed into the aqueous by-product streams. According to Krochta *et al.* (1984), after alkaline thermochemical treatment of cellulose at processing temperatures from 200 - 280 °C, significant yields of formic, acetic, glycolic and lactic acids were observed; at higher temperatures of 280 – 370 °C, the main acids formed were acetic and propionic acids (Krochta *et al.,* 1984). It was shown by Paasikallio *et al.* (2014) that after hydrothermal liquefaction of lignocellulosic biomass, 27 – 40 % of the carbon yield in the aqueous by-product phase consisted of acetic and glycolic acids (Paasikallio *et al.,* 2014).

2.3.2. Catalytic pyrolysis

Catalytic fast pyrolysis or catalytic pyrolysis is a biomass-to-bio-oil pyrolysis with a solid catalyst (Crocker, 2010). The main product is a partially deoxygenated bio-oil containing some solids and water (Crocker, 2010; Black *et al.,* 2016). Synthetic zeolite as a solid catalyst is typically used in catalytic pyrolysis (Paasikallio *et al.,* 2014; Black *et al.,* 2016). Carbon yield of volatile fatty acids accounts for up to 15 % of the aqueous by-product phase generated through catalytic fast pyrolysis of pine sawdust in the presence of a HZSM-5 catalyst (synthetic form of zeolite) (Paasikallio *et al.,* 2014). Catalytic pyrolysis of carbohydrate materials has been shown to generate acetic acid, phenol, hydrozyacetone, acetaldehyde, and sugar-type

compounds (Paasikallio *et al.,* 2014). Acetic, formic, propanoic acids, acetaldehyde, and more than 100 others compound were detected in the aqueous phase after catalytic fast pyrolysis of lignocellulosic feedstock with catalyst ZSM-5 by Black *et al.* (2016).

2.3.3. Fast pyrolysis

Biomass-to-bio-oil fast pyrolysis occurs very quickly at high temperatures from 480 – 520 °C (Crocker, 2010). As the main product of fast pyrolysis, the bio-oil composition is very complex and represents mixtures of oxygenated hydrocarbons with solids and water, similar to the bio-oil obtained through catalytic fast pyrolysis were the catalyst is present (Paasikkallio *et al.,* 2014; Black *et al.,* 2016). For use as biofuel, the bio-oil received after fast pyrolysis need to be refined. Organics (~ 60 %), water (~ 15 %), gas (~ 13 %), and char (~ 12 %) are products of fast pyrolysis of wood biomass feedstock (Bridgwater, 2012). Carbon yield of volatile fatty acids accounts for 5 - 10 % of the aqueous by-product phase generated through fast pyrolysis. The major compounds present are acetic acid, ethylene glycol, propylene glycol, and phenol (Paasikkallio *et al.,* 2014).

2.3.4. Value-added applications for aqueous by-product streams

The organic material present in aqueous by-product streams has potential for recovery and value-added applications. The aqueous by-product stream generated after thermochemical conversion of biomass has an extremely complex composition.

For example, researchers reported the presence of up to 400 compounds, mainly furan derivatives, carboxylic acids, phenolic compounds and other components in the aqueous by-product stream after thermochemical conversion of lignocellulosic materials (Garcia-Perez *et al.,* 2010).

Some applications for valorization of aqueous by-product streams/phases received through various thermochemical conversion processes have been demonstrated. For instance, it was shown that the organics in the aqueous byproduct stream that are generated during bio-oil production can be successfully processed to hydrogen (Leiva-Candia *et al.,* 2014; Magrini, 2015) or methane (Demirel & Yenigun, 2002). The recovery of acetic acid (up to 84 %) by reactive extraction using tri-*n*-octylamine in 2-ethyl-hexanol from the aqueous phase obtained through generation of pyrolysis oil was demonstrated by Rasrendra *et al.* (2011). Production of succinic acid using the transgenic *Escherichia coli* strain grown in modified media containing 20 % (v/v) aqueous phase from bio oil production was investigated by Wang *et al.* (2013). The conversion of organics in the aqueous phase obtained through pyrolysis for bio-oil production to acetic acid, acetol, glycolaldehyde and phenolic derivatives has also been demonstrated (Vitasari *et al.,* 2015). It has also been shown that crude glycerol present in the aqueous by-product phase after hydrothermal treatment of oils and fats (Espinosa-Gonzalez, 2014b) could serve as the sole carbon source for both yeast *C. curvatus* and microalgae *C. protothecoides* growth. It was reported by Lian *et al,* (2012) that the carboxylic acids present in the aqueous phase after lignocellulosic biomass pyrolysis could be used for yeast lipid production. Among the many different carbon molecules produced through

pyrolysis, carboxylic acids was chosen as a main carbon source for yeast fermentation. Before fermentation was started, the aqueous phase generated after lignocellulosic biomass pyrolysis was subjected to detoxification and adjustment. It was reported that after 144 h of cultivation of *C. curvatus* in the aqueous phases with 20 g/L of acetate received after pyrolysis of lignocellulosic biomass, the amount of biomass produced was 6.9 g/L and the amount of lipids was 2.2 g/L (Lian *et al.,* 2012).

2.4. Conclusion

As waste substrates, yellow and brown greases, as well as biosolids, are considered as non-food carbon feedstocks with a low- and negative- value, respectively. These types of wastes can potentially be used as the lipid and water feedstocks for application in a lipid pyrolysis technology to produce biofuels. The present concerns about disposal, utilization, and recycling of the aqueous by-product waste streams obtained after hydrolysis and pyrolysis may be addressed by using these streams as biorefinery feedstocks.

As an alternative to the first- and second- generation feedstocks, oleaginous microorganisms, especially yeast and microalgae, are the most promising third-generation feedstocks for renewable fuel production. Oleaginous microorganisms are able to utilize the waste substrates to produce biofuels, their high ability to accumulate lipids, their ability to survive the toxic environment of the fermentation feedstock, and their high salt and temperature tolerance.

Because of the extremely complex composition of the aqueous streams received after biofuel conversion technologies, the applications of these streams to grow oleaginous microorganisms are very limited thus far. It has been demonstrated that the main carbon-containing compounds in the aqueous streams after numerous biofuel conversion technologies are volatile fatty acids. As described above, recent studies indicate that the different volatile fatty acids can be applied as the single carbon source, as well as in mixtures, for cultivation and lipid production of oleaginous microorganisms. In this study, we are planning to compare and analyze the volatile fatty acids for oleaginous microorganisms' cultivation and lipid accumulation in specific by-product streams from lipid pyrolysis. In addition, evaluation of the aqueous by-product streams obtained after hydrolysis of 4% biosolids, brown grease, and their mixtures as nutrients for oleaginous yeast *C. curvatus* and microalgae *C. prototheco*ides growth and lipid accumulation will be done. As of yet, there has been no report describing the characterization of lipid pyrolysis aqueous by-product streams, and their subsequent application for growth and lipid accumulation in the oleaginous yeast *C. curvatus* and microalgae *C. prototheco*ides.

CHAPTER 3. MATERIALS AND METHODS

3.1. Oleaginous microorganisms

As lipid producers, the yeast *Cryptococcus curvatus* and microalgae *Chlorella protothecoides* were used in this study. Yeast extract peptone dextrose (YPD) agar media was used for the storage of the yeast *Cryptococcus curvatus*. The yeast inoculum was grown in YPD broth for 24 h at 30 °C and shaking at 200 rpm.

Microalgae *Chlorella protothecoides* were stored for long term at room temperature with a light/dark cycle in APT (All purpose Tween 80) agar media. The microalgae inoculum was grown in APT broth for 72 h at 25 °C, and shaking at 140 rpm to produce algae starter cultures. *C. protothecoides* was grown heterotrophically in all experiments.

3.2. Materials

Base mineral media were used for yeast (Hassan *et al.,* 1993) and algae (Espinosa-Gonzalez *et al.,* 2014b) cultivation with glucose and urea as sources of carbon and nitrogen, respectively. Salts composition in the base mineral media and the company that they were purchased from are stated in Tables 3.1 and 3.2.

Difco YPD broth and Difco APT broth were obtained from Becton, Dickinson and Company (Sparks, MD, USA), agar was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Glucose (99.5 %), urea (98 %), formic acid (88 %), 1-hexanol (99.5 %), N, N-dimethylformamide (anhydrous, 99.6%), and

Table 3.1. Essential minerals used for growth of *C. curvatus*.

Name, Formula	Supplier Purity	Concentration of salt used in base mineral media	Essential mineral	Amount of essential minerals in base mineral media	Amount of essential minerals in biosolids aqueous phase *	Added amount of essential minerals **
Potassium phosphate monobasic, $KH_2\ PO_4$	Fisher Scientific Fairlawn, NJ, USA, 99 %	7.0 g/L	K^+	2.01 g/L	0.41 g/L	5.6 g/L
Sodium phosphate dibasic dodecahydrate, $Na_2HPO_4 \cdot 12H_2O$	ACROS Organics, New Jersey, USA, 99 %	2.0 g/L	Na^+	0.26 g/L	0.18 g/L	0.6 g/L
Magnesium sulfate heptahydrate, $MgSO_4 \cdot 7H_2O$	Fisher Scientific Fairlawn, NJ, USA, 99 %	1.5 g/L	Mg^{2+}	0.15 g/L	0.06 g/L	0.9 g/L
Calcium chloride dehydrate, $CaCl_2 \cdot 2H_2O$	Fisher Scientific Fairlawn, NJ, USA, 99 %	0.1 g/L	Ca^{2+}	0.03 g/L	0.36 g/L	N/A
Iron (III) chloride hexahydrate, $FeCl_3 \cdot 6H_2O$	ACROS Organics, New Jersey, USA, 98 %	24 mg/L	Fe^{3+}	4.96 mg/L	12.1 mg/L	N/A
Copper (II) sulfate, $CuSO_4$	Sigma-Aldrich St. Luis, MO, USA, 99 %	7 mg/L	Cu^{2+}	2.78 mg/L	0.08 mg/L	6.8 mg/L

Zinc sulfate heptahydrate, $ZnSO_4 \cdot 7H_2O$	Sigma-Aldrich St. Luis, MO, USA, 99 %	5 mg/L	Zn^{2+}	1.14 mg/L	4.04 mg/L	N/A
Manganese (II) sulfate monohydrate, $MnSO_4 \cdot H_2O$	Fisher Scientific Fairlawn, NJ, USA, 98 – 101 %	2 mg/L	Mn^{2+}	0.65 mg/L	2.71 mg/L	N/A
Ammonium chloride NH_4Cl	Fisher Scientific Fairlawn, NJ, USA, 99 %	0.645 g/L				0.645 g/L
Thiamine hydrochloride, (Vitamin B_1)	Sigma-Aldrich St. Luis, MO, USA, 99 %	40 µg/L				40 µg/L

* amount of essential minerals in biosolids aqueous by-product phase was analyzed with an Inductively Coupled Plasma Mass Spectrometry at the Canadian Center for Isotopic Microanalysis, University of Alberta

** added amount of essential minerals was calculated based on difference between amount of essential minerals in base mineral media and amount of essential minerals in biosolids aqueous phase and recalculated to actual salt concentration

N/A - not added

Table 3.2. Essential minerals used for growth of *C. protothecoides*.

Name, Formula	Supplier Purity	Amount of salt used in base mineral media	Essential minerals	Amount of essential minerals in base mineral media	Amount of essential minerals in biosolids aqueous phase *	Added amount of essential minerals **
Potassium phosphate monobasic, $KH_2\ PO_4$	Fisher Scientific Fairlawn, NJ, USA, 99 %	2.8 g/L	K^+	2.01 g/L	0.2 g/L	2.5 g/L
Potassium phosphate dibasic, K_2HPO_4	Fisher Scientific Fairlawn, NJ, USA, 99 %	2.0 g/L	K^+	0.89 g/L	0.2 g/L	1.5 g/L
Magnesium sulfate heptahydrate, $MgSO_4 \cdot 7H_2O$	Fisher Scientific Fairlawn, NJ, USA, 99 %	1.2 g/L	Mg^{2+}	0.15 g/L	0.06 g/L	0.7 g/L
Manganese (II) chloride tetrahydrate, $MnCl_2 \cdot 4H_2O$	Sigma-Aldrich St. Luis, MO, USA, 99 %	7.2 g/L	Mn^{2+}	1.99 g/L	0.002 g/L	7.2 g/L
Iron (II) sulfate heptahydrate, $FeSO_4 \cdot 7H_2O$	Sigma-Aldrich St. Luis, MO, USA, 99 %	48 mg/L	Fe^{2+}	9.64 mg/L	12.1 mg/L	N/A
Boric acid, H_3BO_3	Fisher Scientific Fairlawn, NJ, USA, 99.5 %	11.6 mg/L	B^{3+}	2.03 mg/L	1.89 mg/L	0.8 g/L
Calcium chloride dehydrate, $CaCl_2 \cdot 2H_2O$	Fisher Scientific Fairlawn, NJ, USA, 99 %	10 mg/L	Ca^{2+}	2.73 mg/L	365 mg/L	N/A

Zinc sulfate heptahydrate, $ZnSO_4 \cdot 7H_2O$	Sigma-Aldrich St. Luis, MO, USA, 99 %	0.88 mg/L	Zn^{2+}	0.2 mg/L	4.04 mg/L	N/A
Copper(II) sulfate, $CuSO_4$	Sigma-Aldrich St. Luis, MO, USA, 99 %	0.32 mg/L	Cu^{2+}	0.13 mg/L	0.08 mg/L	0.12 mg/L
Molybdenum(VI) oxide, MoO_3	Sigma-Aldrich St. Luis, MO, USA, 99.5 %	72 µg/L	Mo^{3+}	0.048 µg/L	0.002 µg/L	69 µg/L
Thiamine hydrochloride (Vitamin B_1)	Sigma-Aldrich St. Luis, MO, USA , 99 %	40 µg/L				40 µg/L

* amount of essential minerals in biosolids aqueous by-product phase was analyzed with an ICPMS at the Canadian Center for Isotopic Microanalysis, University of Alberta

** added amount of essential minerals was calculated based on difference between amount of essential minerals in base mineral media and amount of essential minerals in biosolids aqueous phase and recalculated to actual salt concentration

N/A - not added

acetonitrile (anhydrous, 99.8 %) were obtained from Sigma-Aldrich (St. Louis, MO). Glycerol (glycerin > 95 %) and propionic acid (95 %) were purchased from Fisher Scientific (Fairlawn, NJ, USA). *n*-butyric acid (99 %) was procured from Fisher Scientific (Rochester, NY, USA). The volatile free acid mix was purchased from Supelco Analytical (Bellefonte, PA). To prepare media and solutions, Milli-Q water was used (Milli-Q, Millipore SAS, Molsheim, France).

Samples of the aqueous by-product streams were directly received in the Biorefining Conversions and Fermentation Laboratory, University of Alberta, as prepared and stored in airtight containers to prevent loss of volatiles at 4 °C until needed. They were then were used "as-is" with no additional pre-treatment.

3.3. Growth determination

Two types of growth assessment were used in this study. The first one was applied to samples grown under standard conditions in colorless media. The growth of yeast and algal cultures was determined using the optical density (OD 600 nm) parameter with a UV/Visible Spectrophotometer Ultrospec 4300 Pro made by GE Healthcare, Piscataway, NJ, USA. To create standard curves, cell dry weight (g/L) was measured and correlated with the optical density readings (Sutton, 2011).

For yeast: $y = 0.8138\ x - 0.1002$ ($R^2 = 0.9983$)

For algae: $y = 1.43913\ x - 0.13306$ ($R^2 = 0.9995$)

where x = OD 600 nm and y = cell dry weight (g/L).

The second one was used to determine the growth of yeast and algae cells in colored media and in the media with high concentrations of acetic, propionic and butyric acids. Briefly, the cell dry weight was determined by traditional oven drying method. Final cell dry weight was recalculated per g per L.

Medium pH was determined directly with a pH meter (Model AB 15 Plus Fisher Scientific (Fairlawn, NJ)).

3.4. Screening experiments

The tolerance of *C. protothecoides* and *C. curvatus* to the aqueous by-product after pyrolysis was analyzed using screening procedures adapted from Espinosa-Gonzalez *et al*. (Espinosa-Gonzalez *et al.,* 2014c). For screening experiments, 96-well microplates were used. Briefly, 190 μL of previously filtered (0.22 μm) aqueous by-product stream (produced through pyrolysis) was added to each well. The same amount of base mineral media was added to the wells to individual lanes (n=12) of a microplate in different dilutions. The positive control in these experiments was a well containing the base mineral media supplemented with glucose as a carbon source (rather than the aqueous by-product stream). As a control, cultures were also grown using glucose as the carbon source; the amount of available carbon in this control was equivalent to the amount of carbon from volatile fatty acids in the well.

The pH of the aqueous by-product stream after lipid pyrolysis was 2.4 (original sample). For screening experiments, the pH of the taken feedstock was

adjusted to 5.4 and 6.4 for yeast and microalgae, respectively, using 5 N NaOH (adjusted sample). 10 μL of yeast or microalgae inoculum (5 % v/v) was used for microplate well inoculation. The inoculum was prepared using cells washed twice with corresponding phosphate buffer to ensure no carry over of nutrients. Microplates were incubated at 25 °C and shaking at 750 rpm in a microplate vibratory shaker (Heidolph, Schwabach, Germany) for 72 h for yeast and 120 h for microalgae cells, in the dark. Initial and final absorbances (OD 600 nm) were measured using a microplate reader (Biotek, Winooski, VT). Experimental replicates were monitored for contamination microscopically.

3.5. Batch experiments

In batch experiments, *C. protothecoides* and *C. curvatus* were grown in 50 mL of cultivation media in 250 mL Erlenmeyer flasks. Acetic acid, propionic acid, and butyric acid, and a mixture of volatile fatty acids were used in flask cultures with the base mineral media. The amount of volatile fatty acids used was chosen based on characterization of the aqueous by-product streams acquired after hydrolysis. The concentrations of acetic acid, propionic acid, and butyric acid, and mixtures of volatile fatty acids are specified in Chapters 3.5.1. and 3.5.2. As a control, cultures were grown using glucose as the carbon source; the amount of available carbon in this control was equivalent to the amount of carbon from volatile fatty acids in the experimental flasks. In other experiments, "original" aqueous byproduct streams obtained after hydrolysis of 4 % biosolids, brown grease, and a mixture of the two (1/1 (w/w) of the 4 % biosolids and brown grease; mixed before hydrolysis) were

used to grow microorganisms. To be used as inoculum, 5 % (v/v) of cells were washed twice using phosphate buffer (pH of the buffer corresponding to the pH of cultivation media), and then re-suspended in the same buffer for subsequent inoculation. Yeasts were grown aerobically at 30°C, 200 rpm, 120 h, and microalgae were grown in the dark for 168 h at 23°C, 140 rpm. The growth of *C. curvatus* and *C. protothecoides* were assessed by measuring cell dry weights. Experimental replicates were monitored for contamination microscopically.

3.5.1. Tolerance of *C. curvatus* and *C. protothecoides* to acetic, propionic, and butyric acids

Base mineral media was supplemented by 0 – 10 g/L of acetic acid, 0 – 3.5 g/L of propionic acid, and/or 0 – 2.5 g/L of butyric acid for yeast and algae cultivation. The pH of the cultivation media was adjusted through addition of 5 N NaOH to 5.4 and 7.0 for microalgae and yeast, respectively. As a basis for comparison, *C. curvatus* and *C. protothecoides* were also grown using glucose as the sole carbon source. In this case, the amount of glucose added to the cultivation media was equal to the amount of volatile fatty acids added (in terms of available carbon) to maintain a carbon to nitrogen ratio of 100/1.

3.5.2. Oleaginous microorganisms growth and lipid accumulation with acetic, propionic, and butyric acids as a single and mixed carbon sources

To examine growth of oleaginous *C. curvatus* and *C. protothecoides* using volatile fatty acids, base mineral media was supplemented with acetic acid (5, 10, 20, or 30 g/L), propionic acid (1.5, 2, or 2.5 g/L), or butyric acid (0.5, 1, or 1.5 g/L). In these experiments two different ratios, 1:1:1 and 20:3.3:1 (acetic acid: propionic acid: butyric acid, accordingly), were used. In the ratio 1:1:1, the concentration of each volatile fatty acids was 1 g/L in the base mineral media. Additionally, as a mixed carbon source feedstock, a ratio of 20:3.3:1 was used along with the base mineral media, with the concentrations of the volatile fatty acids set at 60 g/L for acetic acid, 10 g/L for propionic acid and 3 g/L for butyric acid (stock solution). The dilutions of volatile fatty acids (20:3.3:1) by one (1:1), two (1:2), and three times (1:3) were done according to the initial concentration of each of volatile fatty acids, and the amount of base mineral media remains constant throughout. As a control, *C. curvatus* and *C. protothecoides* were also grown using glucose as a carbon source, where the amount of glucose added to the cultivation media was equal to the amount of carbon of added via volatile fatty acids to maintain a carbon to nitrogen ratio of 100/1.

3.5.3. Oleaginous microorganisms growth and lipid accumulation with the aqueous by-product streams

Biomass production and lipid accumulation were assessed for *C. curvatus* and *C. protothecoides* in the aqueous by-product streams recovered after hydrolysis of 4 % biosolids, brown grease, and mixtures of the two (50/50 (w/w) of 4 % biosolids and brown grease mixed before hydrolysis). At the Natural Resources Analytical Laboratory, University of Alberta, total organic carbon and total nitrogen in the aqueous by-product streams were determined using a Shimadzu TOC-V (Shimadzu Corporation, Analytical &Measuring Instrument Division, Nakagyo-ku, Kyoto, Japan). The pH of the aqueous by-product streams was adjusted to 7.0 with 5 N NaOH before its use as a growth medium for both, yeast and algae. *C. curvatus* was grown for a total of 120 h or 168 h at 30 °C, 200 rpm. *C. protothecoides* was grown heterotrophically for a total of 168 h or 240 h at 25 °C, 140 rpm.

The aqueous by-product stream after hydrolysis of the mixed feedstock was supplemented by glycerol adding 234.0 g/L and 121.0 g/L to adjust the carbon to nitrogen ratio to 50/1 and 30/1, respectively. Glycerol consumption was measured and *C. curvatus* and *C. protothecoides* growth and lipid accumulation were evaluated after 168 h and 240 h of cultivation, respectively. Contamination was monitored microscopically during experiments.

3.6. Analytical methods

The water content of the aqueous by-product streams was quantified using a gas chromatograph BRUCKER 456 GC equipped with an autosampler CP-8400, a

thermal conductivity detector (TCD), and a 4' x 1/8' Sulfinert Haye Sep R coated stainless steel column (Chromatographic Specialities Inc., Brockville, ON, Canada) packed with 60/80 mesh (180 to 250 μm) porous polymer packing material. Sample preparation and analysis was done according to ASTM D3792-05. Anhydrous acetonitrile was used as an internal standard.

Volatile fatty acids were analyzed by gas chromatography coupled with flame ionization detector (GC-FID) as published by Asomaning *et al.* (2014a) and Espinosa-Gonzalez *et al.* (2014a). It was done on an Agilent 7890A GC with an Agilent 7693 series autosampler and injector with a Stabilwax-BA (RESTEK) column (30 m × 0.53 mm × 0.5 μm). As a standard was used volatile free acid mix, as an internal standard was used 1 % 1-hexanol. Acidification using formic acid was done for volatile fatty acid analysis by GC-FID (Nollet, 2000). The amount of added formic acid was adjusted according to sample volume to maintain pH levels at 2.9 - 3.0.

The total fatty acid composition was evaluated by esterification with 3N methanolic HCl followed by GC–FID analyses (Espinosa-Gonzalez *et al.,* 2014b). Nonadecanoic acid methyl ester was used as an internal standard. For *C. curvatus* and *C. protothecoides*, lipid accumulation was analyzed by measured fluorescence using Nile Red as described by De la Hoz (2012), using a reference standard curve. For this measurement, microalgae biomass of known lipid content (14.1 – 42.6 % dry weight basis) and yeast biomass of known lipid content (13.1 – 52.4 % dry weight basis) were used to produce a standard curve (fluorescence signal vs. lipid percentage) for each experiment.

The glycerol concentration was determined using high performance liquid chromatography coupled with refractive index detector (HPLC-RID) and a Bio-Rad Aminex HPX87H column following the method published by Asomaning *et al.* (2014a) and Espinosa-Gonzalez *et al.* (2014a).

Total amino acids were analyzed using high performance liquid chromatography coupled with a fluorescent detector (HPLC-FD) (excitation at 340 nm, emission at 450 nm) using freeze-dried *C. curvatus* and *C. protothecoides* biomass. *O*-phthaldialdehyde was used for direct derivatization of the free amino acids (Sedgwick *et al.,* 1991).

The amount of essential minerals in the aqueous stream obtained through hydrolysis of 4 % biosolids was analyzed with an Inductively Coupled Plasma Mass Spectrometry at the Canadian Center for Isotopic Microanalysis, University of Alberta. When used in growth media, essential minerals were added to the aqueous by-product stream to ensure similar levels as when base mineral media was used for general cultivation of *C. curvatus* (Table 3.1) and *C. protothecoides* (Table 3.2).

3.7. Calculations

Statistical analysis of variance of data for biomass production, turbidity (OD 600 nm), lipid accumulation, water concentration, glycerol composition, volatile fatty acids composition and amino acids composition were done using the unpaired Tukey test ($p < 0.05$). Significant differences are indicated throughout using asterisks (Fay & Gerow, 2013).

CHAPTER 4. RESULTS

4.1. *C. curvatus* and *C. protothecoides* growth in original and neutralized aqueous by-product streams after lipid pyrolysis

The aqueous by-product streams were taken for initial characterization after the hydrolysis and pyrolysis stages of a lipid pyrolysis process. All aqueous by-product streams examined had an acidic pH from 2.7 to 4.1 for samples after hydrolysis, and a pH from 2.0 to 2.5 for samples after pyrolysis. Table 4.1 indicates general characterization (volatile fatty acids, glycerol and water content) of the two aqueous by-product streams from the lipid pyrolysis process generated through hydrolysis of brown grease and pyrolysis of yellow grease-derived fatty acids. These results express the means of multiple sample characterizations of the different lipid pyrolysis runs.

It was determined that the aqueous by-product stream after hydrolysis of yellow grease samples contained 88.9 ± 0.2 % water. The water content in the samples of the aqueous by-product stream after pyrolysis of yellow grease-derived fatty acids was 91.9 ± 3.9 %. The composition of the aqueous by-product streams from pyrolysis technology process depends on the feedstocks and operation conditions of the pilot plant.

Table 4.1. Characterization of the sample composition of the aqueous by-product streams after lipid hydrolysis and pyrolysis.

	Aqueous by-product streams	
Composition	After hydrolysis	After pyrolysis
Volatile fatty acids	0.09 ± 0.01 %	7.8 ± 3.7 %
Glycerol	10.3 ± 0.2 %	0
Other compounds *	0.7 ± 0.2 %	0.3 ± 0.2 %
Water	88.9 ± 0.2 %	91.9 ± 3.9 %

* calculated by difference: Other compounds (%) = 100 % – Volatile fatty acids (%) – Glycerol (%) – Water (%)

Using GC-FID, it was determined that hydrolytic aqueous by-product streams received through bench scale application of the lipid pyrolysis process contain volatile fatty acids 0.09 ± 0.01 %, predominantly acetic acid (0.27 ± 0.02 g/L), propionic acid (0.15 ± 0.04 g/L), and valeric acid (0.14 ± 0.01 g/L) (Figure 4.1). Similarly, it was determined that pyrolytic aqueous by-product streams received through the pyrolysis process contain volatile fatty acids as well (7.8 ± 3.7 %), predominantly acetic acid (62.1 ± 3.2 g/L), propionic acid (10.9 ± 1 g/L), and butyric acid (3.6 ± 0.8 g/L) (Figure 4.2).

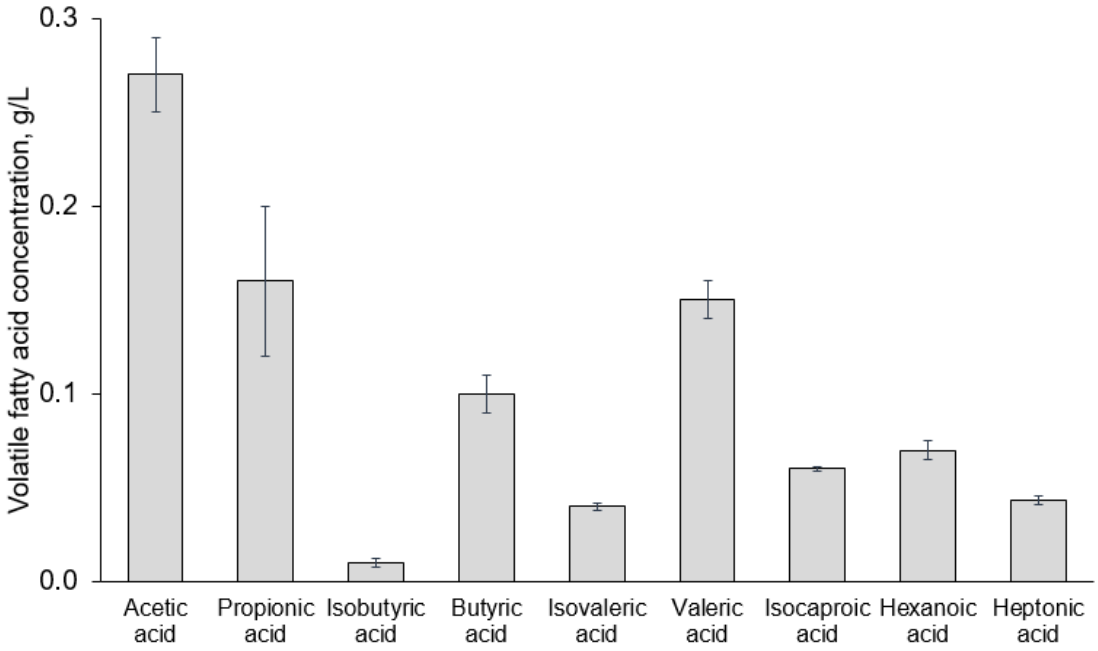

Figure 4.1. Volatile fatty acids content in the aqueous by-product stream after hydrolysis of brown grease detected by GC-FID.

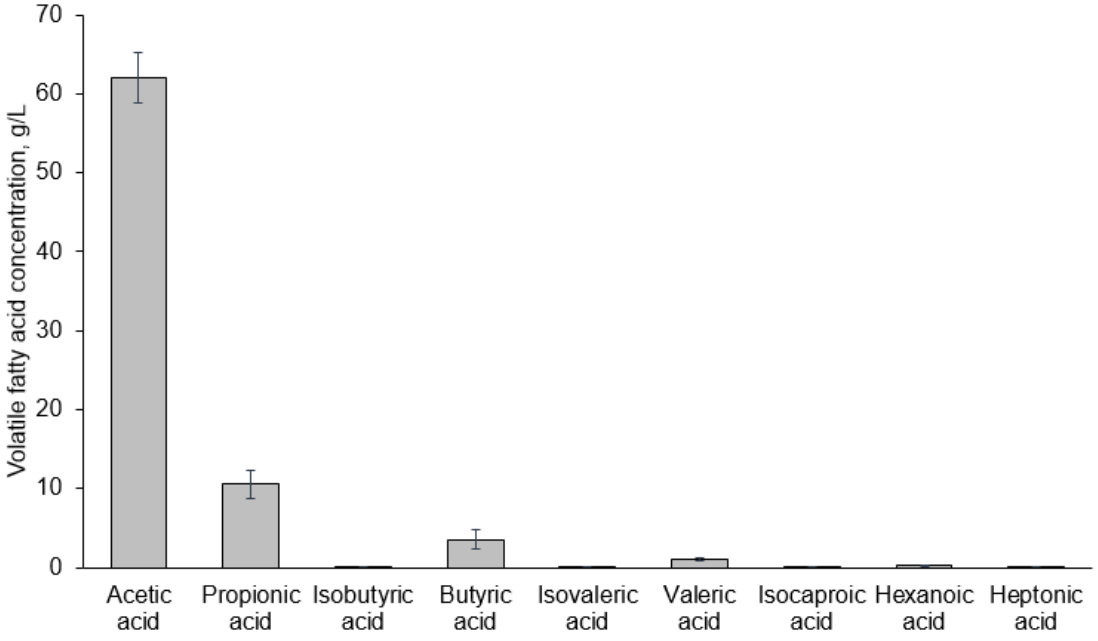

Figure 4.2. Volatile fatty acids content in the aqueous by-product stream after the pyrolysis step detected by GC-FID.

To examine the impact of the volatile fatty acids present in the aqueous by-product stream after lipid pyrolysis on the growth of the *C. curvatus* and *C. protothecoides*, the pH was adjusted to 5.4. The initial screening was done using the microplate cultivation method. As a control, glucose was used at a concentration such that the moles of carbon were the same as were present in the wells containing volatile fatty acids. In Figures 4.3 and 4.4, the light bars represent growth on base mineral medium and various concentrations of glucose. For the dark bars, the same amount of the base mineral medium was used, but the glucose has been replaced with different concentrations of the aqueous by-product stream after pH adjustment. In this case, the glucose was substituted with an equivalent amount of carbon, based on the volatile fatty acids levels determined by GC-FID. The aqueous by-product stream was used either as-is (original) or after pH adjustment with sodium hydroxide (neutralized).

The data gathered from screening experiments of studying the tolerance of *C. curvatus* and *C. protothecoides* to aqueous by-products obtained after the pyrolysis step showed that oleaginous yeast and algae can grow after dilution of the aqueous stream 1:64 to 1:256 and 1:128 to 1:256, respectively (Figures 4.3A and 4.4A). However, after the pH was adjusted, the growth of *C. curvatus* and *C. protothecoides* was observed in the 1:4 and 1:2 dilution, respectively, as shown on Figure 4.3B and 4.4B. Relative to control data, the growth of both microorganisms was significantly inhibited. Such growth inhibition suggested presence of the other inhibitory compounds. Similarly, the same tendency was observed for microorganisms

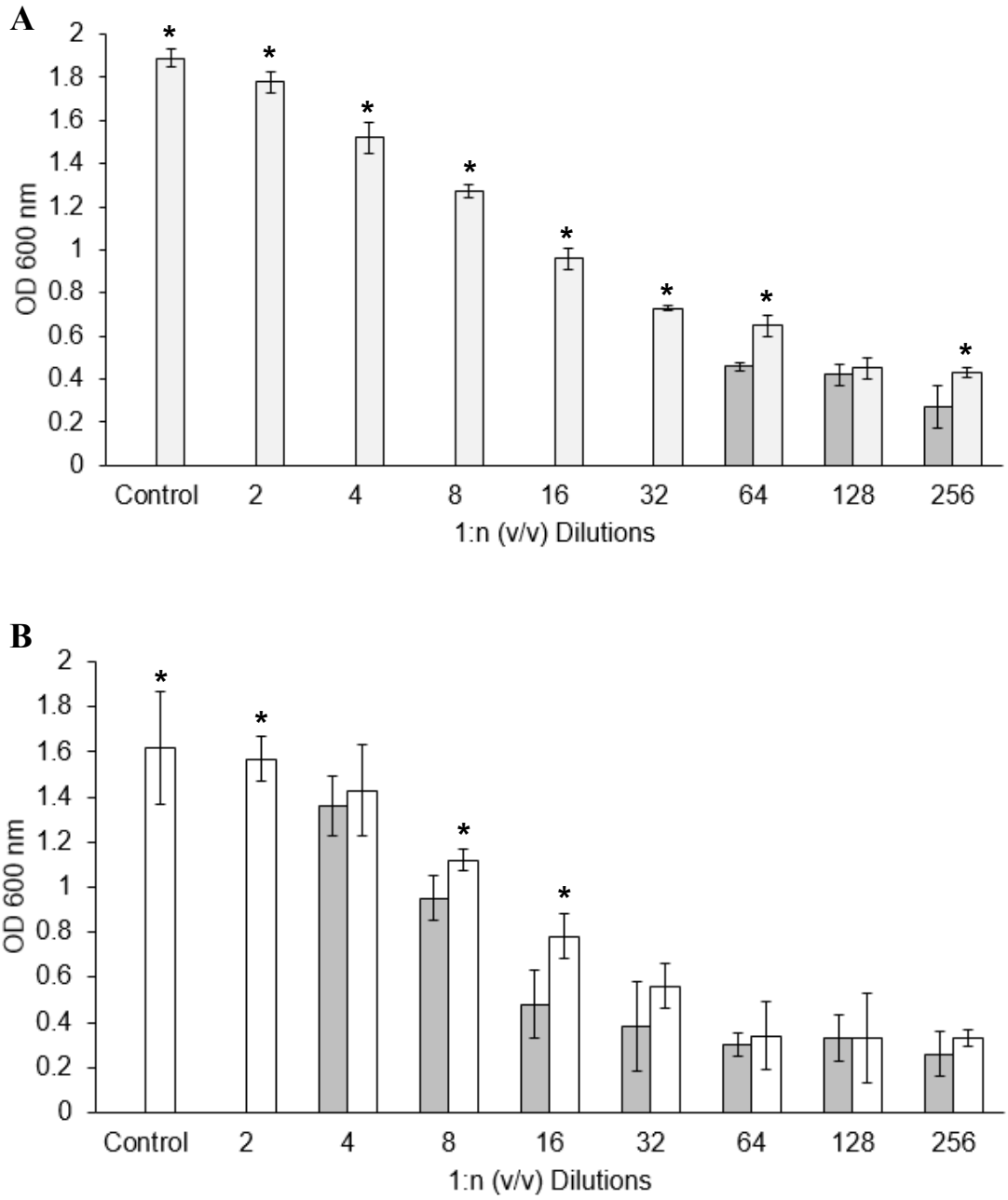

Figure 4.3. The growth of *C. curvatus* with the aqueous stream obtained after pyrolysis (dark bars) or glucose (positive control, white bars). At a given dilution, the moles of carbon from volatile fatty acids present in the pyrolytic aqueous by-product stream was equal to the moles of carbon in the glucose medium. The experiment was performed using pyrolytic aqueous streams that were either used "as is" (A), or adjusted to pH 5.4. Control cultures were grown with undiluted media. Asterisk (*) indicated differences in yeast growth relative to the positive control, white bars at given dilution that were statistically significant ($p < 0.05$), done in replicate (n=12).

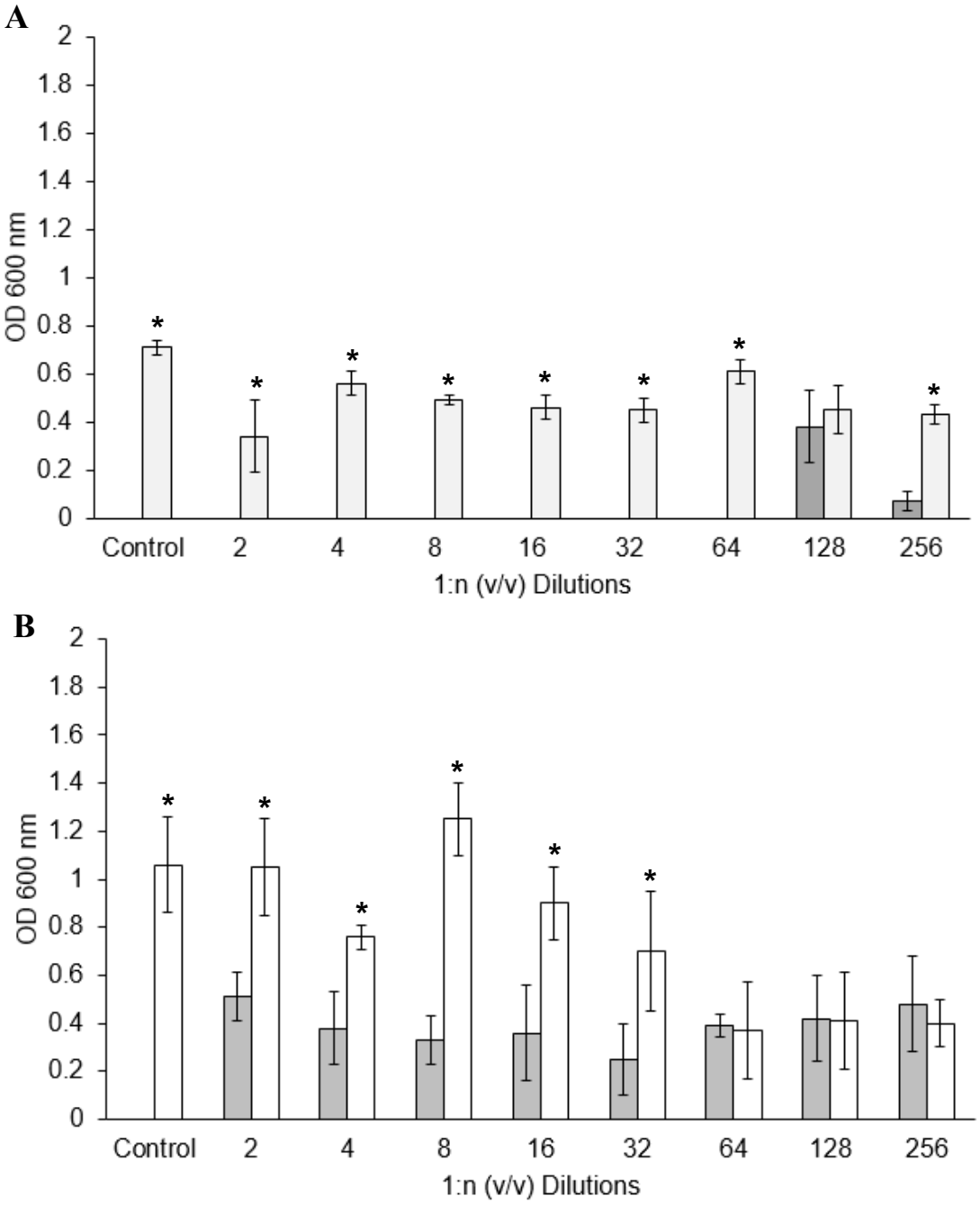

Figure 4.4. The tolerance of *C. protothecoides* to the aqueous by-product stream obtained after pyrolysis (dark bars) or glucose (positive control, white bars). The experiment was performed using pyrolytic aqueous streams that were either used "as is" (A), or adjusted to pH 6.4. Control cultures were grown with undiluted media. At a given dilution, the moles of carbon from volatile fatty acids present in the pyrolytic aqueous stream was equal to the moles of carbon in the glucose medium. Asterisk (*) indicated differences in algae growth relative to the positive control, white bars at given dilution that were statistically significant ($p < 0.05$), done in replicate (n=12).

growth in samples grown after pH neutralization at the high concentrations of aqueous by-products 1:4 to 1:64 (yeast cells) and 1:2 to 1:32 (algae cells). Further dilution led to leveling off of the influence of other inhibitory compounds present in the aqueous by-product stream.

This investigation has clearly indicated the sample composition of the aqueous by-product stream after the hydrolysis and pyrolysis steps of lipid pyrolysis. It was noted that the large amounts of volatile fatty acids are present in the samples of the aqueous by-product stream after the lipid pyrolysis stage: acetic, propionic and butyric acids in ratio 20:3.3:1. Based on these data, we decided to use these volatile fatty acids as the carbon source for fermentation of *C. curvatus* and *C. protothecoides* in subsequent experiments.

4.2. Acetic, propionic and butyric acids as single and mixed carbon sources to grow oleaginous yeast

Volatile fatty acids obtained from a chemical supplier (acetic, propionic and butyric acids) were used to grow oleaginous yeast biomass. In this study, we determined biomass growth and biomass yield on acetic, propionic, and butyric acids at two different pHs, 5.4 and 7.0. For all three acids examined, the biomass production of *C. curvatus* was significantly reduced when cells were grown at pH 5.4 compared to pH 7.0, for all studied acetic acid concentrations (Figures 4.5A), for 1.0 g/L – 2.5 g/L propionic acid concentrations (Figures 4.6A), for 0.5 g/L – 0.75 g/L butyric acid concentrations (Figures 4.7A). As shown in Figure 4.5A, the growth of *C. curvatus* at pH 5.4 was significantly reduced compared to pH 7.0 for acetic acid

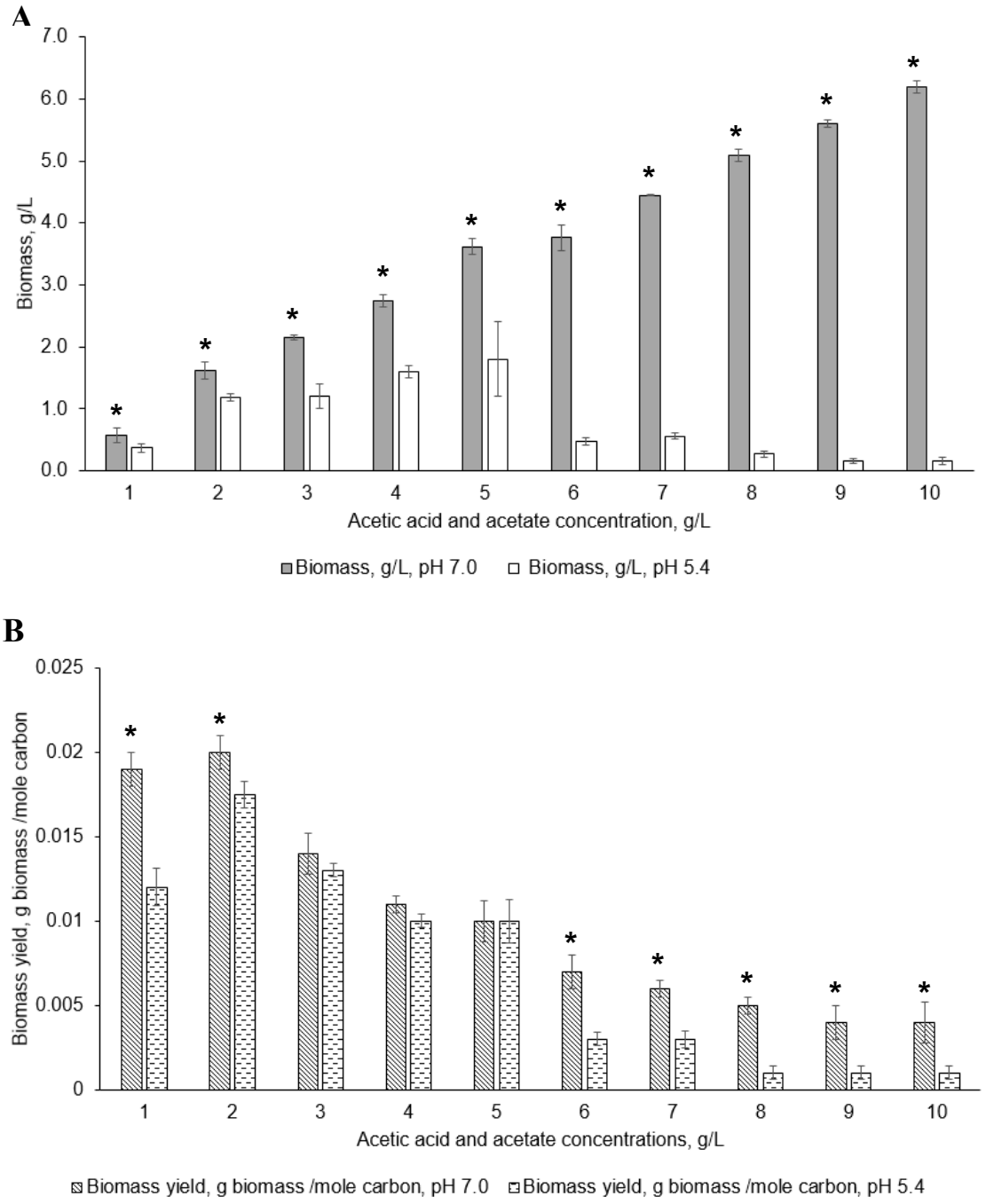

Figure 4.5. *C. curvatus* growth (A) and biomass yield (B) as produced biomass per utilized substrate in base mineral media supplemented with different amounts of acetic acid at pH 5.4 and pH 7.0. Asterisk (*) are indicated differences in biomass growth or biomass yield at pH 5.4 relative to growth or yield at pH 7.0 that were statistically significant ($p < 0.05$), done in replicate (n=3).

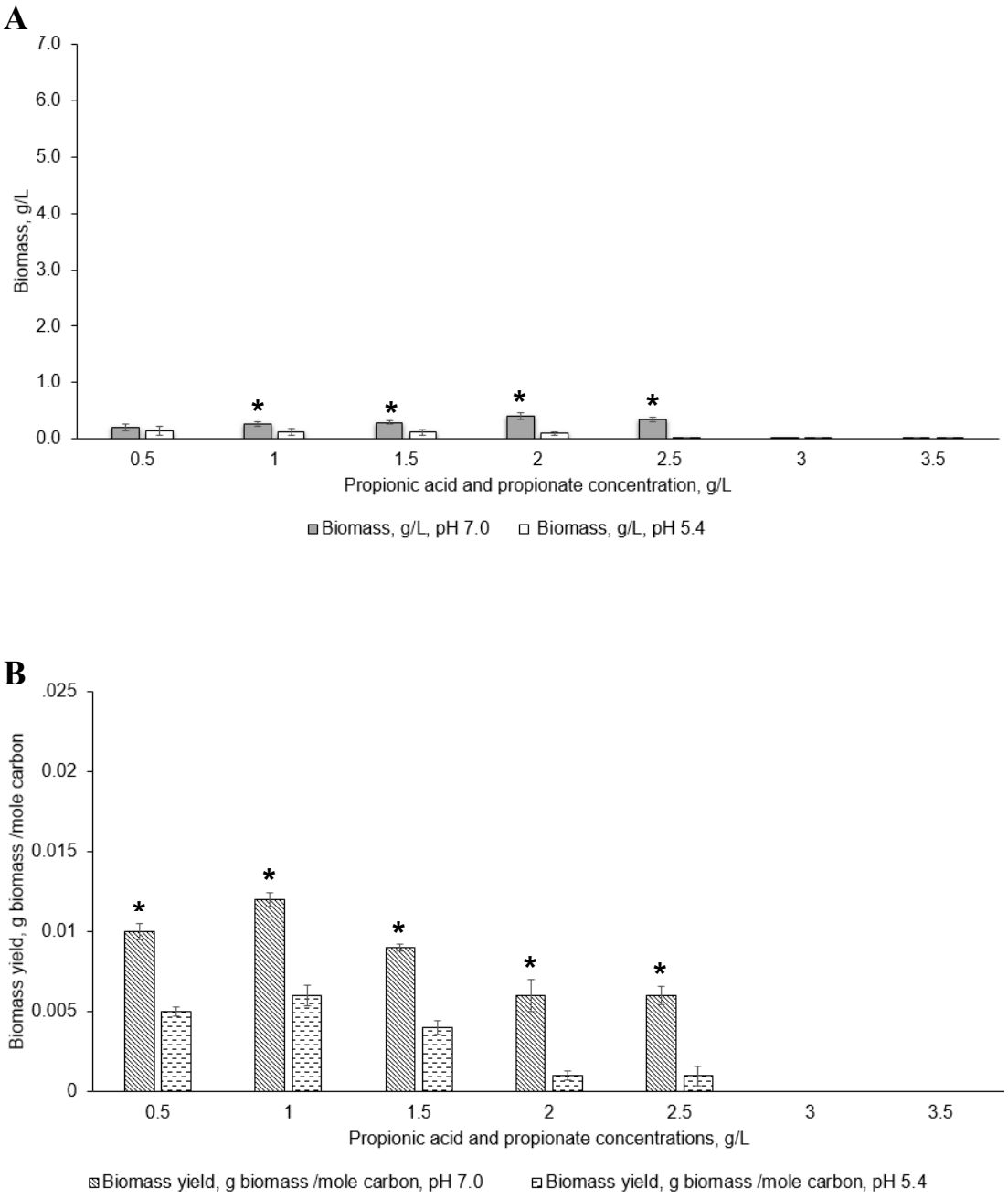

Figure 4.6. Biomass growth (A) and biomass yield (B) as produced biomass per utilized substrate of *C. curvatus* in base mineral media supplemented with different amounts of propionic acid at pH 5.4 and pH 7.0. Asterisk (*) are indicated differences in biomass growth or biomass yield at pH 5.4 relative to growth or yield at pH 7.0 that were statistically significant ($p < 0.05$), done in replicate (n=3).

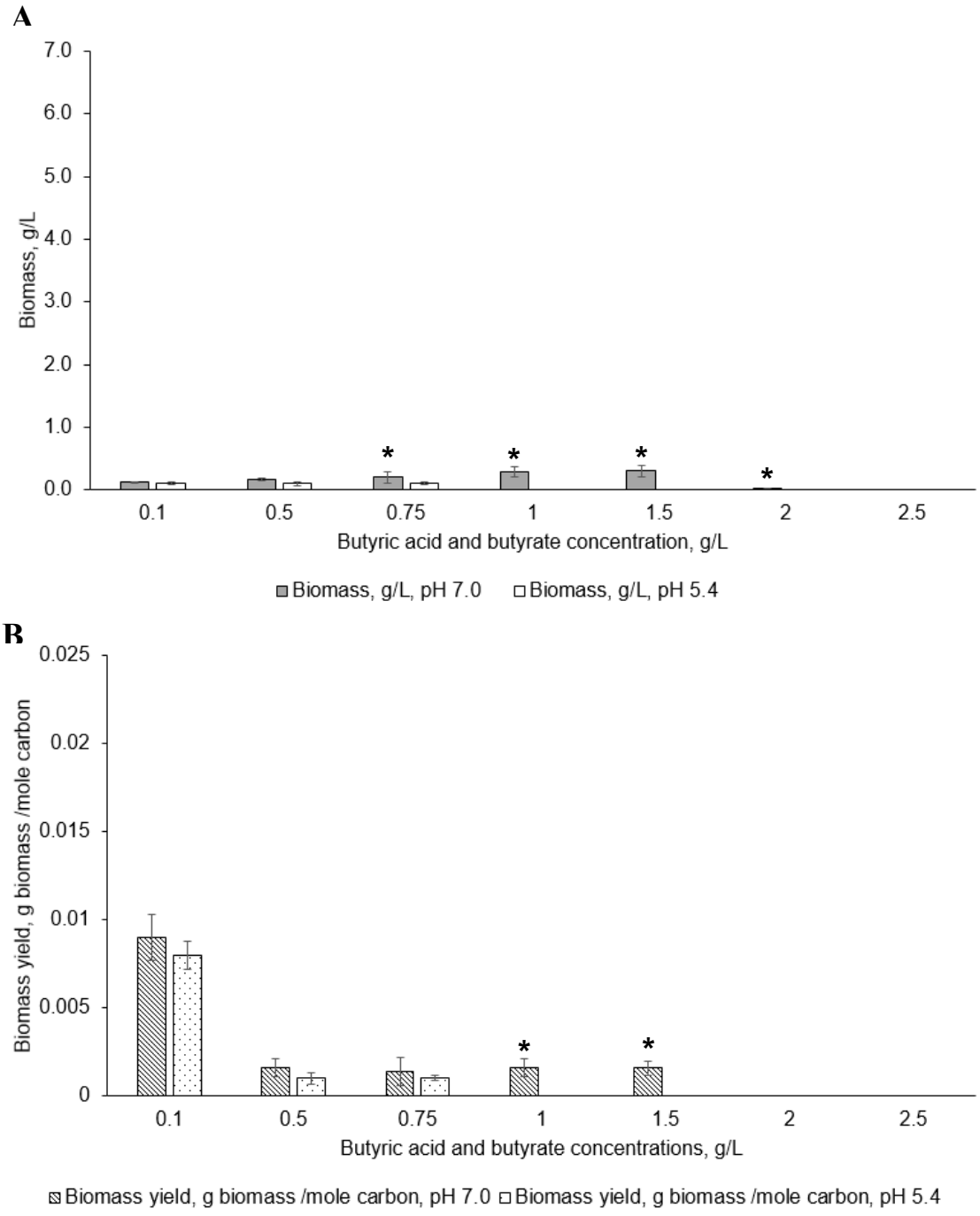

Figure 4.7. Impact of butyric acid at pH 5.4 and pH 7.0 on *C. curvatus* growth (A) and biomass yield (B) as produced biomass per utilized substrate. Asterisk (*) are indicated differences in biomass growth or biomass yield at pH 5.4 relative to growth or yield at pH 7.0 that were statistically significant ($p < 0.05$), done in replicate (n=3).

concentrations of 2.0 g/L and higher. At pH 7.0, growth of *C. curvatus* increased steadily and with increasing amounts of acetic acid. When the acetic acid concentration was increased from 5.0 to 6.0 g/L at pH 5.4, there was a large decrease in biomass yield (Figures 4.5B). No significant difference in biomass yield was observed, when *C. curvatus* grown at both pHs at 5.0 g/L of acetic acid.

At pH 5.4, a propionic acid concentration of 2.5 g/L in the media inhibited *C. curvatus* growth. Similarly, 3.0 g/L of propionic acid appears to inhibit growth when the pH was 7.0. The highest biomass yields were detected in the presence of 1.0 g/L of propionic acid in the media (Figure 4.6). For butyric acid, concentrations of 1.0 g/L and 2.0 g/L seemed to inhibit growth in the pH 7.0 and 5.4 systems, respectively. In terms of biomass yield, the two pH systems displayed very similar trends, with the highest biomass yield observed at 0.1 g/L butyric acid.

The pH 7 system outperformed the pH 5.4 system in terms of biomass accumulation, with the best statistically similar growth occurring with 10.0 g/L of acetic acid, 1.5 - 2.5 g/L of propionic acid or 0.75 - 1.5 g/L of butyric acid. To provide insight into the consumption of acids by *C. curvatus*, we performed GC-FID to quantify the amount of acids in the growth media before and after 120 h of growth (Table 4.2). The GC-FID analyses showed that for all of the conditions examined, after 120 h of growth, the acids were entirely consumed by the yeast, with the exception of 10.0 g/L of acetic acid, for which the amount of consumption was 96.2 ± 0.2 %.

Table 4.2. The acetic, propionic and butyric acids consumption of *C. curvatus*. Done in replicate (n=3).

	Theoretical amount, g/L	Before growth, g/L	After growth, g/L	% consumed
Acetic acid	5.0	5.1 ± 0.6	0	100
	10.0	10.5 ± 1.0	0.4 ± 0.1	96.2 ± 0.6
Propionic acid	1.5	1.26 ± 0.1	0	100
	2.0	1.89 ± 0.2	0	100
	2.5	2.54 ± 0.4	0	100
Butyric acid	0.5	0.46 ± 0.1	0	100
	1.0	0.92 ± 0.1	0	100
	1.5	1.42 ± 0.1	0	100

The actual aqueous by-product stream produced after pyrolysis contains a mix of volatile fatty acids, with the major volatile fatty acids being acetic, propionic and butyric acids. To investigate the influence of a mixed carbon source for biomass production, the ratio of volatile fatty acids was initially set at 1:1:1 (acetic acid: propionic acid: butyric acid), with an initial concentration of each volatile fatty acids of 1.0 g/L (total amount 3.0 g/L) and supplemented with base mineral media. The potential of *C. curvatus* to grow and utilize acetate, propionate and butyrate as a carbon source in the base mineral media were compared to the growth on glucose.

As represented in Figure 4.8A, a difference in growth was observed depending on which carbon source was used for *C. curvatus* cultivation. Maximum biomass concentration was higher when yeast used glucose as a carbon source

compared to the volatile fatty acids mixture. The growth of *C. curvatus* on glucose achieved stationary phase at 48 h, with a maximal biomass concentration of 2.2 ± 0.1 g/L (Figure 4.8A). Conversely, yeast growth on volatile fatty acids during the 72 h confirmed consumption of the volatile fatty acids up to 80 % (Figure 4.8B) and

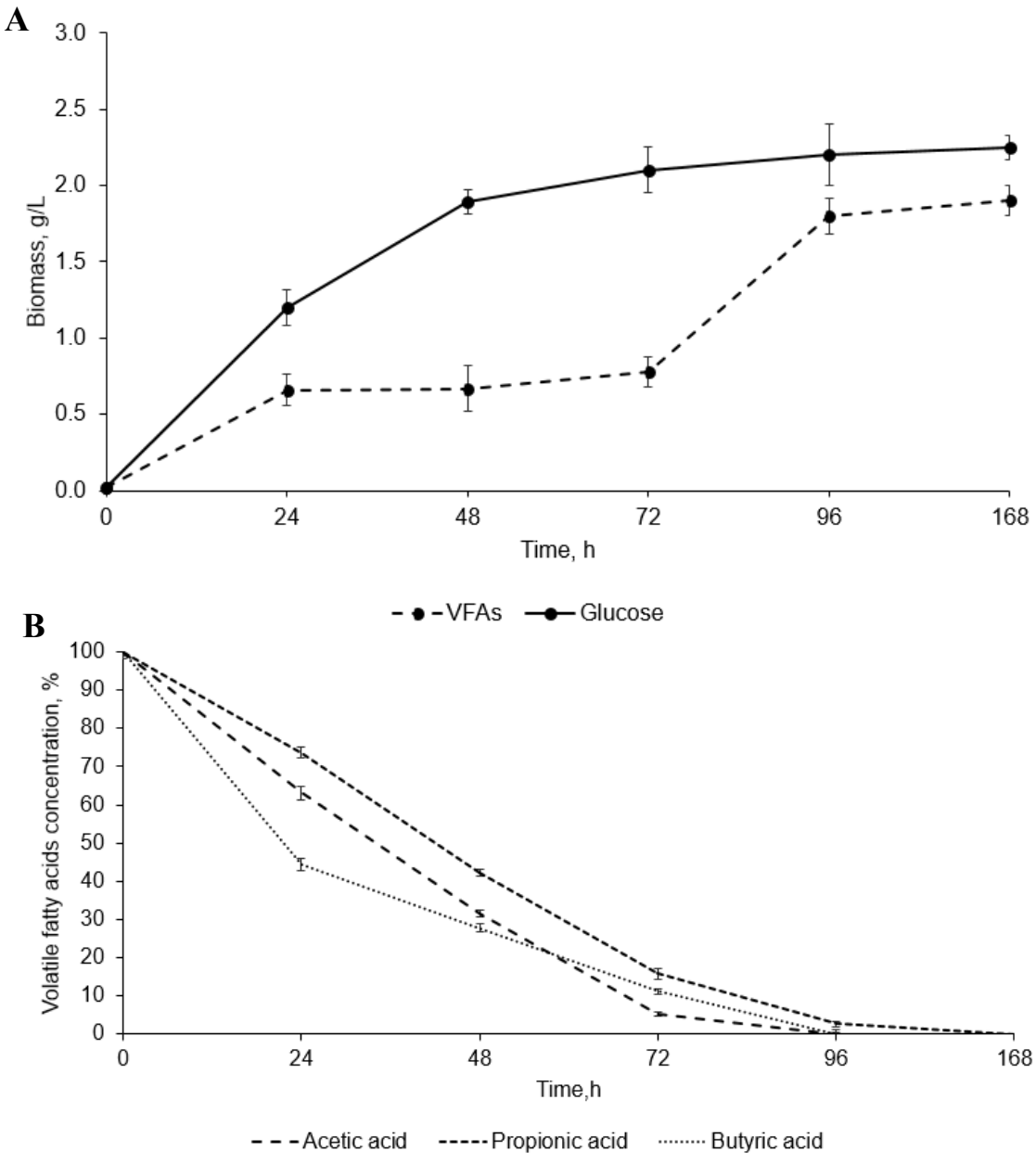

Figure 4.8. Biomass production (A) and volatile fatty acids substrate consumption (B) of *C. curvatus.* Volatile fatty acids were added in the ratio 1:1:1. Done in replicate (n=3).

production of 0.9 ± 0.1 g/L biomass (Figure 4.8A). Over the next 24 h, yeast cell consumed the remaining 20 % of volatile fatty acids (Figure 4.8B), and biomass growth roughly doubled to 2.0 ± 0.2 g/L (Figure 4.8A). In addition, for the first 24 h of yeast growth, nearly 40 % of the initial volatile fatty acids placed in the media were consumed, with preferred consumption of butyric acid, followed by acetic acid, and finally propionic acid (Figure 4.8B). From 24 to 72 h, consumption of the volatile fatty acids steadily increased, with similar consumption rates observed for acetic and propionic acids, both of which were higher than that of butyric acid (Figure 4.8B). Taken together, these data demonstrate that *C. curvatus* could grow on a volatile fatty acids mix.

Next, we investigated *C. curvatus* growth at higher initial concentrations of volatile fatty acids, added at a ratio of 20:3.3:1 (acetic acid: propionic acid: butyric acid), supplemented with base mineral media and at pH 7.0. The maximum amounts of volatile fatty acids used for yeast fermentation were 60.0 g/L for acetic acid, 10.0 g/L for propionic acid and 3.0 g/L for butyric acid (Figure 4.9; Undiluted). The dilutions of the initial volatile fatty acids concentrations by one (1:1), two (1:2) and three (1:3) fold were done to decrease the initial amounts of volatile fatty acids. This was done to determine the concentration of volatile fatty acids in the ratio 20:3.3:1 that would allow *C. curvatus* to grow and accumulate lipids. As a control, glucose was also used as a carbon source; the moles of carbon in this control were identical to that of the 1:3 dilution mix of volatile fatty acids. The carbon to nitrogen ratio was adjusted to 100/1 using urea as a source of nitrogen for all experiment and control samples.

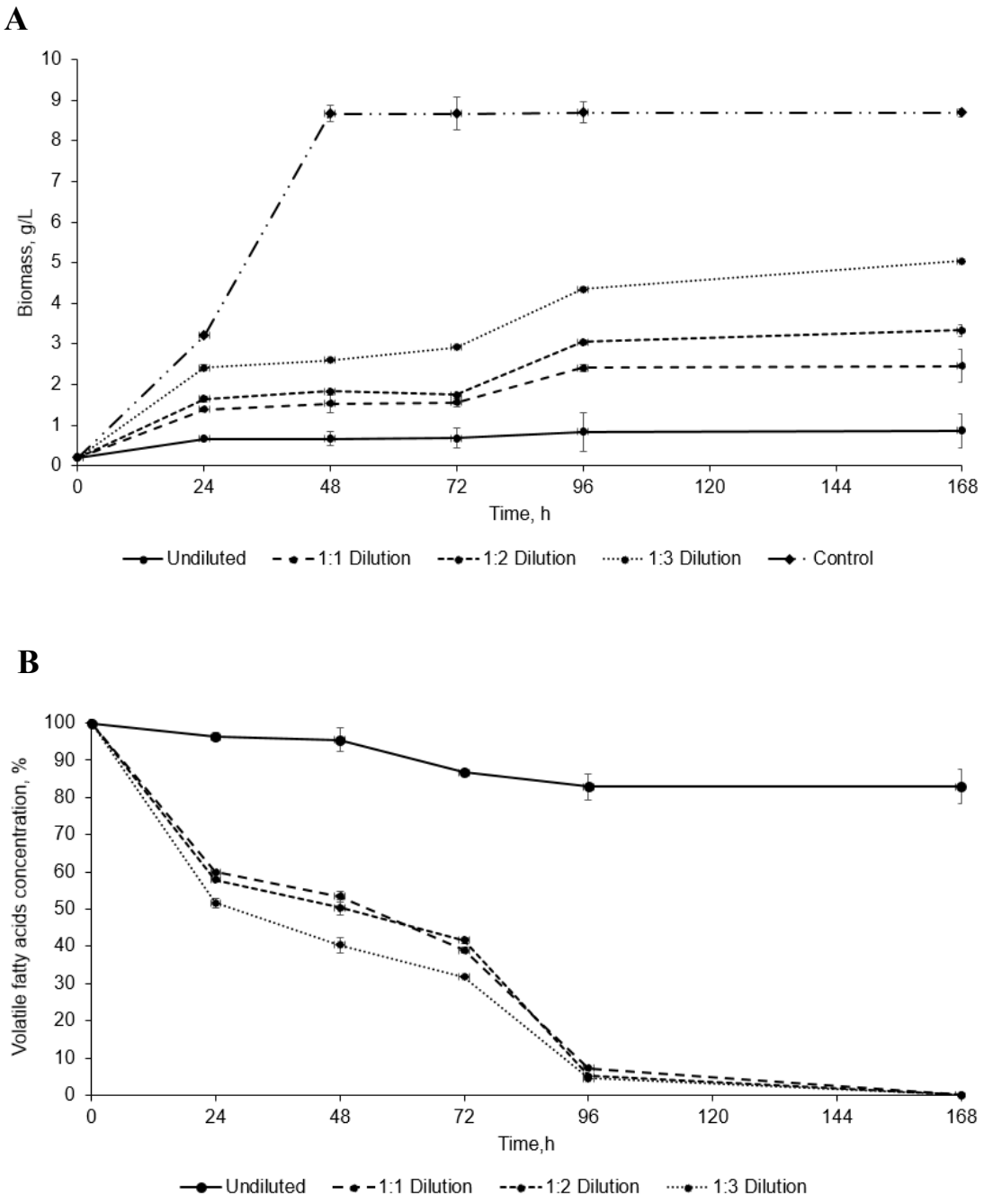

Figure 4.9. Biomass production (A) and substrate consumption (B) of *C. curvatus.* The consumption of the undiluted volatile fatty acid mix (acetic, propionic and butyric acids ratio 20:3.3:1), and with three different dilutions (1:1, 1:2 and 1:3) were plotted relative to time (h). Done in replicate (n=3).

The influence of the initial volatile fatty acids concentrations on *C. curvatus* growth was determined (Figure 4.9A). The amounts of produced yeast biomass, after 48 h of growth, for all samples grown using volatile fatty acids were significantly lower than what was observed using a glucose substrate. The amount of volatile fatty acids in the "undiluted" medium was too high to allow *C. curvatus* to grow as virtually no biomass accumulation was observed. This is supported by the fact that the lowest total consumption of volatile fatty acids was observed for the "undiluted" culture: 18 ± 2 % consumption after 168 h of growth (Figure 4.9B). According to the results shown in Figure 4.9A, significant biomass growth after 96 h of cultivation was observed for the three dilutions compared with "undiluted" media, with the 1:3 dilution generating the best results (Figure 4.9A). The highest amount of biomass, 4.3 ± 0.04 g/L, was produced through use of the 1:3 dilution of the original volatile fatty acid solution (Figure 4.9A). Amounts and rates of the consumed volatile fatty acids from 0 h to 96 h of cultivation showed the direct dependence on the produced amounts of *C. curvatus* biomass (Figure 4.9B). After 96 h of cultivation, approximately the same amounts of volatile free fatty acids were consumed in all three cases, but there were significant difference in biomass accumulation. The 1:3 dilution resulted in better overall biomass yields compared to other tested conditions. It is of interest to note that after 96 hours, the amount of volatile fatty acids remaining is the same in all three dilutions, but the biomass produced is so much higher in the 1:3 dilution relative to the 1:2 and 1:1 dilutions.

To investigate the specific contributions of acetic, propionic and butyric acids in the volatile fatty acids mix on yeast growth, their concentrations were determined

every 24 h by GC-FID (Figure 4.10 and Figure 4.11). The initial ratio of acetic, propionic and butyric acids was 20:3.3:1 in the volatile fatty acids mix, with a carbon to nitrogen ratio of 100/1.

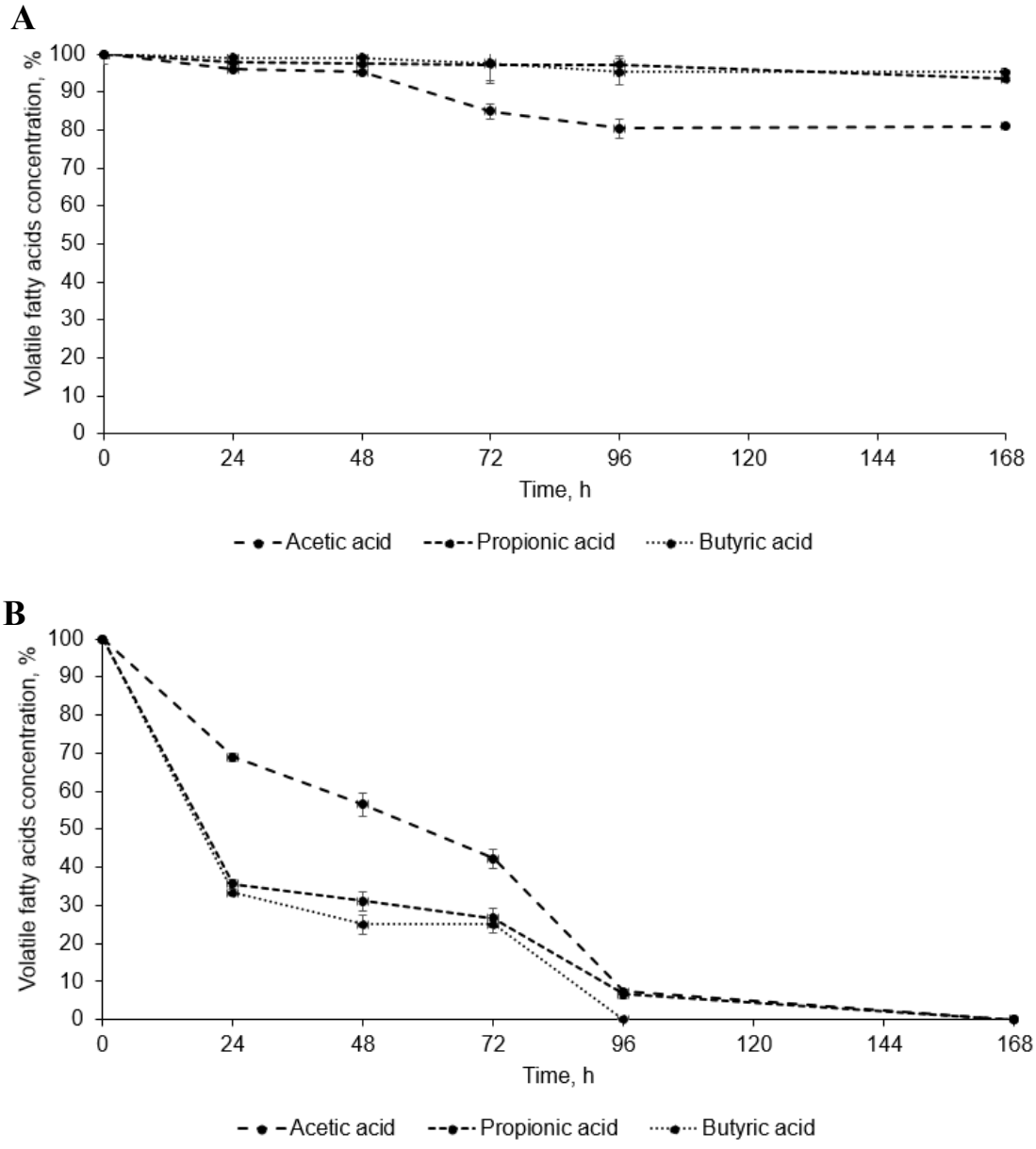

Figure 4.10. Volatile fatty acids consumption of *C. curvatus*. The consumption of volatile fatty acids was examined in growth media containing undiluted (A) 1:1 diluted (B) volatile fatty acid mix (acetic acid: propionic acid: butyric acid in a ratio of 20:3.3:1) and were plotted relative to time (h), done in replicate (n=3).

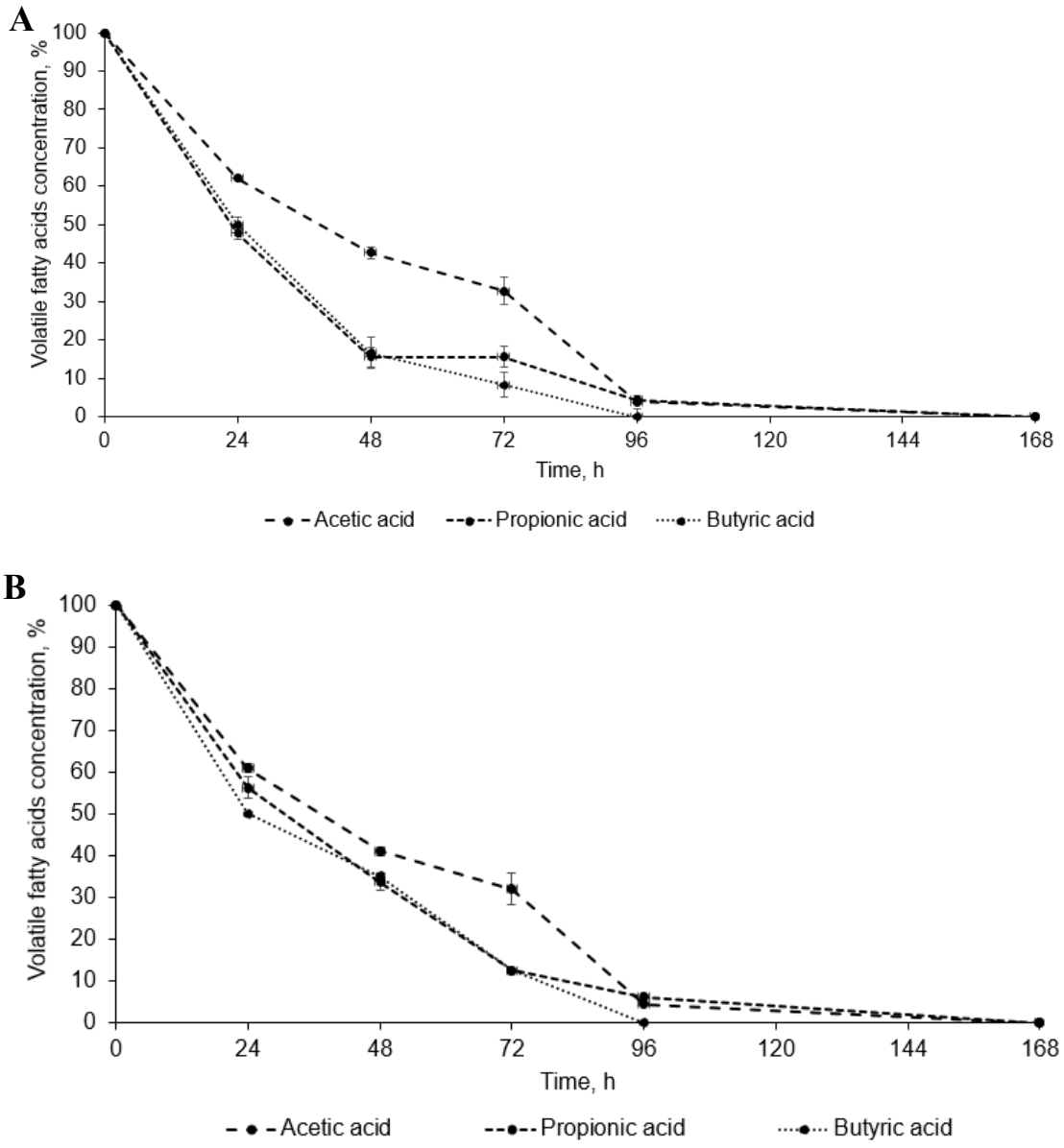

Figure 4.11. Volatile fatty acids consumption of *C. curvatus*. The consumption of volatile fatty acids was examined in growth media containing 1:2 diluted (A), and 1:3 diluted (B) volatile fatty acid mix (acetic acid: propionic acid: butyric acid in a ratio of 20:3.3:1) and were plotted relative to time (h), done in replicate (n=3).

A similar trend of volatile fatty acids consumption was detected for 1:1 (Figure 4.10B), 1:2 and 1:3 dilutions (Figure 4.11A and 4.11B). For instance, in the first 96 h, butyric and propionic acids were generally consumed faster than acetic

acid. After 96 h of growth, the amount of all acids left in the media was less than 10 %. In all three of the dilution systems, the main contributor to *C. curvatus* growth from 72 h to 96 h was acetic acid. Similarly, acetic acid was essentially the only volatile fatty acid consumed in the "undiluted" system, with consumption occurring between 48 h to 96 h. It should be noted that for all four systems examined, the actual consumption of acid per g per L was highest for acetic acid because the initial loading of acetic acid was significantly higher compared to propionic and butyric acids.

Our studies clearly demonstrated the ability of *C. curvatus* to grow and tolerate acetic, propionic and butyric acids as single and mixed carbon source. This includes growth facilitated by aqueous by-product streams, which for *C. curvatus* was significantly better when the system was pH 7.0 rather than pH 5.4. The GC-FID analysis of volatile fatty acids consumption during *C. curvatus* fermentation showed that high initial concentrations of volatile fatty acids led to growth inhibition. It was observed that use of the 1:3 dilution of volatile fatty acids resulted in the highest amount of *C. curvatus* biomass.

4.3. Lipid production by *C. curvatus* using acetic, propionic and butyric acids as single and mixed carbon sources

The acetic, propionic and butyric acids were added as a single or mixed carbon sources to the base mineral media. In the lipid accumulation study, the results of the *C. curvatus* growth using varying amounts of acetic, propionic and butyric acids as single and mixed carbon sources were compared to those of grown in control

experiments using glucose. Biomass growth, biomass yield, total lipids accumulation, and lipid yield after 120 h of growth are shown in Figure 4.12, 4.13, and 4.14.

In general, based on the data on biomass growth and amount of total lipids, acetic acid was better carbon source then propionic or butyric acids to grow *C. curvatus*. The amounts of biomass produced at 120 h of growth for all studied acetic, propionic and butyric acids concentrations were significantly lower compared to control samples with glucose (Figure 4.12A, 4.13A and 4.14A, respectively). The use of acetic acid concentrations 5.0 g/L and 30.0 g/L, compared to the glucose loading with equal moles of carbon, resulted in a significantly decreased biomass yield. However, use of higher acetic acid concentrations (10.0 g/L and 20.0 g/L) resulted in biomass yields that were statistically similar to those obtained using the appropriate glucose control (Figure 4.12A). Significantly higher amounts of total lipids were observed when 10.0, 20.0 and 30.0 g/L of acetic acid were used as a carbon source for *C. curvatus* cells when compared with control data using glucose (Figure 4.12A). At the same time, no significant differences were observed in the amounts of total lipids produced using propionic (Figure 4.13A) and butyric (Figure 4.14A) acids, and control loadings using glucose. Using volatile fatty acids, the highest amounts of total lipids in cells were produced when they were grown in 20.0 g/L and 30.0 g/L of acetic acid, reaching levels of 32.0 ± 0.6 % and 38.4 ± 0.4 %, respectively (Figure 4.12A). The total amount of lipids in the control cells using glucose as a carbon source at comparable moles of carbon to the 20.0 g/L and 30.0 g/L acetic acid

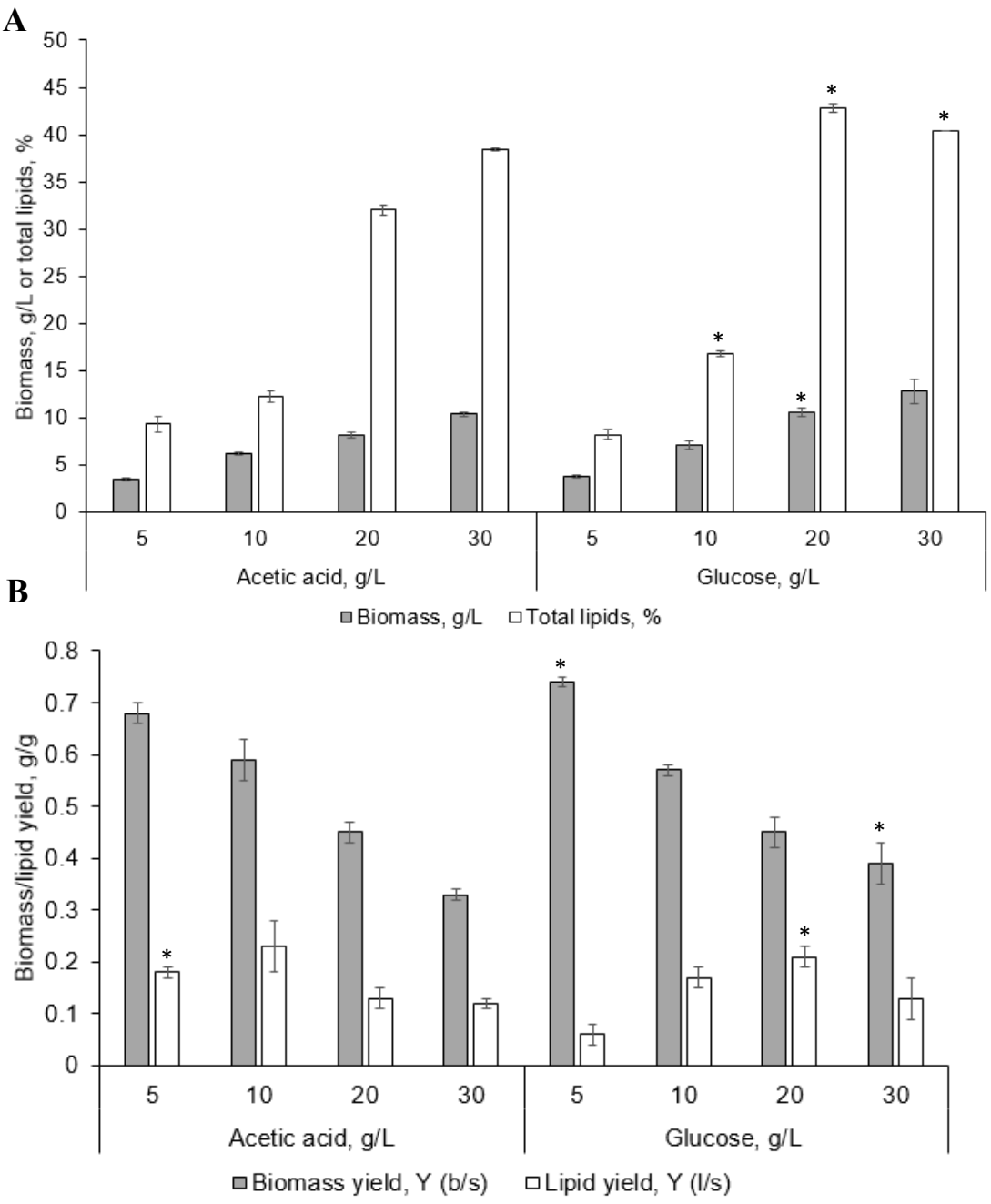

Figure 4.12. The performance of *C. curvatus* grown in acetic (A, B) acid at 168 hours of growth, pH 7.0 and C to N ratio of 100/1. Biomass yield ($Y_{b/s}$) = Biomass (g)/Utilized substrate (initial – residual) (g). Lipid yield ($Y_{l/s}$) = Lipid (g)/Utilized substrate (initial – residual) (g). Glucose was used as a control for lipid accumulation where the amount of the glucose in control samples corresponds (in terms of moles of carbon) to the stated amounts of the volatile fatty acids as indicated. Asterisk (*) are indicated differences in the biomass and total lipids produced, biomass and lipid yields using volatile fatty acids relative to the glucose control that were statistically significant ($p < 0.05$), done in replicate (n=3).

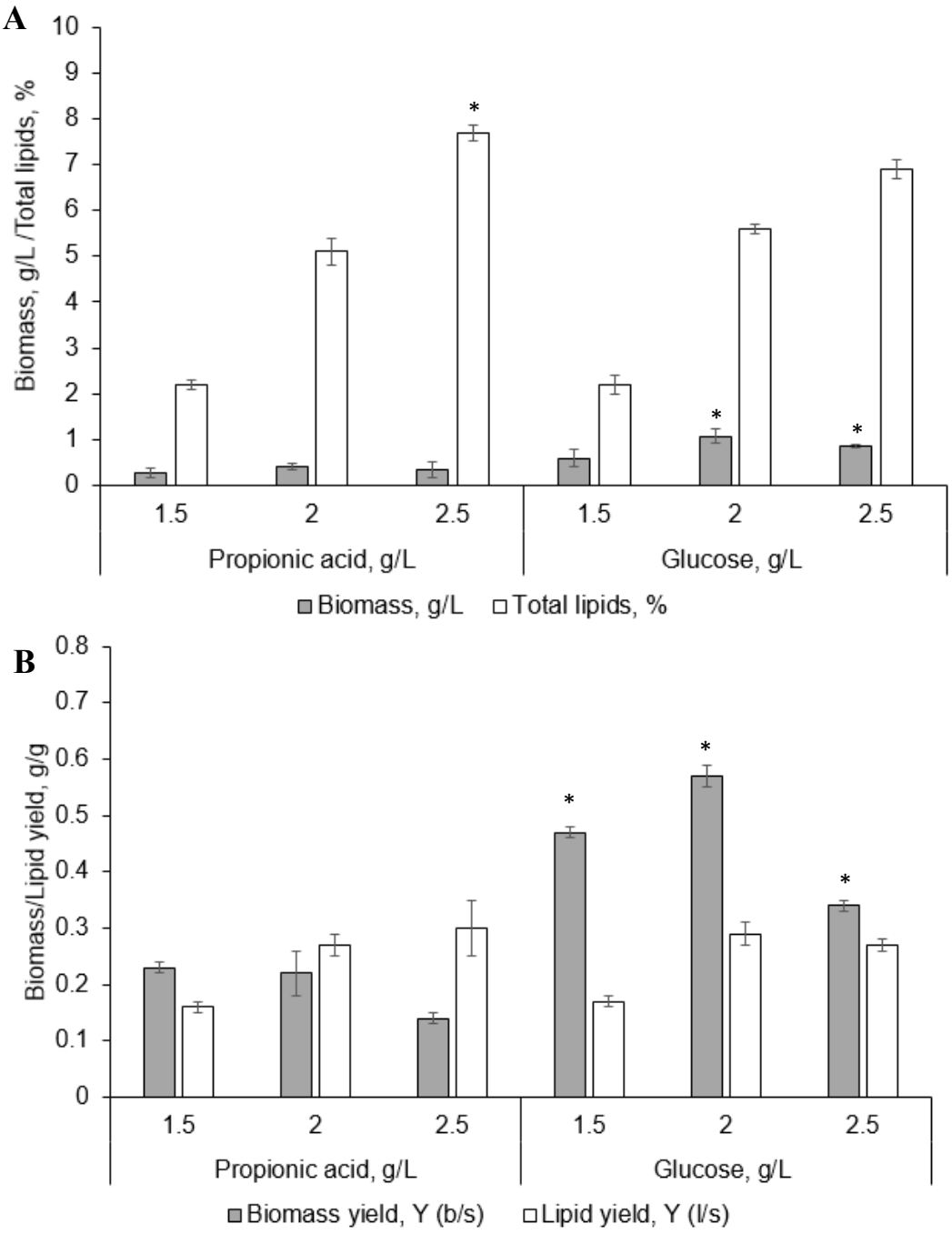

Figure 4.13. The performance of *C. curvatus* grown in propionic (A, B) acid at 168 hours of growth, pH 7.0 and C to N ratio of 100/1. Biomass yield ($Y_{b/s}$) = Biomass (g)/Utilized substrate (initial – residual) (g). Lipid yield ($Y_{l/s}$) = Lipid (g)/Utilized substrate (initial – residual) (g). Glucose was used as a control for lipid accumulation where the amount of the glucose in control samples corresponds (in terms of moles of carbon) to the stated amounts of the volatile fatty acids as indicated. Asterisk (*) are indicated differences in the biomass and total lipids produced, biomass and lipid yields using volatile fatty acids relative to the glucose control that were statistically significant ($p < 0.05$), done in replicate (n=3).

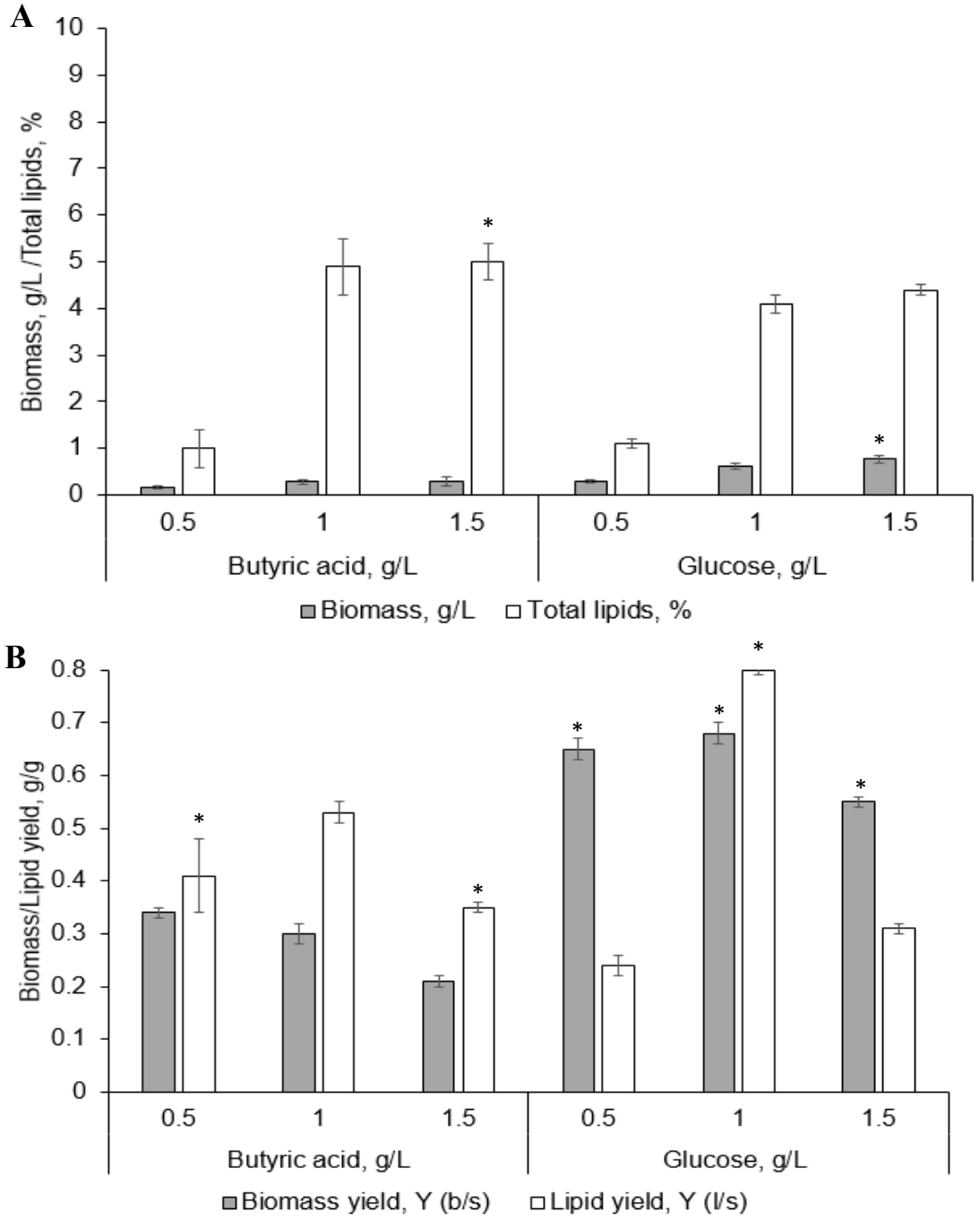

Figure 4.14. The performance of *C. curvatus* grown in butyric (A, B) acid at 168 hours of growth, pH 7.0 and C to N ratio of 100/1. Biomass yield ($Y_{b/s}$) = Biomass (g)/Utilized substrate (initial – residual) (g). Lipid yield ($Y_{l/s}$) = Lipid (g)/Utilized substrate (initial – residual) (g). Glucose was used as a control for lipid accumulation where the amount of the glucose in control samples corresponds (in terms of moles of carbon) to the stated amounts of the volatile fatty acids as indicated. Asterisk (*) are indicated differences in the biomass and total lipids produced, biomass and lipid yields using volatile fatty acids relative to the glucose control that were statistically significant ($p < 0.05$), done in replicate (n=3).

systems was significantly higher, displaying total lipid accumulations of 42.8 ± 0.5 % and 40.4 ± 0.8 %, respectively.

Interestingly, significantly higher lipid yields were detected in the 5.0 g/L and 10.0 g/L acetic acid systems relative to the appropriate glucose control systems (Figure 4.12B). At the same moment, the biomass yield was significantly higher when 5.0 g/L of acetic acid was used for cultivation. Conversely, for propionic and butyric acids, the biomass yields were significantly lower than glucose controls for all concentrations examined (Figure 4.13B and 4.14B). No significant difference in the performance of *C. curvatus* based on lipid yield were detected when 1.5, 2.0 and 2.5 g/L propionic acid and 0.5 and 1.5 g/L of butyric acid were used as a carbon source compare to the glucose.

The high amounts of acids present in the undiluted volatile fatty acids mix media significantly inhibited biomass growth and the lipid yield (Table 4.3). Despite this fact, there was an amount of lipid produced (25.9 ± 0.8 %) as well as 0.16 ± 0.02 g/g lipid yield in the cells that were able to grow (Table 4.3).

In general, the lipid yield increased with greater dilution factors, moving from 0.09 ± 0.01 g/g in the 1:1 diluted system to 0.26 ± 0.01 g/g in the 1:3 dilution system. The lipid yield for the 1:3 dilution system was significantly higher than control data evaluated after consumption of glucose (with equal moles of carbon). Furthermore, the 1:3 diluted media showed the highest amount of total lipids produced at 49.2 ± 1.2 %, which was again significantly higher than that of the control. It should be noted that a key difference between the control and 1:3 dilution systems is the

amount of biomass generated, with 8.7 ± 0.1 g/L and 5.1 ± 0.2 g/L of biomass being produced, respectively.

Table 4.3. The performance of *C. curvatus* cultures grown in volatile fatty acids. The control (glucose) corresponds (in terms of moles of carbon) to the amounts of the volatile fatty acids in 1:3 diluted media. Lipid yield = Lipid (g)/Utilized substrate (initial – residual) (g), done in replicate (n=3).

Volatile fatty acids mix	Carbon amount, g/L	Biomass, g/L	Total lipids, %	Lipid yield, g lipid/g utilized substrate
Undiluted	30.48	0.8 ± 0.2	25.9 ± 0.8	0.06 ± 0.02
1:1 Dilution	15.24	2.5 ± 0.2	30.2 ± 1.6	0.09 ± 0.01
1:2 Dilution	10.20	3.3 ± 0.1	33.1 ± 2.8	0.14 ± 0.02
1:3 Dilution	7.62	5.1 ± 0.2	49.2 ± 1.2	0.17 ± 0.02
Control (glucose)	7.62	8.7 ± 0.1	42.8 ± 0.5	0.21 ± 0.02

In general, the lipid yield increased with greater dilution factors, moving from 0.06 ± 0.02 g/g in the undiluted system to 0.17 ± 0.02 g/g in the 1:3 dilution system. The lipid yield for the 1:3 dilution system was significantly lower than control data evaluated after consumption of glucose. Furthermore, the 1:3 diluted media showed the highest amount of total lipids produced at 49.2 ± 1.2 %, which was again significantly higher than that of the control. It should be noted that a key difference between the control and 1:3 dilution systems is the amount of biomass generated, with 8.7 ± 0.1 g/L and 5.1 ± 0.2 g/L of biomass being produced, respectively.

The composition of the fatty acids in the lipids accumulated by *C. curvatus* was analyzed by CG-FID after esterification (Figure 4.15). This was done to track

possible changes in the fatty acid profile promoted by using different concentrations of the volatile fatty acid mix as a feedstock. The yeast cells in control experiments were grown using glucose added at concentrations where the amount of carbon in glucose equalled the amount by carbon as in the 1:3 diluted volatile fatty acids mix of acetic, propionic and butyric acids. The fatty acid profile of *C. curvatus* grown in glucose was found to consist mainly of oleic (C18:1) 33.3 ± 0.5 %, linoleic (C18:2) 27.5 ± 1.6 %, palmitic C16:0 26.5 ± 2.4 %, stearic (C18:0) 9.8 ± 1.5 %, and linolenic (C18:3) 2.9 ± 0.1 % acids.

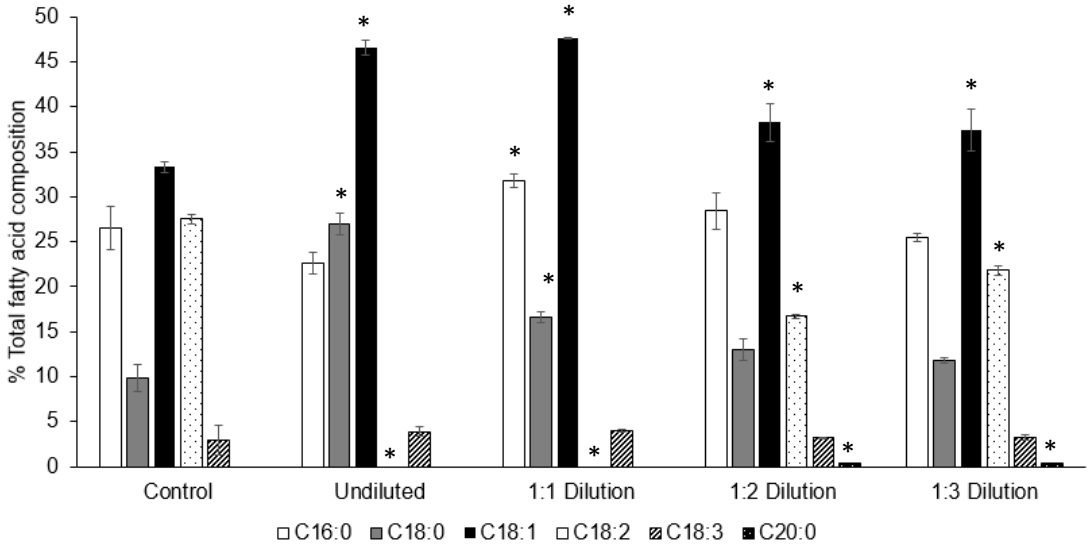

Figure 4.15. Fatty acid composition of *C. curvatus* grown in volatile fatty acids. Asterisk (*) are indicated fatty acid levels that differ significantly ($p < 0.05$) between the control culture (used glucose as a carbon source) and the culture supplemented with volatile fatty acids. C16:0 - palmitic acid, C18:0 - stearic acid, C18:1- oleic acid, C18:2 - linoleic acid, C18:3 - linolenic acid, and C20:0 - arachidic acid, done in replicate (n=3).

Significant differences in the composition of fatty acids were determined for the five different systems examined. Oleic acid (18:1) levels were significantly increased when volatile fatty acids of various dilutions were used instead of glucose. Conversely, while stearic acid (18:0) levels increased significantly relative to the control when the undiluted or 1:1 diluted volatile fatty acids were used, further dilution of the fatty acid mixture led to stearic acid levels similar to that observed in the control. For palmitic acid (16:0), only the 1:1 diluted volatile fatty acid system displayed levels that were statistically different from the control, increasing from 26.5 ± 1.5 % to 31.8 ± 0.8%. Linoleic acid (18:2) was not detected in cells grown using the undiluted or 1:1 diluted volatile fatty acids, while 27.5 ± 1.6 % was observed in the profile observed for the control condition. Similarly, significant decreases in linoleic acid to 16.7 ± 0.2 % and 21.8 ± 0.6 % were observed when using the 1:2 and 1:3 dilutions, respectively. There were no significant differences in the amount of α-linolenic acid (18:3) and arachidic acid (20:0) in any of the systems, with levels below 4.0 ± 0.1 % and 0.4 ± 0.1 %, respectively.

In summary, our studies on lipid production by *C. curvatus* grown on a mixture of volatile fatty acids demonstrated that the amount of total lipids produced as well as the lipid yield were significantly higher when the cells were grown in the 1:3 diluted volatile fatty acids mix. Subsequent analysis of the fatty acid composition of *C. curvatus* grown using volatile fatty acids revealed changes, with significantly higher levels of oleic acid in all systems, relative to the control.

4.4. Acetic, propionic and butyric acids as single and mixed carbon sources to grow oleaginous microalgae

To study the ability of a microalgae, *C. protothecoides*, to use as a carbon source acetic, propionic and butyric acids, biomass production was evaluated using these volatile fatty acids at pH 5.4 or pH 7.0 (Figure 4.16, 4.17, and 4.18). The biomass production and biomass yield were assessed after 168 h of heterotrophic growth where the base mineral media was supplemented with 1.0 – 10.0 g/L of acetic acid at pH 5.4 or 7.0. As shown in Figure 4.16A, low levels of *C. protothecoides* biomass (up to 0.5 ± 0.0 g/L biomass) can grow in the presence up to 2.0 g/L of acetic acid in media at pH 5.4. No growth was observed at concentrations of acetic acid higher than 2.0 g/L at pH 5.4. At pH 7.0, the microalgae biomass increased gradually as the acetic acid concentration in the media increased from 1.0 to 7.0 g/L. No significant difference was observed for biomass growth when the acetic acid concentration was 7.0 – 10.0 g/L at pH 7.0; the amount of biomass produced was roughly 3.9 ± 0.04, 3.85 ± 0.1, 3.85 ± 0.2, 3.85 ± 0.18 g/L, respectively, for all four conditions examined.

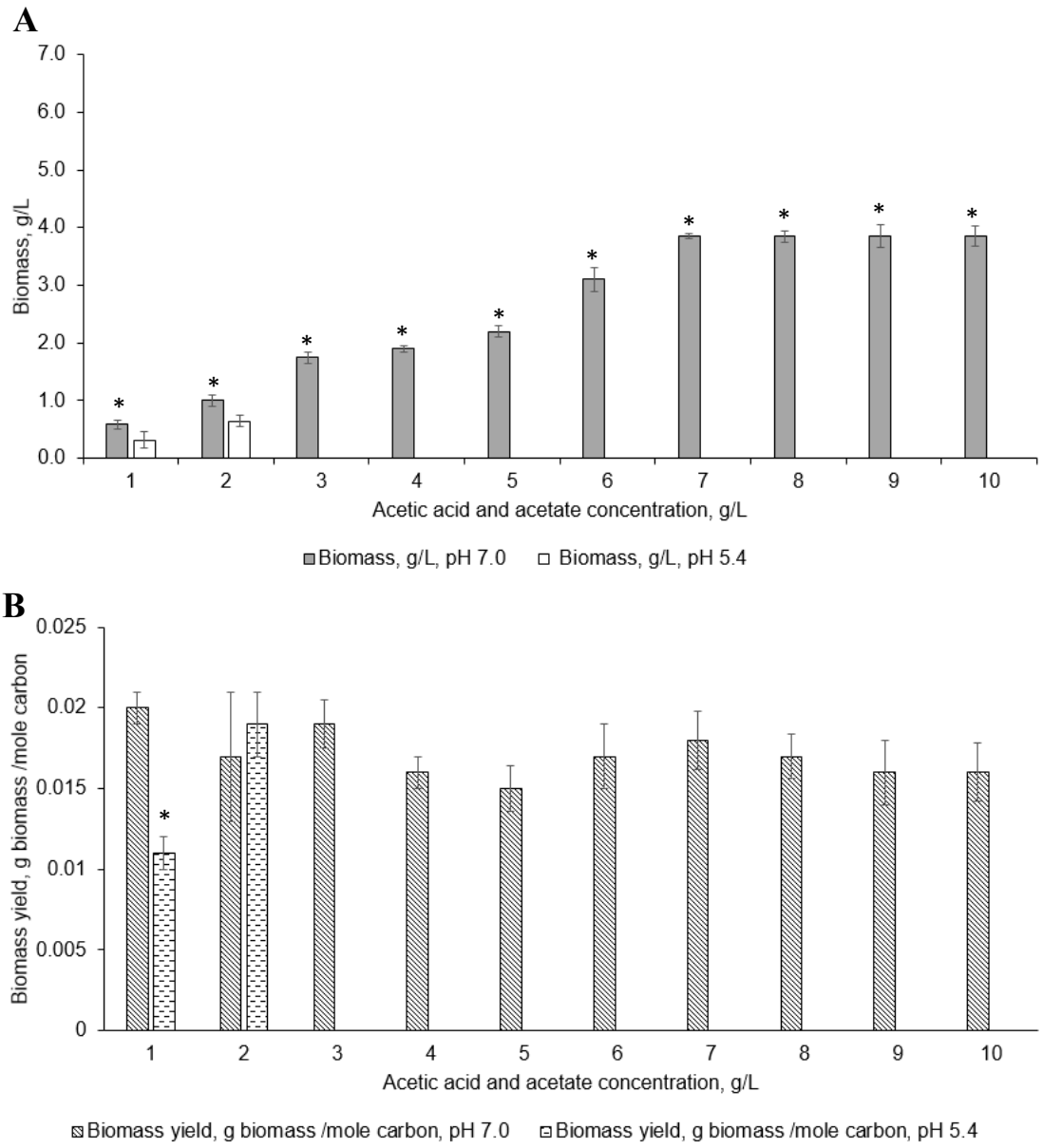

Figure 4.16. *C. protothecoides* growth (A) and biomass yield (B) in base mineral media supplemented by different amounts of acetic acid at pH 5.4 and pH 7.0. Asterisk (*) are indicated differences in biomass growth or biomass yield at pH 5.4 relative to growth or yield at pH 7.0 that were statistically significant ($p < 0.05$), done in replicate (n=3).

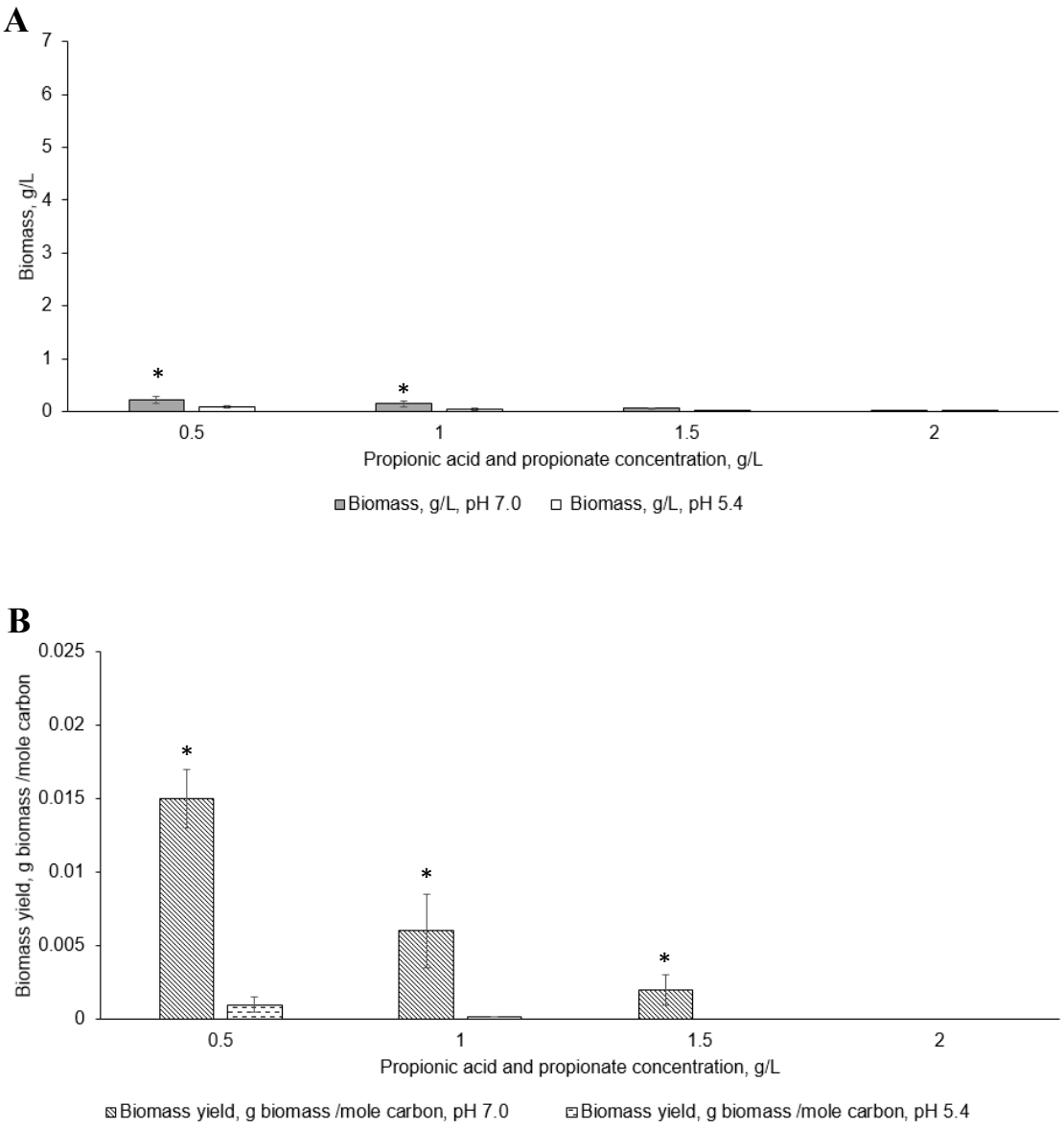

Figure 4.17. *C. protothecoides* growth (A) and biomass yield (B) in base mineral media supplemented by different amounts of propionic acid at pH 5.4 and pH 7.0. Asterisk (*) are indicated differences in biomass growth or biomass yield at pH 5.4 relative to growth or yield at pH 7.0 that were statistically significant ($p < 0.05$), done in replicate (n=3).

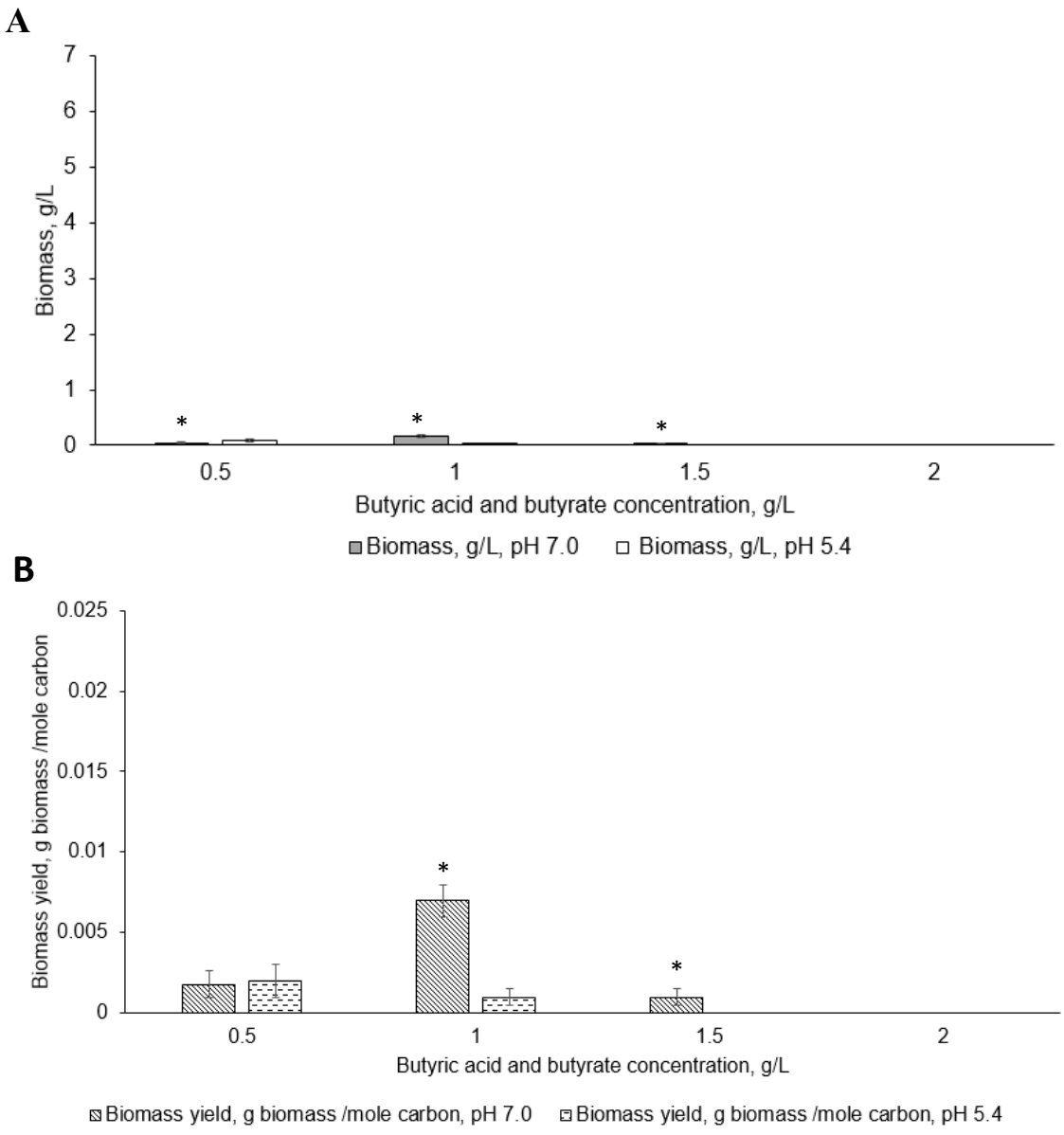

Figure 4.18. *C. protothecoides* growth (A) and biomass yield (B) in base mineral media supplemented by different amounts of butyric acid at pH 5.4 and pH 7.0. Asterisk (*) are indicated differences in biomass growth or biomass yield at pH 5.4 relative to growth or yield at pH 7.0 that were statistically significant ($p < 0.05$), done in replicate (n=3).

The results of *C. protothecoides* biomass growth in base mineral media supplemented by propionic acid shown in Figure 4.17A and 4.17B, and by butyric acid shown in Figure 4.18A and 4.18B. As shown in Figure 4.17A and 4.18A, the biomass growth decreased significantly when propionic or butyric acid was present in the media compared to *C. protothecoides* biomass growth in acetic acid (Figure 4.16A.).

At pH 5.4, the highest microalgae biomass amounts produced in the three different volatile fatty acid systems were achieved using 2.0 g/L of acetic acid (0.65 ± 0.01 g/L), 0.5 g/L of propionic acid (0.1 ± 0.02 g/L), or 0.5 g/L of butyric acid (0.09 ± 0.01 g/L). Conversely, at pH 7.0 *C. protothecoides* growth was highest using 7.0 – 10.0 g/L of acetic acid (from 3.85 ± 0.1 g/L to 3.9 ± 0.02 g/L), 0.5 g/L of propionic acid (0.21 ± 0.04 g/L) or 1.0 g/L of butyric acid (0.16 ± 0.01 g/L). As shown in Figure 4.16B, 4.17B and 4.18B, the biomass yield was significantly higher at pH 7.0 than at pH 5.4 for growth in the presence of 3.0 – 10.0 g/L of acetic acid, 0.5 – 1.5 g/L of propionic acid, or 1.0 g/L of butyric acid. At pH 7.0 no significant difference was observed for biomass yield of *C. protothecoides* grown in 1.0 – 10.0 g/L of acetic acid; the cells can reach roughly 0.02 g biomass per mole of consumed carbon (Figure 4.16B). In addition, the same biomass yield was determined for growth at pH 5.4 and 2.0 g/L of acetic acid in media. In contrast, the propionic acid concentration of 0.5 g/L (Figure 4.17B) or 1.0 g/L of butyric acid (Figure 4.18B) at pH 7.0 generated the highest biomass yields of their respective systems of 0.015 ± 0.001 g/g and 0.005 ± 0.001 g/g, respectively.

In addition, the results of acetic, propionic and butyric acids consumption after 168 h of growth of *C. protothecoides* were evaluated. The data from GC-FID analysis indicate that acids were not fully consumed. As shown in Table 4.4, in the presence of 5.0 g/L and 10 g/L of acetic acid, algae cells used 82.2 ± 0.9 % and 62.8 ± 0.1 % of the volatile fatty acids, respectively. In addition, 68.5 ± 0.7 % and 39.5 ± 0.9 % of propionic acid was used from initial amount 0.5 g/L and 1.0 g/L respectively. The amounts of butyric acid used when initial loadings were 1.0 g/L and 1.5 g/L were 75.0 ± 0.8 % and 65.9 ± 0.2 %, respectively.

Table 4.4. The acetic, propionic and butyric acids consumption of *C. protothecoides* after 168 h of growth determined by GC-FID, done in replicate (n=3).

	Theoretical amount, g/L	Experimental amount, g/L		% consumed*
		Before growth (C_b)	After growth (C_a)	
Acetic acid	5.0	4.93 ± 0.3	0.88 ± 0.1	82.2 ± 0.9
	10.0	9.56 ± 0.5	3.56 ± 0.2	62.8 ± 0.1
Propionic acid	0.5	0.46 ± 0.1	0.14 ± 0.1	68.5 ± 0.7
	1.0	0.94 ± 0.3	0.55 ± 0.1	39.5 ± 0.9
Butyric acid	1.0	0.98 ± 0.1	0.25 ± 0.1	75.0 ± 0.8
	1.5	1.52 ± 0.2	0.52 ± 0.1	65.9 ± 0.2

* - % consumed acid = 100 % - (C_b*100/C_a), C_b – acid concentration before growth, C_a – acid concentration after growth.

The potential of *C. protothecoides* to grow and utilize acetate, propionate and butyrate as a carbon source in the base mineral media were compared to its growth on glucose. The ratio of volatile fatty acids was 1:1:1 (acetic acid: propionic acid: butyric acid) used to evaluate the contribution for biomass production of *C. protothecoides* based on equal minimal loading. As represented in Figure 4.19A the

difference in growth observed depended on the carbon source used for *C. protothecoides* cultivation. The amount of glucose used (black line) was calculated

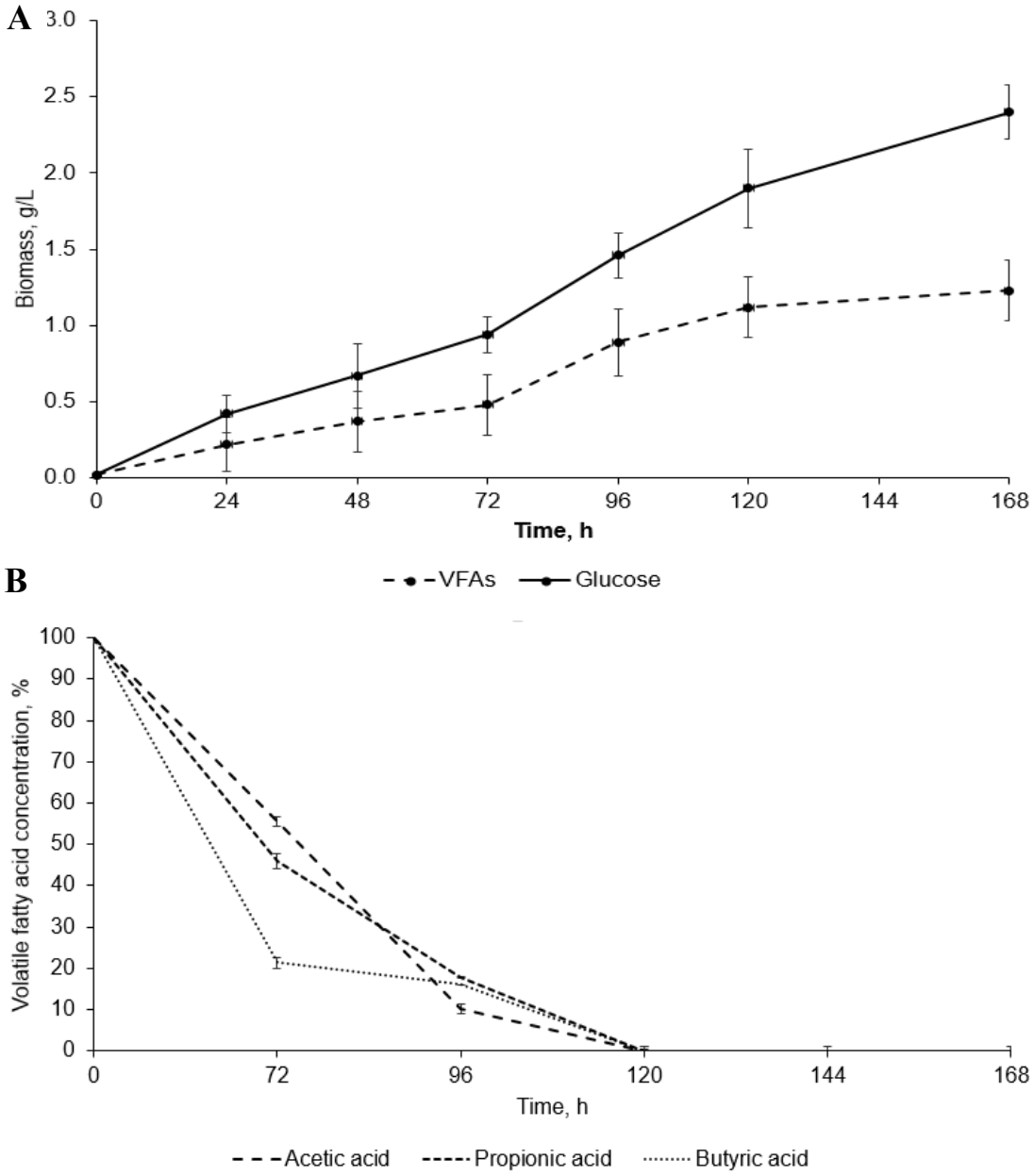

Figure 4.19. *C. protothecoides* biomass production (A) and volatile fatty acids substrate consumption (B) used at ratio 1:1:1 (acetic acid: propionic acid: butyric acid), done in replicate (n=3).

such that the moles of carbon present was identical to the amount found in the volatile fatty acids (dotted line). The carbon to nitrogen ratio was maintained at 100/1 using urea as the nitrogen source. This study showed that *C. protothecoides* could grow on volatile fatty acids mix. Significant differences in biomass production were observed after 72 h of growth. High biomass production was detected when glucose was used as a carbon source. Significant decreases in algae biomass production were noticed when volatile fatty acids were used as a feedstock. During the first 72 h of growth, ~45 % of acetic acid, ~54 % of propionic acid and ~78 % of butyric acid were consumed (Figure 4.19B). For this period, the *C. protothecoides* biomass was 0.5 ± 0.16 g/L. The remaining amount of volatile fatty acids was entirely consumed after 120 h of growth.

C. protothecoides fermentation was then performed using volatile fatty acids in undiluted media at a ratio of 20:3.3:1 (Figure 4.20). Dilution of the initial volatile fatty acids mixture by one (1:1), two (1:2) and three (1:3) times was done to decrease the initial concentrations of acetic, propionic and butyric acids (Figures 4.20B). As a control, glucose was used at a concentration where the moles of carbon were the same amount as in the 1:3 dilution of volatile fatty acids. The carbon to nitrogen ratio was adjusted to 100/1 using urea as a source of nitrogen for all experiment and control samples.

The biomass growth results in Figure 4.20A indicate that the biomass concentration obtained in all four volatile fatty acids systems showed significant decreases compared to algae growth in control media where glucose was used as a carbon source. Biomass production depended on the initial concentrations of acetic,

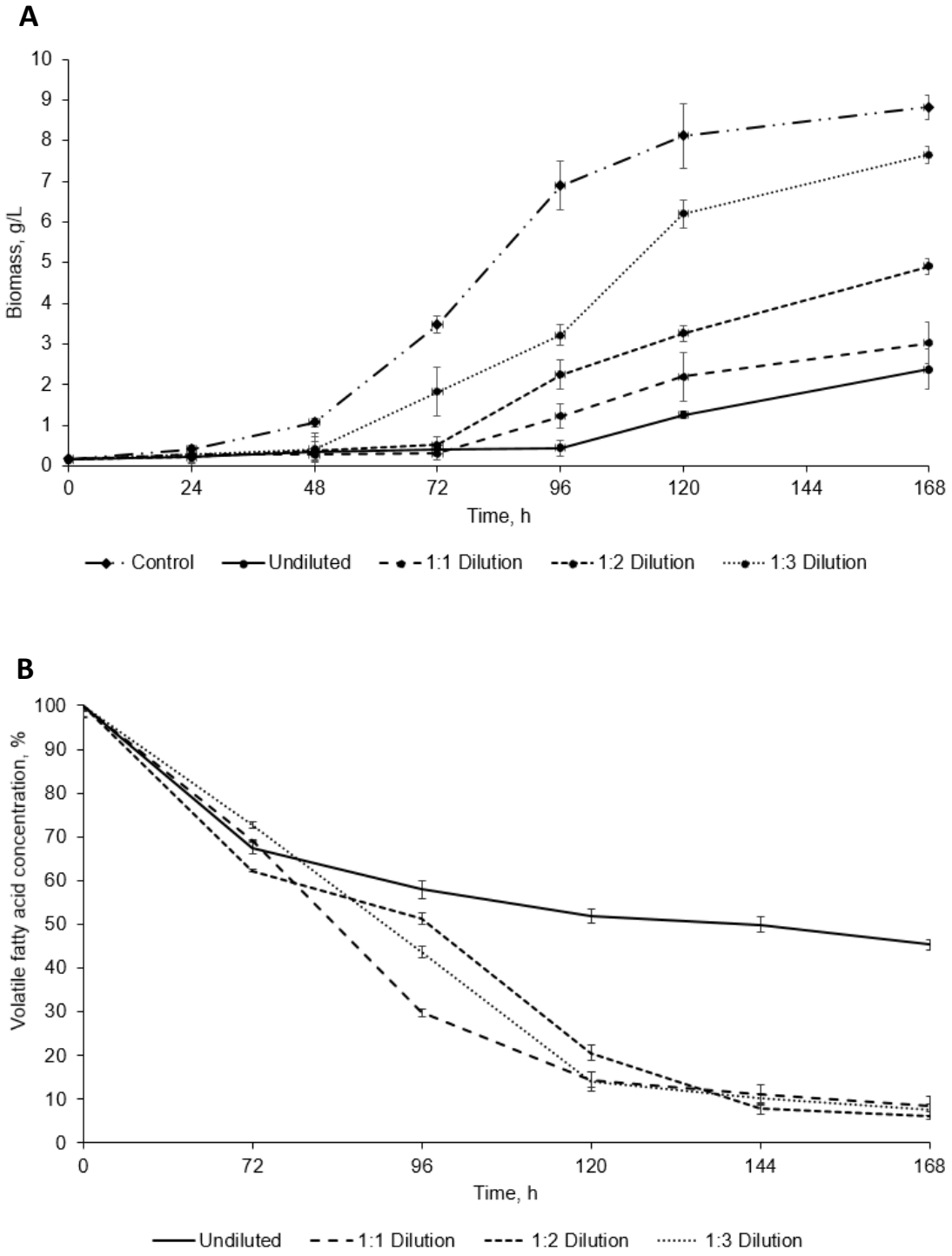

Figure 4.20. Biomass production (A) and substrate consumption (B) of *C. protothecoides*. Total consumption of an undiluted volatile fatty mix (acetic acid: propionic acid: butyric acid; ratio 20:3.3:1), and with 1:1, 1:2 and 1:3 dilutions, were plotted relative to time (h), done in replicate (n=3).

propionic and butyric acids (i.e. the dilution of volatile fatty acids). Although all media supported algae growth, *C. protothecoides* produced higher biomass (8.4 ± 0.1 g/L) in the 1:3 diluted system than in all other systems containing fatty acids. In these systems, growth was indirectly proportional to the concentration of fatty acids. As shown in Figure 4.20B, by 168 h of cultivation in undiluted media, up to 53 % of volatile fatty acids were consumed. No significant differences in volatile fatty acids consumption were observed by 168 h of growth for the 1:1, 1:2 and 1:3 media dilutions.

To investigate the consumption of each acid, GC-FID was performed using samples taken every 24 h. The distribution of consumption rates was similar for all volatile fatty acids containing media. For the first 72 h of growth, up to 70 % of propionic acids was used while the acetic and butyric acids were consumed only up to 30 % (Figure 4.21 and Figure 4.21). For the next 72 h of growth, algae used mainly acetic and to a lesser extent, butyric acids (Figure 4.21, 4.22A). Based on the 1:3 dilution system (Figure 4.22B), by 144 hours, *C. protothecoides* consumed ~80 % of the propionic acid, ~98 % of the acetic acid, and ~70 % of the butyric acid.

The GC-FID analysis of volatile fatty acids consumption during *C. protothecoides* fermentation showed that initially high concentrations of volatile fatty acids led to growth inhibition. It was observed that the 1:3 dilution of volatile fatty acids was the best dilution to grow the highest amount of *C. protothecoides* biomass. Based on this observation of the volatile fatty acids consumption during fermentation of C. *protothecoides,* it was noticed that the preference as a carbon source depends on fermentation time, ratio and their initial concentration.

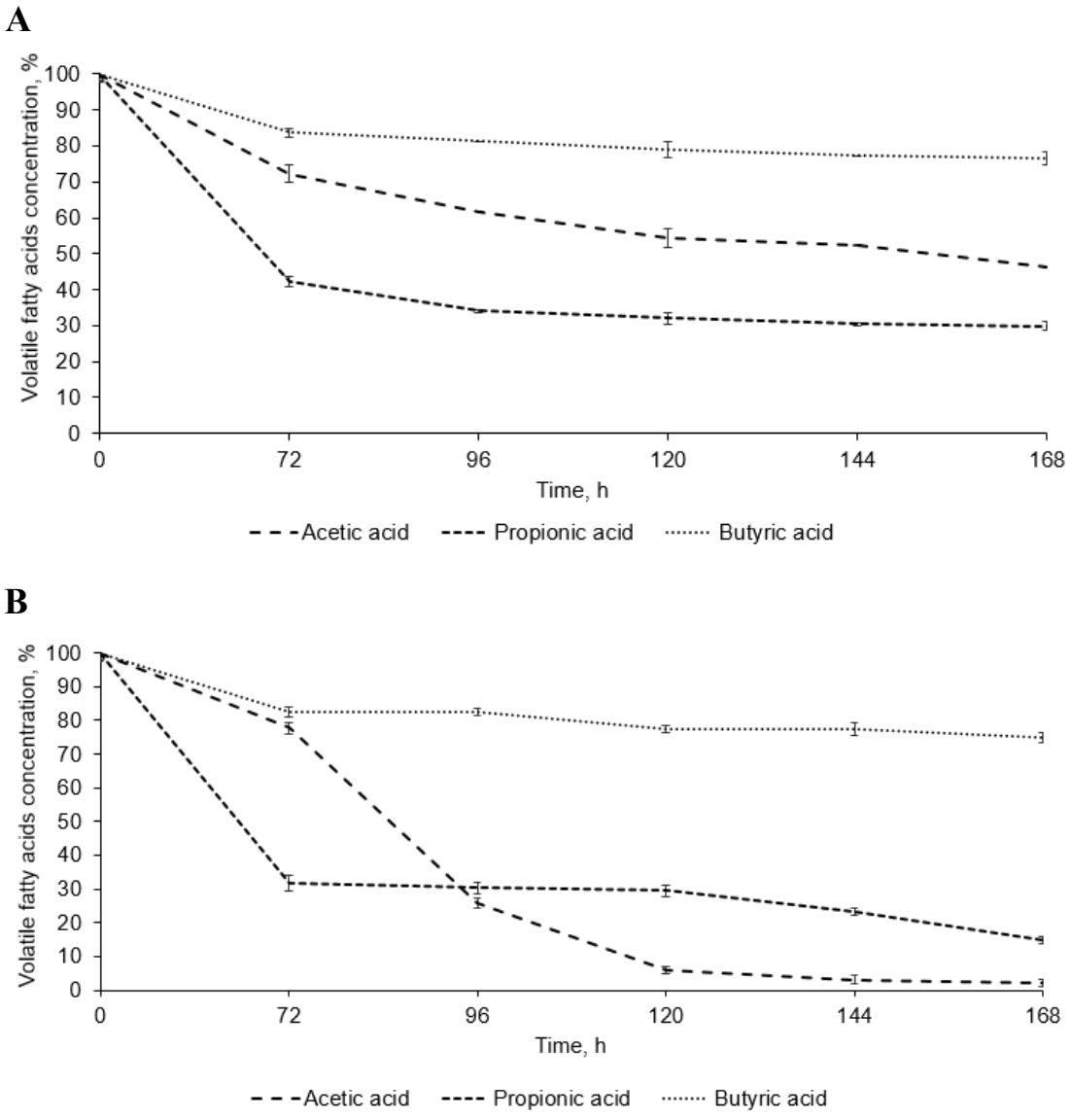

Figure 4.21. Volatile fatty acids consumption of *C. protothecoides*. Total consumption of undiluted (A) volatile fatty mix (acetic acid: propionic acid: butyric acid; ratio 20:3.3:1), with dilution 1:1 (B) were plotted relative to time (h), done in replicate (n=3).

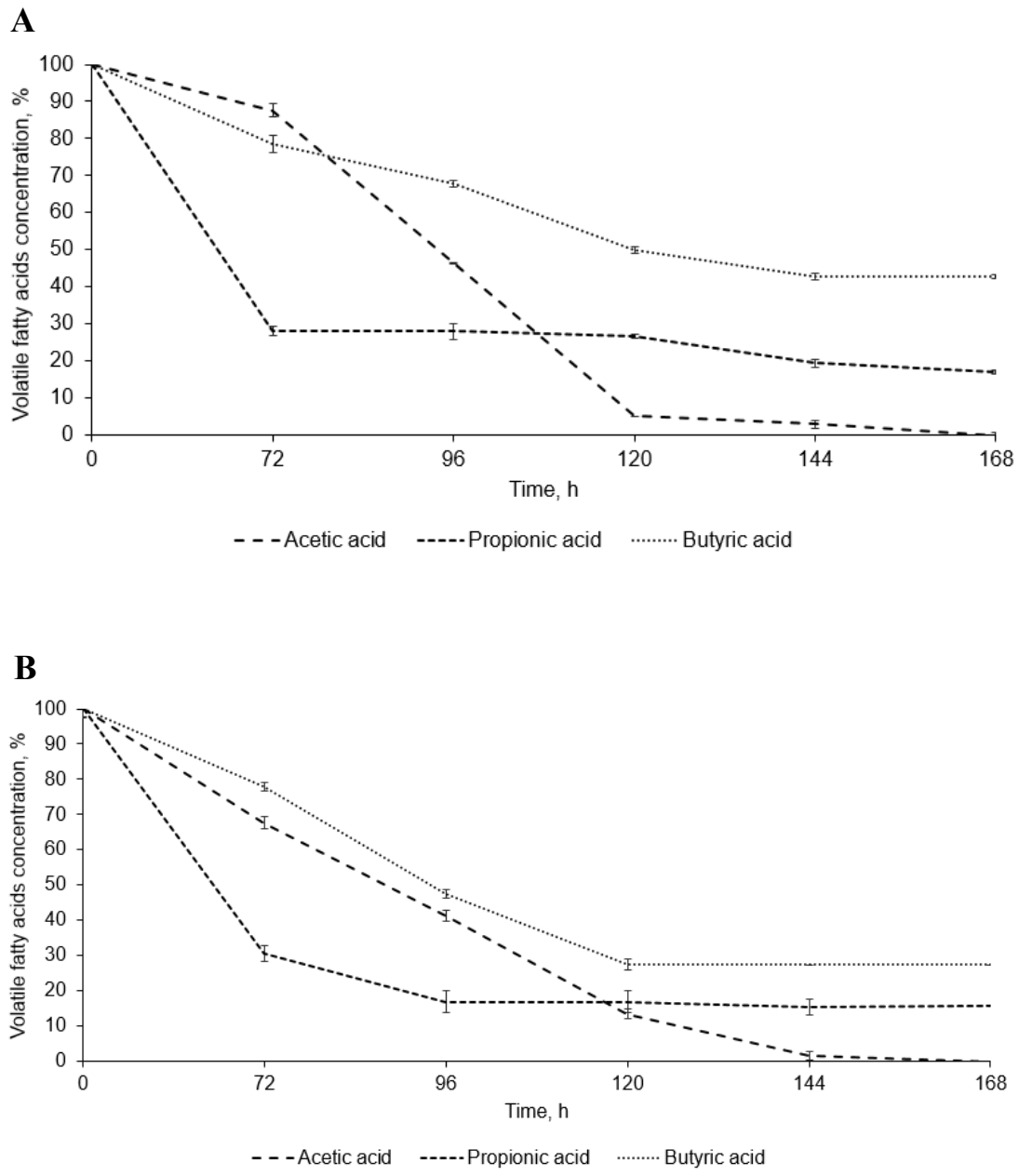

Figure 4.22. Volatile fatty acids consumption of *C. protothecoides*. Total consumption of volatile fatty mix (acetic acid: propionic acid: butyric acid; ratio 20:3.3:1), with dilution 1:2 (A) and 1:3 (B) were plotted relative to time (h), done in replicate (n=3).

4.5. Lipid production by *C. protothecoides* using acetic, propionic and butyric acids as single and mixed carbon sources

In the lipid accumulation study, the results of growing *C. protothecoides* were compared to growth in the control experiments after the same period of cultivation. The performance of *C. protothecoides* in glucose was used as a control for lipid accumulation. To examine the impact of using various volatile fatty acids on lipid accumulation, cells were cultivated in the dark for 168 h at 25°C with shaking at 140 rpm using different concentrations of acetic acid, propionic acid, or butyric acid. Biomass growth, biomass yield, total lipids, and lipid yield are shown in Table 4.5. The biomass growth of *C. protothecoides* was reduced when the cells were cultivated with different concentrations of acetic, propionic and butyric acids in the cultivation media (Table 4.5). Glucose was added such that the moles of carbon was identical with that of the 30.0 g/L acetic acid system (1 C mol/L).

The biomass growth (Figure 4.23), biomass yield (Table 4.5) and total amount of produced lipids (Figure 4.24) were significantly decreased for any volatile fatty acids system as compared to that of the control. The lipid yield was significantly decreased when algae were cultivated in 5.0 – 30.0 g/L of acetic acid as compared to that of the control. In contrast, lipid yield of *C. protothecoides* grown in propionic and butyric acids was significantly increased to 0.54 - 0.57 g/g and by 0.42 - 0.76 g/g, respectively (Table 4.5). It should be noted that the amount of biomass produced in these systems were significantly lower than the control (Figure 4.23).

Table 4.5. The performance of *C. protothecoides* grown in acetic, propionic, and butyric acids at 168 hours of growth, pH 7.0 and carbon to nitrogen ratio of 100/1, where Y(b/s) = biomass (g)/utilized substrate (initial – residual) (g) and Y(l/s) = lipid (g)/utilized substrate (initial – residual) (g), done in replicate (n=3).

Carbon source	Carbon amount, mol/L	Biomass yield, Y (b/s)	Lipid yield, Y(l/s)
Acetic acid, g/L			
5.0	0.17	0.44 ± 0.00	0.14 ± 0.01
10.0	0.33	0.40 ± 0.02	0.13 ± 0.02
20.0	0.67	0.34 ± 0.02	0.11 ± 0.03
30.0	1	0.37 ± 0.04	0.11 ± 0.03
Propionic acid, g/L			
1.5	0.06	0.54 ± 0.01	0.57 ± 0.07
2.0	0.08	0.46 ± 0.01	0.54 ± 0.02
2.5	0.1	0.44 ± 0.01	0.55 ± 0.05
Butyric acid, g/L			
0.5	0.02	0.44 ± 0.01	0.76 ± 0.07
1.0	0.05	0.35 ± 0.04	0.52 ± 0.02
1.5	0.07	0.22 ± 0.02	0.42 ± 0.01
Glucose, g/L			
30.0	1.0	0.58 ± 0.01	0.17 ± 0.04

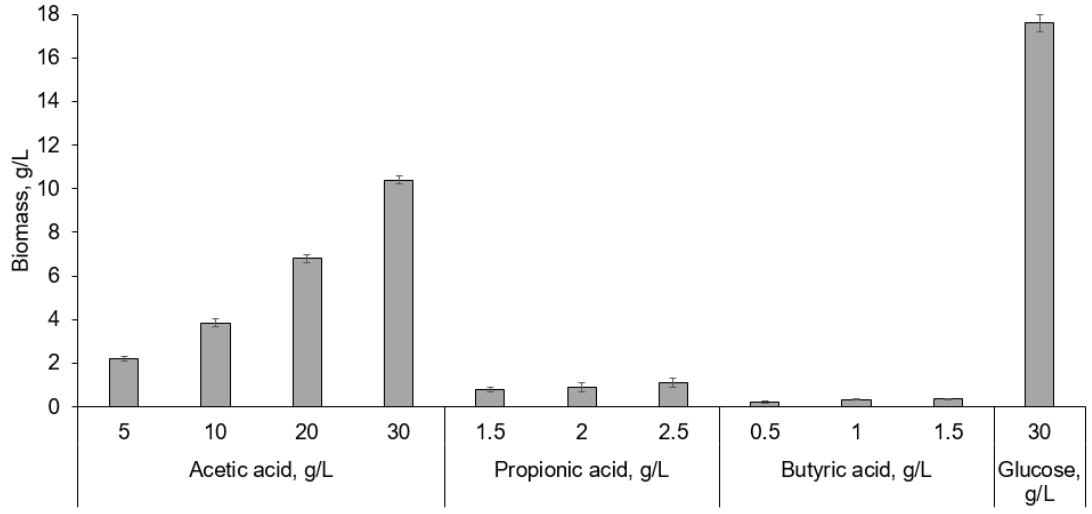

Figure 4.23. Biomass produced by *C. protothecoides* under the different loadings of acetic, propionic and butyric acids at 168 hours of growth, pH 7.0 and carbon to nitrogen ratio of 100/1, done in replicate (n=3).

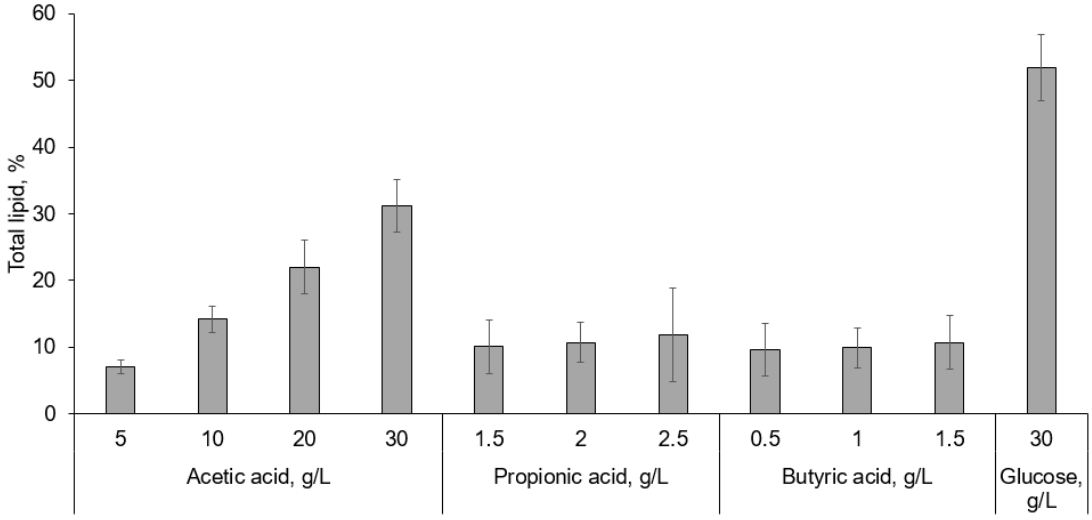

Figure 4.24. Lipid amount produced by *C. protothecoides* under the different loadings of acetic, propionic and butyric acids at 168 hours of growth, pH 7.0 and carbon to nitrogen ratio of 100/1, done in replicate (n=3).

Biomass production, total lipid accumulation and lipid yield shown for *C. protothecoides* grown in the undiluted volatile fatty acid mix (acetic, propionic and butyric acids ratio 20:3.3:1), or the 1:1, 1:2, and 1:3 dilutions are shown in Table 4.6. The control was standardized according to the amount of carbon loaded into 1:3 dilution culture media recipe for lipid production by *C. protothecoides.* The biomass production and total lipid accumulation were reduced when the algae cells were cultivated in volatile fatty acids media. For the volatile fatty acid systems, the maximum biomass production (8.6 ± 0.2 g/L) and lipid accumulation (48.6 ± 0.8 %) were achieved when algae were grown using the 1:3 dilution. In comparison, the control with glucose as a carbon source resulted in biomass production of 11.1 ± 0.3 g/L and total lipids of 52.1 ± 1.5 %.

Table 4.6. The performance of *C. protothecoides* cultures grown using mixtures of volatile fatty acid. The control (glucose) corresponds (in terms of moles of carbon) to the amounts of the volatile fatty acids in 1:3 diluted media. Lipid yield = Lipid (g)/Utilized substrate (initial – residual) (g), done in replicate (n=3).

Volatile fatty acids mix	Carbon amount, g/L	Biomass, g/L	Total lipids, %	Lipid yield, g lipid/g utilized substrate
Undiluted	30.48	2.4 ± 0.5	27.3 ± 0.2	0.05 ± 0.1
1:1	15.24	3.0 ± 0.5	30.9 ± 1.0	0.17 ± 0.2
1:2	10.20	4.9 ± 0.2	44.2 ± 0.2	0.18 ± 0.1
1:3	7.62	8.6 ± 0.2	48.6 ± 0.8	0.19 ± 0.0
Control (glucose)	7.62	8.8 ± 0.1	52.1 ± 1.5	0.21 ± 0.5

Lipid yield observed for *C. protothecoides* growth in 1:3 volatile fatty acids dilution to 0.19 ± 0.0 g/g as compared to lipid yield in the control media with glucose 0.21 ± 0.5 g/g was not significantly different. Based on the amount of consumed carbon after 168 h of *C. protothecoides* growth, no significant difference between lipid yields in undiluted volatile fatty acid mix or the 1:1, 1:2, and 1:3 dilutions were observed (Table 4.6).

Changes in the fatty acid profile during growth of *C. protothecoides* using the volatile fatty acid mix as a feedstock were then examined. The quantification of the fatty acids from the lipids accumulated by *C. protothecoides* was done through CG-FID after esterification (Figure 4.25).

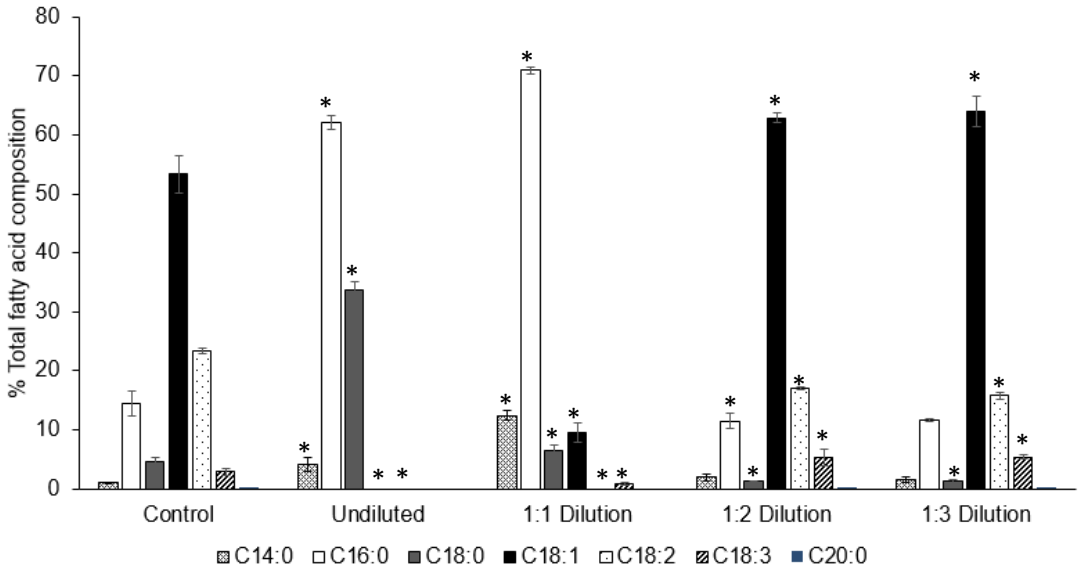

Figure 4.25. Fatty acid composition of *C. protothecoides* grown in volatile fatty acids. Asterisk (*) are indicated fatty acid levels that differ significantly ($p < 0.05$) between the control culture (used glucose as a carbon source) and the culture supplemented with volatile fatty acids, done in replicate (n=3). C14:0 – myristic acid, C16:0 - palmitic acid, C18:0 - stearic acid, C18:1- oleic acid, C18:2 - linoleic acid, C18:3 - linolenic acid, and C20:0 - arachidic acid.

As a control, the fatty acid profile of *C. protothecoides* grown in glucose was examined, where glucose was added to the cultivation media in an amount equivalent (based on moles of carbon) to the 1:3 diluted volatile fatty acids mix. The fatty acid composition of *C. protothecoides* grown in glucose (control) was found to be predominantly oleic (C18:1) at 53.3 ± 0.5 %, linoleic (C18:2) at 23.7 ± 0.6 % and palmitic (C16:0) at 14.4 ± 1.2 % acids with minor amounts of stearic acid (C18:0) at 4.7 ± 0.6 %, linolenic acid (C18:3) at 2.9 ± 0.3 %, myristic acid (C14:0) at 1.4 ± 0.2 % and arachidic acid (C20:0) at 0.3 ± 0.1 % . Significant differences in the fatty acid composition of *C. protothecoides* grown in volatile fatty acids mix were found (Figure 4.25). The major fatty acid in the profile of algae grown in undiluted and the 1:1 diluted media was palmitic acid, reaching 62.1 ± 0.6 % and 70.9 ± 1.2 %, respectively. For the fatty acid profile of *C. protothecoides* grown with undiluted fatty acid mix, we observed significant increases in stearic and myristic acids, and unsaturated fatty acids were not detected. For example, the concentration of myristic acid was significantly increased to 12.4 ± 0.6 %, while the concentration of oleic acid was reduced to 6.4 ± 0.2 %. The concentration of oleic acid was significantly increased to 62.9 ± 0.3 % and 64.0 ± 0.8 % when algae were grown using 1:2 and 1:3 diluted volatile fatty acids mixture, respectively, as compared to that of the control and growth with the undiluted and 1:1 diluted fatty acid mix. Conversely, compared to the control, use of the 1:2 and 1:3 diluted fatty acid mixtures resulted in significantly lower proportions of linoleic acid.

In the study of lipid production by *C. protothecoides* grown with a volatile fatty acids mix, we demonstrated that the amount of total lipids produced, as well as

lipid yield, were significantly higher when the cells were grown in the 1:3 diluted volatile fatty acids mix, compared to other volatile fatty acid systems examined. Analysis of the fatty acid composition of *C. protothecoides* grown in higher concentration of volatile fatty acids present in the fermentation media (undiluted and 1:1 diluted) displayed enhanced amounts of saturated fatty acids. Additionally, enhanced amounts of unsaturated fatty acids were observed during fermentation of *C. protothecoides* in 1:2 and 1:3 diluted media.

4.6. Evaluation of feasibility of the aqueous streams produced through hydrolysis of biosolids, brown grease, and their mixtures as feedstocks for oleaginous microorganisms growth and lipid accumulation

The aqueous by-product stream after hydrolysis of biosolids (4%) was characterized; the water content, volatile fatty acids content (Table 4.7), amino acid content (Figure 4.26, 4.27), and amounts of total organic carbon and total nitrogen (Figure 4.28) were determined. The aqueous by-product stream after hydrolysis of 4 % biosolids contains 99.8 ± 0.1 % water as determined by GC-TCD analysis.

Determination of volatile fatty acids in the aqueous by-product streams after hydrolysis of 4 % biosolids, brown grease, and their mixtures were done by GC-FID. The aqueous by-product streams after hydrolysis of 4 % biosolids mainly consists of acetic, propionic, and butyric acids, and the total content of volatile free acids was determined to be 2.55 ± 0.3 g/L. The same main volatile free fatty acids that were predominant in sample composition were observed after hydrolysis of brown grease.

Table 4.7. The volatile free acids content (g/L) in samples after hydrolysis of 4 % biosolids, brown grease and mixtures of 4 % biosolids and brown grease (50/50; w/w), done in replicate (n=3).

	Acetic acid	**Propionic acid**	**Isobutyric acid**	**Butyric acid**	**Isovaleric acid**	**Valeric acid**	**Isocaproic acid**	**Hexanoic acid**
Biosolids	1.02 ± 0.2	0.83 ± 0.1	BDL	0.41 ± 0.1	0.07 ± 0.01	0.12 ± 0.03	BDL	0.11 ± 0.03
Brown grease	0.32 ± 0.03	0.17 ± 0.01	BDL	0.26 ± 0.02	BDL	0.08 ± 0.01	0.11 ± 0.04	BDL
Mix*	2.11 ± 0.1	0.23 ± 0.04	0.07 ± 0.01	0.23 ± 0.04	0.27 ± 0.02	0.07 ± 0.01	BDL	BDL

* - biosolids and brown grease mix (50/50; w/w).

In the aqueous by-product water samples after hydrolysis of brown grease the total concentration of volatile fatty acids was 0.84 ± 0.05 g/L. The aqueous by-product streams produced through hydrolysis of the brown grease/biosolids mixture contained 2.11 ± 0.1 g/L acetic acid, 0.23 ± 0.04 g/L propionic acid as well as 0.23 ± 0.04 g/L butyric acid, and 0.27 ± 0.02 g/L isovaleric acid. The total amount of carbon in the form of volatile fatty acids was 2.98 ± 0.2 g/L in the aqueous by-product streams after hydrolysis of the brown grease/biosolids mixture. The amount of acetic acid in the mixture was significantly higher than the amount of acetic acid in biosolids and brown grease separately. This implies that mixing the biosolids and brown grease and subsequent hydrolysis promotes acetic acid production in the aqueous by-product stream. Conversely, the amount of propionic acid as that found in the aqueous by-product stream after hydrolysis of brown grease or biosolids are significantly lower than in the aqueous by-product stream after hydrolysis of biosolids.

The amino acid composition in the aqueous by-product streams after hydrolysis of 4 % biosolids or brown grease was determined using HPLC coupled with fluorescence detection, and the results are shown in Figures 4.26 and 4.27, respectively. The total amount of amino acids was quantified in the aqueous by-product streams after hydrolysis of 4 % biosolids at 179.9 ± 1.2 mg/L. The major amino acids detected were leucine at 42.2 ± 0.6 mg/L and alanine 40.4 ± 0.4 mg/L. The minor amino acids identified were glutamate, glycine, arginine, tyrosine, methionine, valine, and isoleucine. In addition, the total amount of amino acids was quantified in the aqueous by-product streams after hydrolysis of brown grease at 49.6

± 0.8 mg/L. The major amino acids detected were phenylalanine 36.6 ± 0.1 mg/L, The minor amino acids detected were tyrosine, methionine, glutamate, and valine.

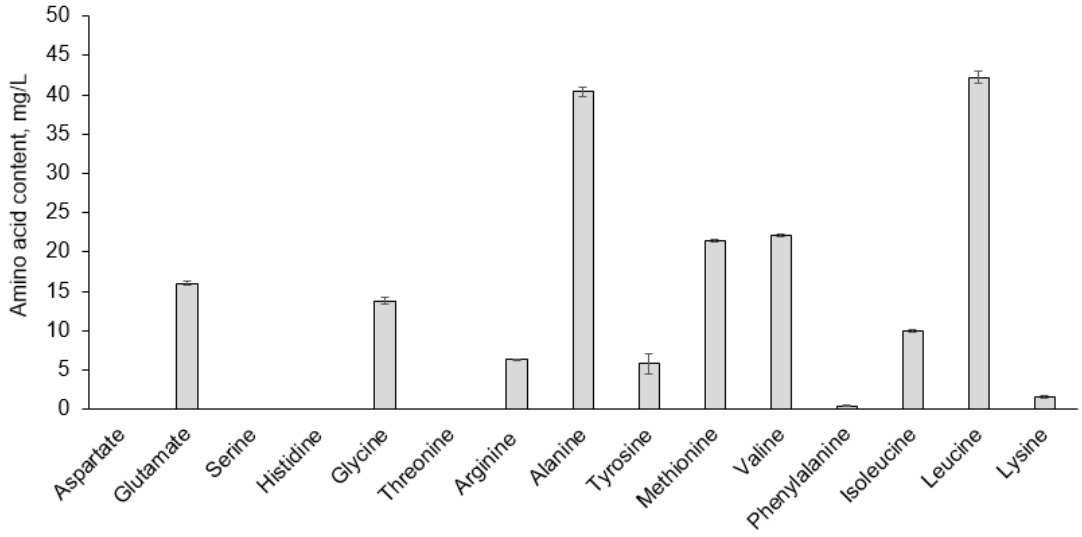

Figure 4.26. Free amino acid content in the aqueous by-product stream after hydrolysis of 4 % biosolids, done in replicate (n=3).

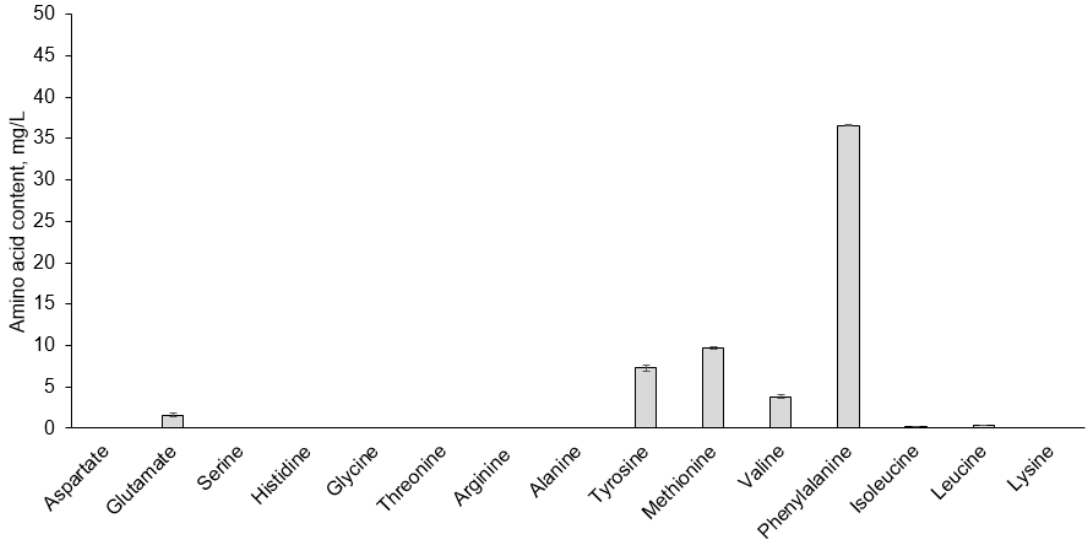

Figure 4.27. Free amino acid content in the aqueous by-product stream after hydrolysis of brown grease, done in replicate (n=3).

As shown in Figure 4.28, the amount of total organic carbon in the aqueous phase after hydrolysis of 4 % biosolids was significantly lower than that detected in the aqueous stream from hydrolysis of brown grease or the biosolids/brown grease mixture. There was a significant decrease of organic nitrogen detected in the aqueous phase after hydrolysis of brown grease, compared to what was observe from the other two systems. The carbon to nitrogen ratio in the aqueous phase after hydrolysis of biosolids was 1.2, that of brown grease was 238.3, and finally, the biosolids/brown grease mixture was 8.5.

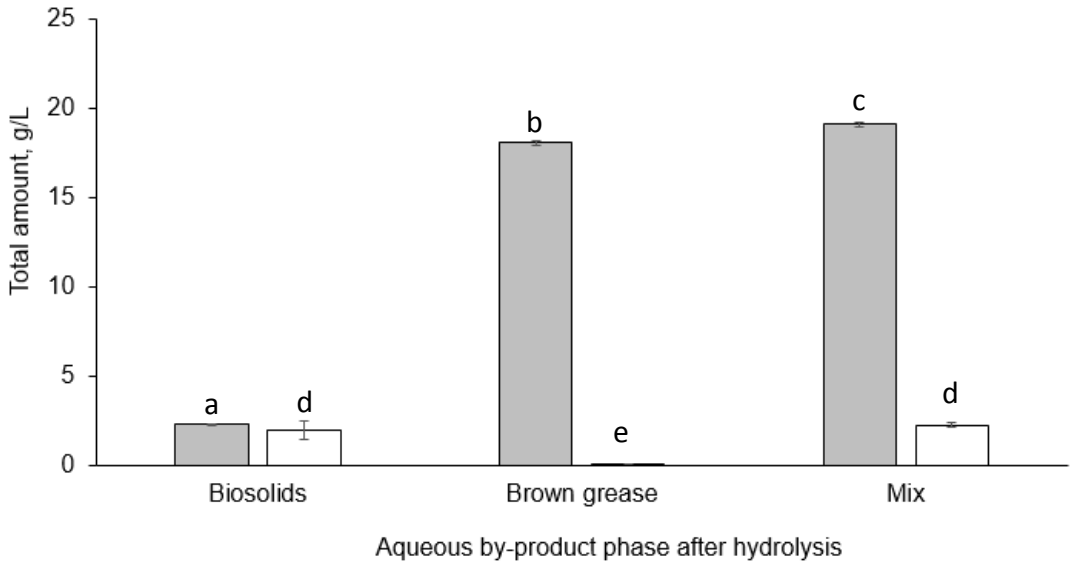

Figure 4.28. Amount (g/L) of total organic carbon (dark gray bar) and total nitrogen (light gray bar) in the aqueous by-product phase obtained after hydrolysis of biosolids, brown grease, or their mixtures. The amount of total organic carbon (a, b, c), total nitrogen (d, e, f) marked with different letters that are significantly different ($p < 0.05$).

The biomass growth and lipid accumulation of *C. curvatus* and *C. protothecoides* cultures were evaluated when the aqueous phases after hydrolysis of

4 % biosolids, brown grease and mixtures of the two (50/50 w/w of 4 % biosolids and brown grease mixed before hydrolysis) were incorporated into the growth medium (Figure 4.29). Yeast (*C. curvatus*) was grown at 30 °C with agitation (200 rpm) for a total of 120 h, while the microalgae *C. protothecoides* was grown at 25 °C with agitation (140 rpm) for a total of 168 h. For both oleaginous microorganisms, significant increases in biomass growth were observed using the aqueous phase after hydrolysis of brown grease and the biosolids/brown grease mixture as compared to the aqueous phase after hydrolysis of 4 % biosolids alone. Yeast biomass experimental results shows that *C. curvatus* can grow and produce 9.7 ± 0.2 g/L and 11.2 ± 0.5 g/L biomass in the aqueous phase after hydrolysis of brown grease and the biosolids/brown grease mixture, respectively. Significantly lower biomass production values were observed for *C. protothecoides* as compared to *C. curvatus* when oleaginous microorganisms were grown in the aqueous phase after hydrolysis of brown grease or the biosolids/brown grease mixture. As shown in Figure 4.29A, the biomass growth for both yeast and microalgae were reduced when cells were grown in the aqueous phase after hydrolysis of 4 % biosolids and no significant differences in biomass production levels were observed between the two organisms.

The aqueous phase after hydrolysis of brown grease was the good feedstock for lipid production. Lipid accumulation of 12.5 ± 0.1 % was detected in yeast cells, and 26.8 ± 3.2 % was observed for microalgae. As shown in Figure 4.29B, the lipid production was significantly reduced when *C. curvatus* and *C. protothecoides* were cultivated in the aqueous phase after hydrolysis of 4 % biosolids and the biosolids/brown grease mixture.

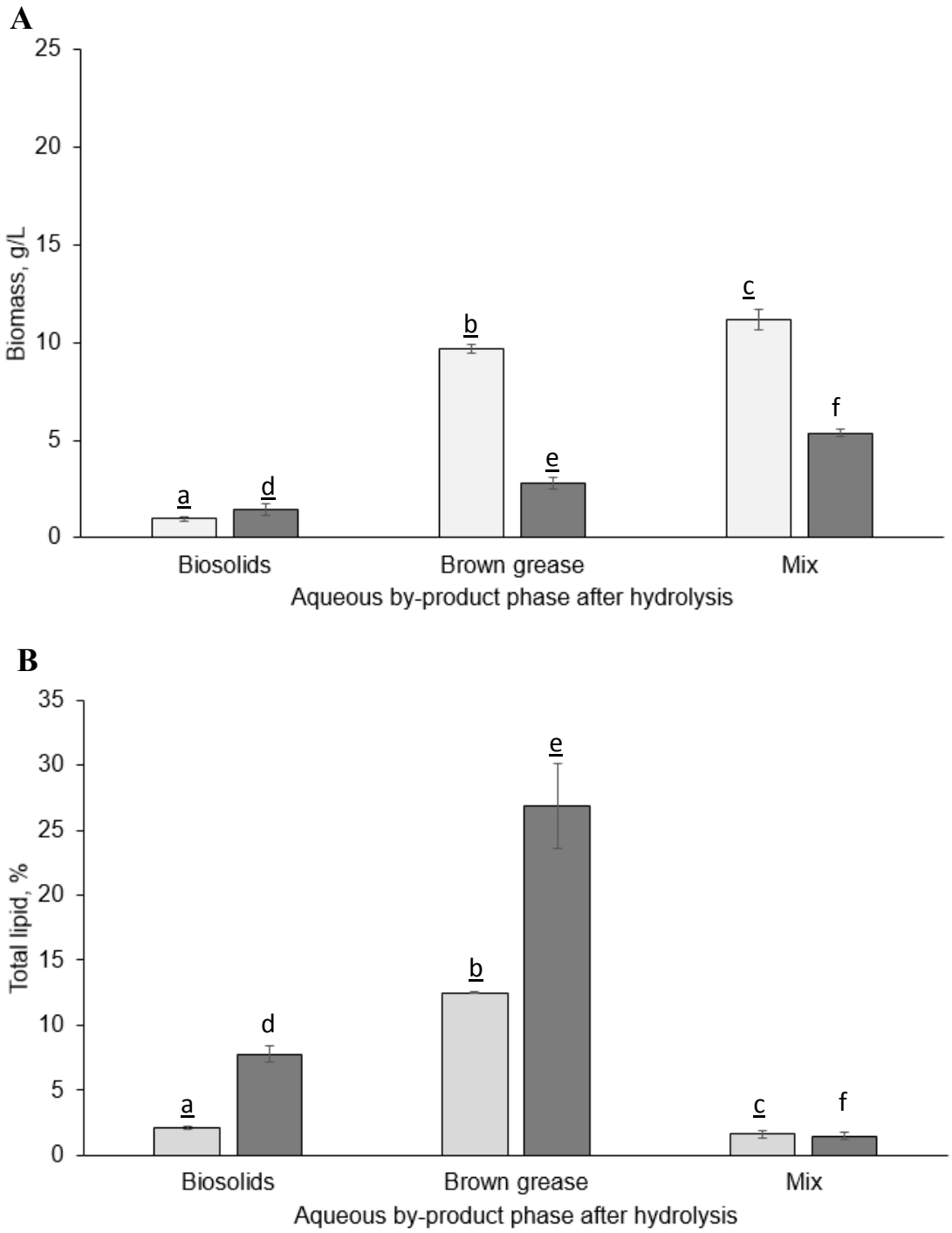

Figure 4.29. The performance of *C. curvatus* (light gray bar) and *C. protothecoides* (dark gray bar) cultures grown in feedstock representing the aqueous phase after hydrolysis of biosolids, brown grease, and mixtures of the two (50/50 (w/w) of biosolids and brown grease mixed before hydrolysis). Oleaginous microorganisms were cultivated 120 h and 168 h, respectively for yeast and algae. The amount of biomass, lipids for *C. curvatus* (a, b, c), *C. protothecoides* (d, e, f) marked with different letters that are significantly different ($p < 0.05$), done in replicate (n=3).

The next cultivation strategy was designed to force the oleaginous yeast and microalgae to produce biomass and accumulate lipids by modification of the aqueous by-product phases after hydrolysis of 4 % biosolids and the mixed feedstock (50/50 (w/w) of 4 % biosolids and brown grease mixed before hydrolysis). Exogenous mineral supplementation of the aqueous by-product phase after hydrolysis of 4 % biosolids did not lead to increased biomass growth in *C. curvatus* (Figure 4.30A). Conversely, increased biomass accumulation was observed for *C. prototheco*ides through exogenous mineral supplementation of the biosolids hydrolysate: biomass production of *C. prototheco*ides increased from 1.4 ± 0.3 g/L in the initial aqueous by-product phase after hydrolysis of 4 % biosolids to 5.3 ± 0.3 g/L in the same system, but supplemented with essential minerals.

Significant increases (~ 3.6 %) in lipid production were observed through supplementation of the growth medium (containing aqueous by-product phase after hydrolysis of 4 % biosolids) with essential minerals for *C. curvatus*. Conversely, for *C. prototheco*ides, supplementation of the aqueous by-product phases after hydrolysis of biosolids with essential minerals led to a reduction in lipid accumulation from 7.8 ± 0.6 % to 1.8 ± 0.2 % (Figure 4.30B), despite the fact that there was an increase in biomass production when essential minerals were added.

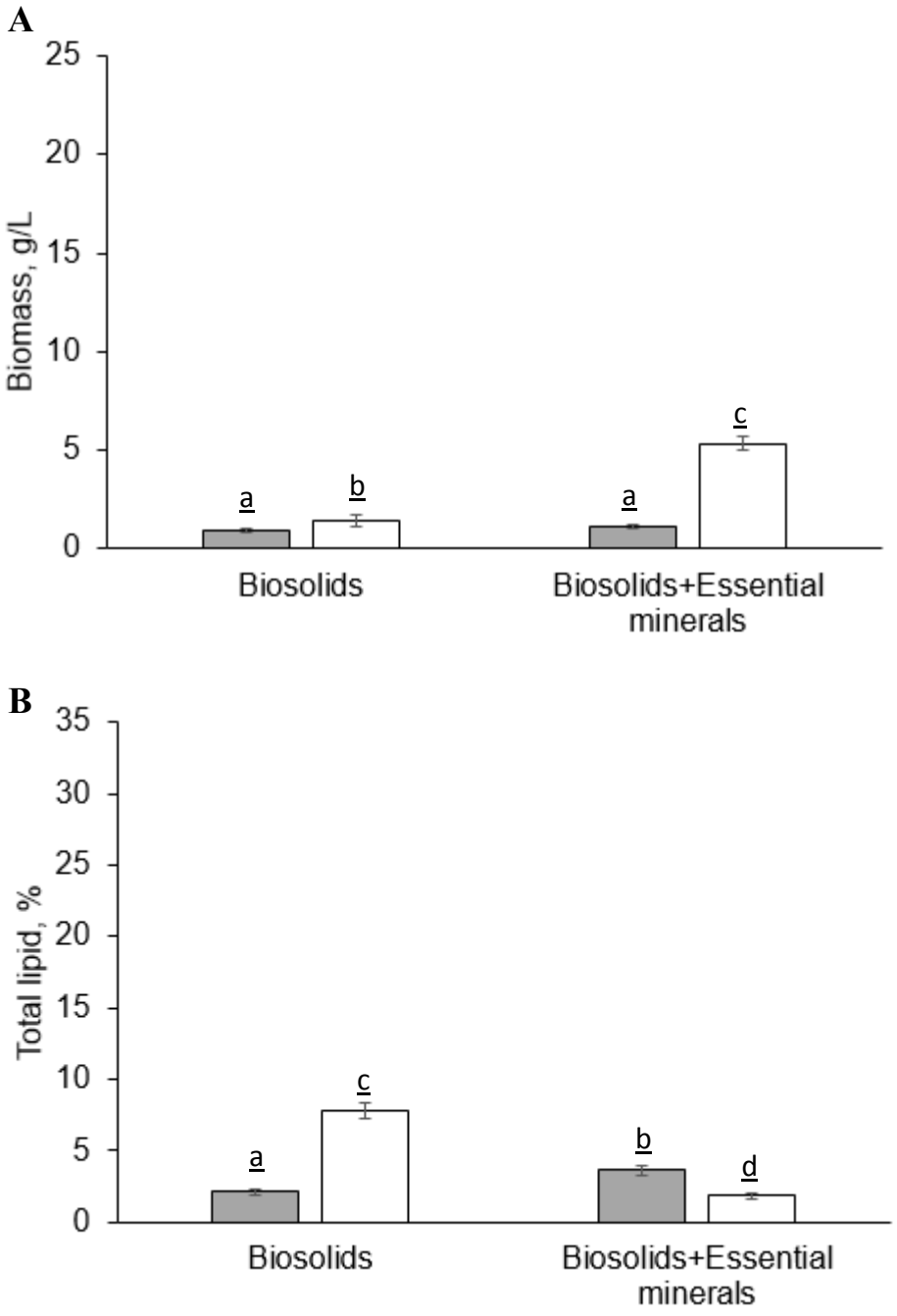

Figure 4.30. Biomass production (A) and lipid accumulation (B) of *C. curvatus* (light grey bar) and *C. protothecoides* (dark grey bar) in the aqueous by-product phase after hydrolysis of biosolids (with/without essential minerals supplementation). The amount of biomass, lipids for *C. curvatus* and *C. protothecoides* marked with different letters that are significantly different ($p < 0.05$), done in replicate (n=3).

To promote the *C. curvatus* and *C. prototheco*ides lipid accumulation, glycerol was added to mix media (50/50 (w/w) of biosolids and brown grease mixed before hydrolysis) to increase the carbon to nitrogen ratio from 8.5/1 to 50/1. After 120 h and 168 h cultivation of *C. curvatus* and *C. prototheco*ides, respectively, the biomass production was decreased significantly for *C. curvatus* and increased significantly for *C. prototheco*ides (Figure 4.31A). No significant differences in lipid accumulation by microalgae *C. prototheco*ides were detected, whereas in yeast *C. curvatus*, total lipid accumulation was increased significantly from 1.6 ± 0.3 % in the aqueous by-product phase after hydrolysis of mixed feedstock to 8.6 ± 0.1 % in the aqueous by-product phase after hydrolysis of mixed feedstock supplemented by glycerol (Figure 4.31B). It was noticed that the amount of consumed glycerol after 120 h of cultivation of *C. curvatus* was 34.5 ± 1.6 % and after 168 h of cultivation for *C. prototheco*ides was 81.6 ± 2.1 %.

To increase substrate consumption and promote lipid accumulation, the cultivation time was extended to 168 h for yeast and 240 h for microalgae. The carbon to nitrogen ratio was also adjusted using glycerol to 30/1 and 50/1 and the media was supplemented with essential minerals. Cultures were grown in the aqueous by-product phase after hydrolysis of mixed feedstock (50/50 w/w of biosolids and brown grease mixed before hydrolysis) as a control. As shown in Figure 4.32A, the yeast biomass production in the aqueous by-product phase after

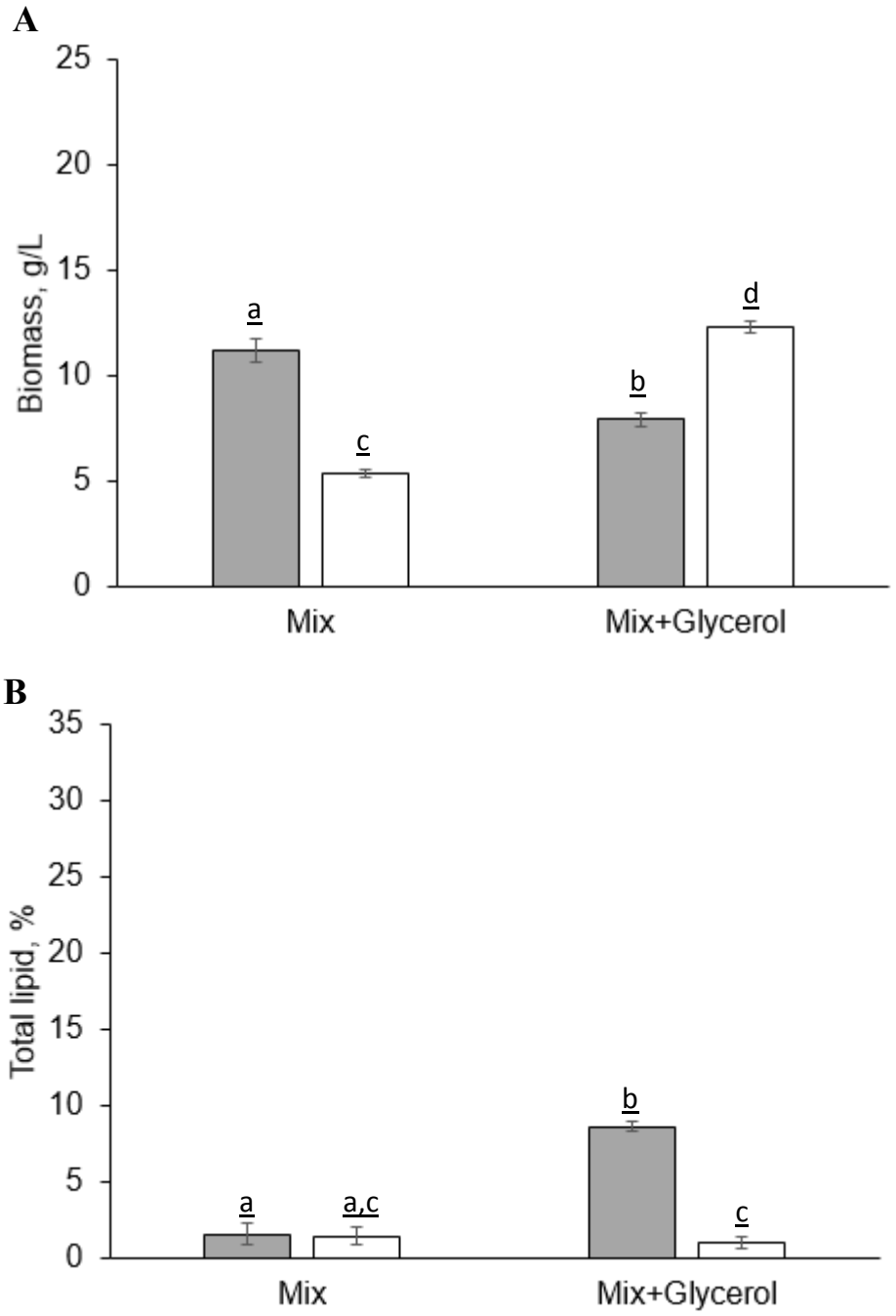

Figure 4.31. The performance of *C. curvatus* (light grey bar) and *C. prototheco*ides (dark grey bar) in the aqueous by-product phase after hydrolysis of the mixed feedstock (with/without glycerol supplementation; carbon to nitrogen ratio was 50/1), where A - biomass production and B - lipid accumulation. The amount of biomass, lipids for *C. curvatus* and *C. prototheco*ides marked with different letters that are significantly different ($p < 0.05$), done in replicate (n=3).

hydrolysis of mixed feedstock supplemented by essential minerals and/or by glycerol as a carbon source (carbon to nitrogen ratio was 50/1 and 30/1) were significantly reduced as compared to the control conditions. Conversely, significant increases in microalgae growth were observed in all systems. The addition of the essential minerals and glycerol (carbon to nitrogen ratio 50/1) in the aqueous by-product phase after hydrolysis of biosolids allowed *C. protothecoides* to produce 20.9 ± 0.4 g/L biomass.

As shown in Figure 4.32B, a similar trend for lipid production by *C. curvatus* and *C. protothecoides* was observed when the cells were grown either in the aqueous by-product phase after hydrolysis of mixed feedstock with glycerol as a carbon source (carbon to nitrogen ratio is 50/1 and 30/1) or in the aqueous by-product phase after hydrolysis of mixed feedstock supplemented by essential minerals and by glycerol as a carbon source (carbon to nitrogen ratio is 50/1 and 30/1). The highest amount of lipids produced by yeast was 10.2 ± 0.1 %, when the aqueous by-product phase after hydrolysis of mixed feedstock was supplemented with essential minerals and glycerol (carbon to nitrogen ratio of 50/1). At the same time, the same cultivation conditions (the aqueous by-product phase after hydrolysis of mixed feedstock was supplemented with essential minerals and glycerol (carbon to nitrogen ratio of 50/1)) led to a significant reduction in lipid production by algae. The highest amount of lipids produced by algae was 9.2 ± 0.2 % when the aqueous by-product phase after hydrolysis of mixed feedstock was supplemented by glycerol (carbon to nitrogen ratio of 30/1). It was noticed that the amount of consumed glycerol after

168 h of cultivation of *C. curvatus* was 46.9 – 57.7 %, and the loaded glycerol was mostly (91.1 – 92.8 %) consumed by *C. protothecoides* after 240 h of cultivation.

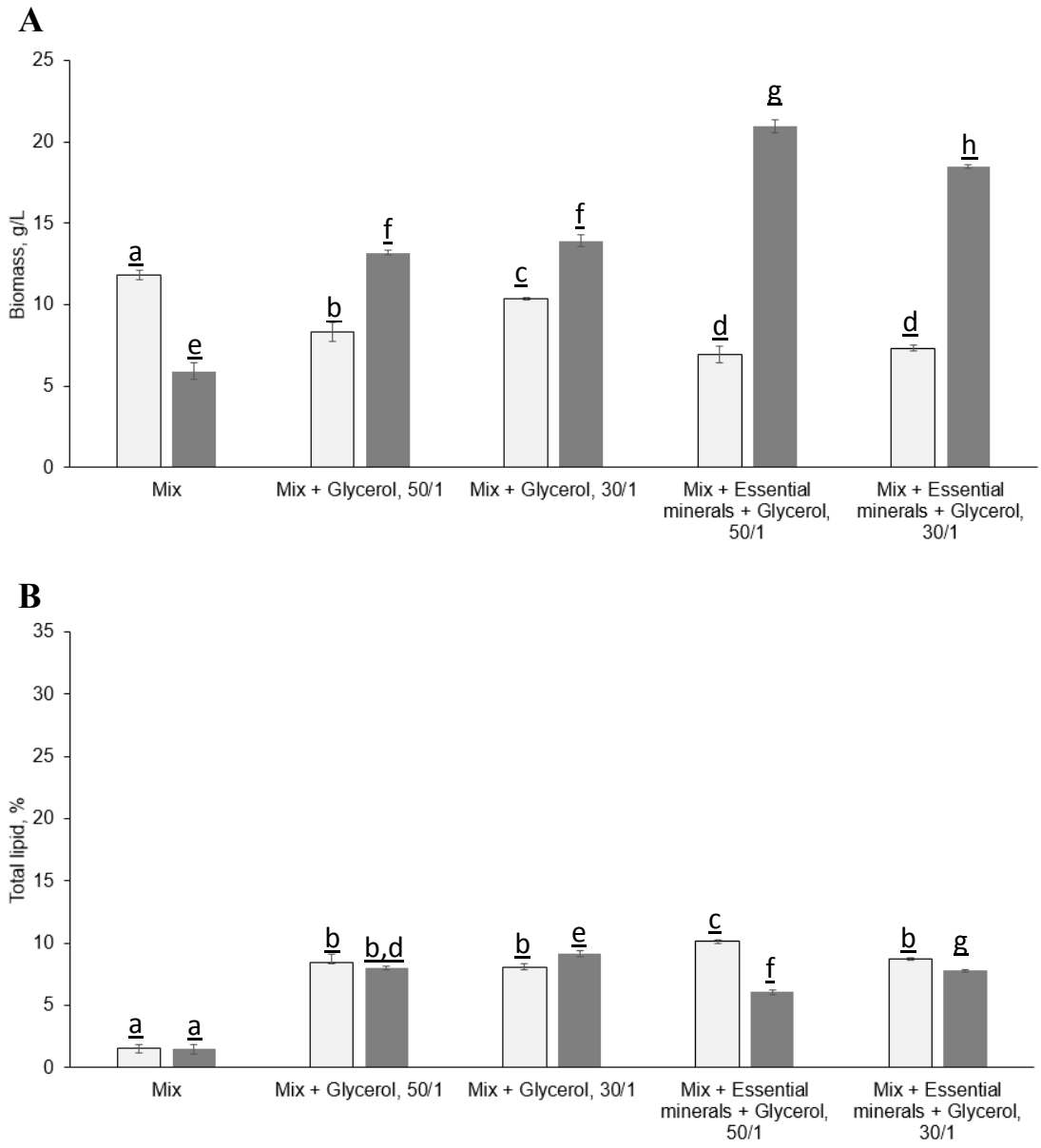

Figure 4.32. Biomass production (A) and lipid accumulation (B) of *C. curvatus* (light gray bar) and *C. protothecoides* (dark gray bar) obtained using the aqueous by-product phase after hydrolysis of a mixed feedstock (biosolids : brown grease at 1:1) supplemented with essential minerals and/or with glycerol (carbon to nitrogen ratio was 50/1 and 30/1). Oleaginous microorganisms were cultivated 120 h and 168 h, respectively for yeast and algae. The amount of biomass, lipids for *C. curvatus* and *C. protothecoides* marked with different letters that are significantly different ($p < 0.05$), done in replicate (n=3).

In order to analyze the possible changes in the fatty acid profile, *C. curvatus* and *C. protothecoides* were grown in the aqueous by-product phase after hydrolysis of mixed feedstock supplemented by essential minerals and by glycerol (carbon to nitrogen ratios of 50/1 or 30/1) after 168 h and 240 h of cultivation, respectively. The quantification of the fatty acids from the lipids accumulated by *C. curvatus* and *C. protothecoides* was determined through CG-FID after esterification (Figure 4.33). The fatty acid profile of yeast and algae grown in the aqueous by-product phase after hydrolysis of the mixed feedstock was established.

The fatty acid profile of *C. curvatus* grown in the aqueous stream from the mixed feedstock hydrolysates was found to contain primarily oleic (42.2 ± 0.6 %), linoleic (17.9 ± 0.5 %), and palmitic (25.4 ± 1.2 %) acids (Figure 4.33A). Significant decreases in the composition of fatty acids were observed for oleic acid in all other systems when compared to the control fatty acids profile. The amount of linoleic acid was significantly increased to 21.7 ± 0.6 % and 19.8 ± 0.4 %, through supplementation with glycerol (carbon to nitrogen ratio of 50/1) or minerals and glycerol (carbon to nitrogen ratio of 30/1), respectively, when compared to control fatty acids profile. For the latter condition, the amount of stearic acid was also significantly increased to 15.2 ± 0.4 % from 11.2 ± 0.5 %.

The fatty acid profile of *C. protothecoides* grown in the aqueous stream from the mixed feedstock hydrolysates was found to be primarily palmitic (43.7 ± 0.6 %) and oleic (51.9 ± 0.8 %) acids (Figure 4.33B). Changes in the fatty acids profile distribution in the experimental systems were tracked compared to the control

(aqueous by-product phase after hydrolysis of mixed feedstock with no supplementation). Significant differences in the oleic acid distribution of *C. protothecoides* grown in supplemented feedstocks were found when compared with the fatty acid profile of the control. For example, the amount of oleic acid was significantly increased to 58.5 ± 0.3 % and 57.8 ± 0.1 % in the aqueous by-product phase after hydrolysis of mixed feedstock with or without essential minerals supplementation, respectively, when glycerol was added to adjust the carbon to nitrogen ratio to 50/1. Interestingly, when the carbon to nitrogen ratio was changed to 30/1, the amount of oleic acid was significantly decreased to 33.8 ± 0.2 % and 41.7 ± 0.1 %, with or without essential minerals supplementation, respectively. Also, significant decreases in the amounts of palmitic acid were observed when the carbon to nitrogen ratio was changed to 30/1 or 50/1, but with no mineral supplementation, or in the aqueous by-product phase after hydrolysis of mixed feedstock supplemented by essential minerals and glycerol (but only at a carbon to nitrogen ratio of 50/1). In the systems using aqueous by-product phase after hydrolysis of mixed feedstock supplemented by glycerol to bring the carbon to nitrogen ratio to 30/1 there were significant increases in the amounts of stearic acid composition: 20.3 ± 0.1 % and 19.0 ± 0.1 %, with or without mineral supplementation, respectively. There were no significant differences in the fatty acid distribution detected for linolenic acid as compared to the control system.

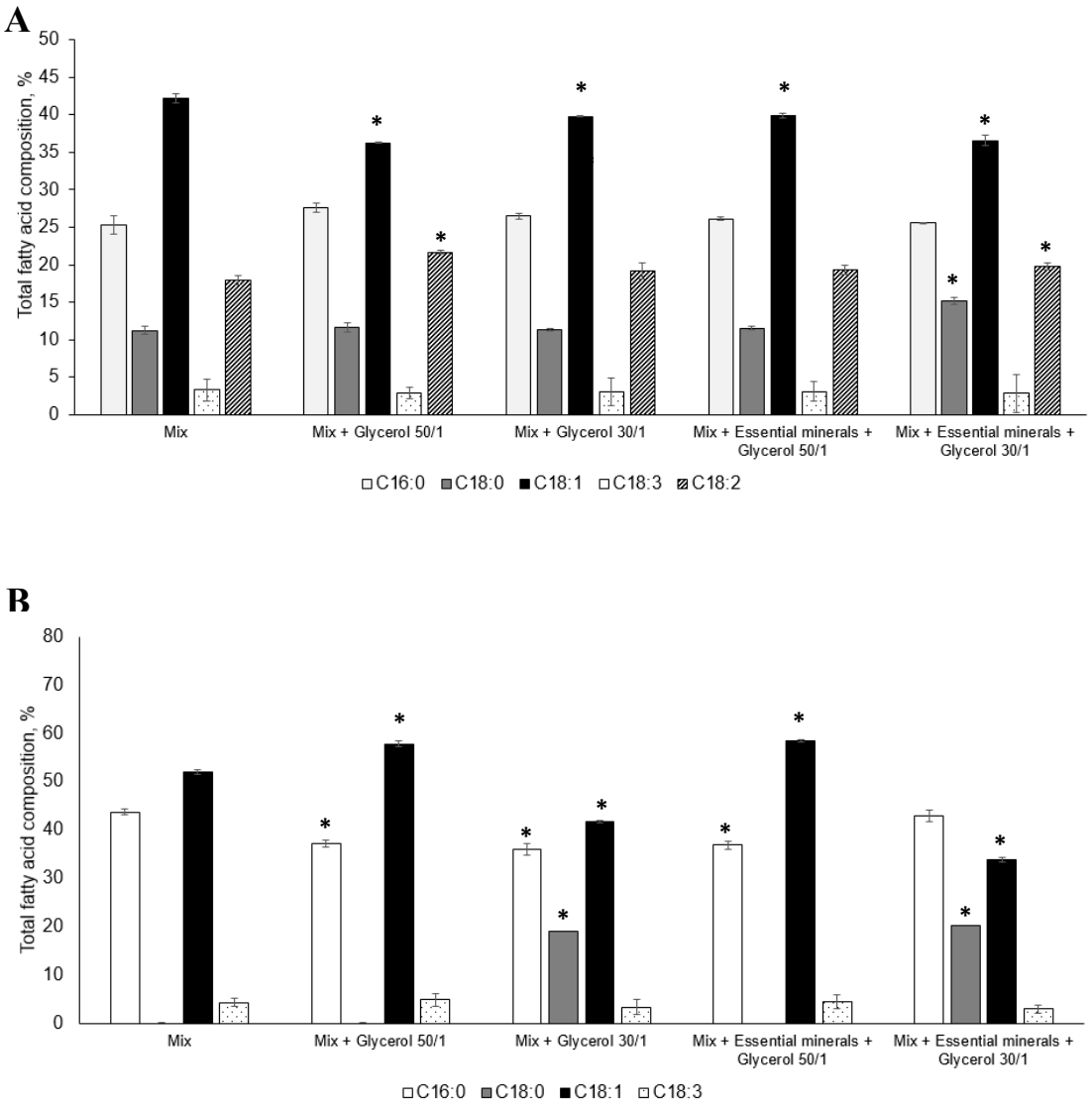

Figure 4.33. Fatty acid composition of *C. curvatus* (A) and *C. protothecoides* (B) grown in aqueous by-product phase after hydrolysis of mixed feedstock supplemented by essential minerals and by glycerol (carbon to nitrogen ratio was 50/1 or 30/1). Asterisk (*) are indicated fatty acid levels that differ significantly ($p < 0.05$) between the control culture (mix) and the culture supplemented mixed feedstock supplemented by essential minerals and/or by glycerol, done in replicate (n=3). C16:0 - palmitic acid, C18:0 - stearic acid, C18:1- oleic acid, C18:2 - linoleic acid, and C18:3 - linolenic acid.

To summarize, testing of exogenous mineral supplementation of the aqueous by-product phase after hydrolysis of 4 % biosolids and increasing carbon to nitrogen ratio for *C. protothecoides* determined that the amount of the produced microalgae biomass was significantly increased, while the amount of produced lipids was generally low, up to 10 %. It was observed for oleaginous yeasts that the exogenous mineral supplementation of the aqueous by-product phase after hydrolysis of 4 % biosolids led to significant increases in lipid production while the amount of produced biomass was significantly low compared to the aqueous phase without supplementation.

CHAPTER 5. OVERALL DISCUSSIONS AND CONCLUSIONS

5.1. Discussion

Addressing the waste by-product streams utilization and receiving the valuable profit from its valorization are the significant motivations and challenges of any industry. The main idea of this study was based on using the aqueous by-product streams from lipid pyrolysis as a cultivation media for growth and lipid accumulation of oleaginous microorganisms. Yeast *Cryptococcus curvatus* and microalgae *Chlorella protothecoides* was chosen as a well-known oleaginous microorganisms that can grow using wide range of waste feedstocks and able to accumulate up to 70 % of lipids by their dry weight (Chang *et al.,* 2010; Fei *et al.,* 2011; Fontanille *et al.,* 2012; Christophe *et al.,* 2012; Wen Q. *et al.,* 2013; Espinosa-Gonzales *et al.*, 2014a; Espinosa-Gonzalez, 2014b; Fei *et al.,* 2015). The first objective was focused on the characterizations of the aqueous by-product streams of lipid pyrolysis as well as determination of the compounds that can be applied as a carbon source for microorganisms fermentation. The preliminary experiments (by GC-FID, HPLC-RID) and compositional analysis (by Maxxam Analytics (Edmonton, AB)) determined the presence of volatile fatty acids and the wide range of other compounds.

The first challenge was to determine the amount of water present in the aqueous by-product streams after application of lipid pyrolysis. Analytical methods of water determination include Karl Fisher titration (colorimetric and volumetric) (Schöffski, Strohm, 2011), azeotropic distillation by the Dean-Stark method (Dean,

Stark, 1920, 2002), oven drying method (Willits, 1951, 2002), ^{1}H NMR spectroscopy (Dalitz *et al.,* 2012), and cobalt (II) complexes method (Zhou, 2011). Unfortunately, these methods are not ideal for determination of water content in the aqueous by-product streams from lipid pyrolysis. The Karl Fisher titration method shows interference with aldehydes, ketones, amines, organic acids, and solvents during water content determination (Schöffski, Strohm, 2011), and azeotropic distillation is influenced by organic compounds soluble in water (Dean, Stark, 1920, 2002). Disadvantages of the oven drying method are the low reaction velocity, bad precision, low sensitivity, and time consumption (Willits, 1951, 2002). In general, these methods can be applied to determine amounts of water less than 50 % (Nollet, 2000). The other methods mentioned are used to analyze water concentration in the low or trace amounts (Dalitz *et al.,* 2012; Zhou, 2011). Preliminary experiments using Karl-Fisher titration method showed that the amount of water present in our sample more than 50 %, the method's detection limit. Additionally, the presence a wide range of all above-mentioned organic compounds in the aqueous by-product samples gave interference in the analysis.

It was found that the GC-TCD method can be used for fast and non-expensive analysis of high water content samples (according to ASTM D3792-05). This method was applied for both types of the aqueous by-product streams (after hydrolysis and pyrolysis) from lipid pyrolysis as a fast, non-expensive, highly accurate, highly precise, and reproducible method to determine the water content. In our case, it was 88.9 ± 0.2 % and 91.9 ± 3.9 % of water present in the aqueous by-product samples after hydrolysis and pyrolysis of the yellow grease, respectively.

These values confirm the amounts of water determined in the aqueous by-product streams produced by conversion technologies used for biofuel production (Lian *et al.,* 2012; Paasikallio *et al.,* 2014; Black *et al*., 2016). For example, water content of 59.6 – 80.6 wt % was determined in samples after fast pyrolysis of lignocellulose biomass and 86.4 – 97.5 wt % water was determined in aqueous fractions received after catalytic fast pyrolysis of lignocellulose biomass (Black *et al*., 2016). It was shown by Paasikallio *et al.,* 2014, that in the product distribution after catalytic fast pyrolysis of the pine sawdust, water accounted for 67.7 wt %. In the aqueous phase after lignocellulosic biomass pyrolysis, there was 58.4 mass % water (Lian *et al.,* 2012).

Based on the aqueous by-product sample compositional analysis it was determined that 7.8 ± 3.7 % volatile fatty acids, predominantly acetic acid, propionic acid, and butyric acid are present after pyrolysis step. That gave the possibility to consider volatile fatty acids in this stream as a carbon source for oleaginous yeast and algae fermentation. The high concentration of volatile fatty acids resulted in high acidity of the stream and it was a major challenge for this study.

Palmqvist and Hahn-Hägaerdal (2000) showed that state of dissociation of the week acids determined their level of the toxicity and influenced the cell growth. In our screening experiments we determined that the low pH of fermentation (2.0 - 2.5) inhibits *Cryptococcus curvatus* as well as *Chlorella protothecoides* growth. This investigation clearly revealed that the pH of the aqueous by-product stream after the pyrolysis step should be neutralized to increase the level of dissociation of the volatile fatty acids, allowing its use to promote growth and lipid accumulation of

microorganisms. Additionally, it should be noted, that the pKa of the main volatile fatty acids present in the aqueous by-product sample as acetic, propionic and butyric are 4.76, 4.87, and 4.82, respectively (Nollet, Toldra, 2013). Adjusting pH to 5.4 and 6.4 led to volatile fatty acid presence in the media in dissociated state and as a result, the fermentation conditions of oleaginous yeast and algae were improved. It was published by Palmqvist and Hahn-Hägaerdal (2000) that at pH 5.4, partial dissociation occurred in the hydrolysates of lignocellulose. It was noticed by Jönsson *et al.* in 2013 that the presence of undissociated acids in the fermentation media was possible at pH 5.5, especially if they are initially loaded in the cultivation media in the high concentrations. The presence of undissociated acids are very unfavourable as this leads to diffusion into the cytosol, decreasing the cytosolic pH (uncoupling theory), and, thus, inhibiting the cell proliferation, viability and growth. Our experiments showed that the pH adjustment was the successful approach that allow oleaginous microorganisms to grow in the aqueous by-product stream after the pyrolysis.

The aqueous by-product stream after the pyrolysis step contained a range of volatile fatty acids and other compounds such as volatiles, phenolic compounds, and alcohols, that apparently could inhibite the biomass growth of the studied oleaginous yeast and algae. Reports have also shown the inhibitory influence of weak acids like acetic acid, furfurals and hydroxymethyl furfurals, and phenolic compounds received after application of methods of hydrolysis of lignocellulosic materials on microorganism's fermentation (Palmqvist, Hahn-Hägaerdal, 2000; Yu *et al.,* 2014; Luque-Moreno, 2015). For example, the presence 1.0 g/L furfural in the

fermentation media with glucose led to inhibition of *C. curvatus* growth by 78.4 % (Yu *et al.,* 2014). The suggested rationale to avoid the inhibitory effect of compounds included dilution, separation, and detoxification (Palmqvist, Hahn-Hägaerdal, 2000). Lian *et al.* (2012) determined that the aqueous phases after pyrolysis of lignocellulosic material could be used to cultivate oleaginous yeast *C. curvatus* after several additional treatments. The first treatment involves neutralization of pH using NaOH, followed by detoxification with activated carbon to remove inhibitory lignin-derived compounds, and finally the dilution of the initially high concentration of carboxylic acids (Lian *et al.,* 2012). Our experiments showed that the dilution approach is successful at lowering concentrations of inhibitors and/or volatile fatty acids to levels that will allow oleaginous microorganisms to grow. Moreover, the significant growth improvement under the high dilution will not be expected based on reduced amount of carbon with each successive dilution.

Based on the observation that the aqueous by-product stream is a complex mixture, the use of media that mimics volatile fatty acids concentrations as a single or mixed carbon source of the original stream are necessary. This allows evaluation of the ability of oleaginous microorganisms to grow and produce lipids as well as to avoid possible inhibition by other chemical compound. In this study, it was observed that the biomass production of *C. curvatus* and *C. protothecoides* was dramatically reduced when cells were grown at pH 5.4 compared to pH 7.0. There was a large decrease in biomass yield, when the acetic acid concentration was increased from 5.0 to 6.0 g/L at pH 5.4, which suggests that at 5.4, this concentration range is where

toxicity has more of an impact than increasing carbon accessibility. Nevertheless, our result showed that for both studied microorganisms, acetic acid was a more suitable carbon source than propionic and butyric acids in the conditions when these acids loaded as a single carbon source.

Previous studies such as those conducted by Garcia-Perez *et al.* (2010), Lian *et al.* (2012), Paasikallio *et al.* (2014), Vitasari *et al.* (2015), and Black *et al.* (2016), showed that in the aqueous by-product stream from conversion technologies used for biofuel production, volatile fatty acids are usually present as mixtures. Because of this, we also considered using different volatile fatty acids as a mixed substrate to grow oleaginous yeast. In some studies, the effect of different volatile fatty acids ratios (acetic: propionic: butyric acids) on yeast growth and lipid production were shown (Fei *et al.,* 2011; Fontanille *et al.,* 2012). For example, Fei *et al.* (2011) studied 6:1:3, 7:2:1, 4:3:3, and 8:1:1 volatile fatty acids ratios to evaluate growth (1.12 g/L, 1.11 g/L, 0.64 g/L and 1.2 g/L, respectively) and lipid production (27.3 %, 26.1 %, 19.8 % and 27.8 % w/w, respectively) by *Candida albidus var. albidus* ATCC 10672. Our results indicated that volatile fatty acids ratios for acetic: propionic: butyric acids 20:3.3:1 and dilutions showed better biomass and lipid production by *C. curvatus* as well as *by C. protothecoides*, in general. Apparently, the increased amount of acetic acid in the volatile fatty acid ratio stimulate biomass and lipid production. This observation was also support our data, as data and conclusions done by Fei *et al.* (2011). Our data showed that the acetic, propionic, and butyric acids were consumed simultaneously when incorporated into the growth medium at ratios of 20:3.3:1 and 1:1:1. The concentration of acetic acid was 8 times

higher in the ratios used by Fei *et al.* (2011, 2015) than propionic and butyric acids. In our experiments, the concentration of acetic acid (undiluted system) was 20 times higher than butyric acid and 6 times higher than propionic acid. The findings of Fei *et al.* (2011), Wen *et al.* (2013a), and Fei *et al.* (2015) indicated that the acetic acid was the more preferable source for biomass and lipid production of *C. curvatus* and *C. protothecoides.* Interestingly, in our system where acetic, propionic, and butyric acids were used at a 1:1:1 ratio for a 24 h, butyric acid was consumed at high rate in the first 96 h; at 96 h, *C. curvatus* completely consumed acetic and butyric acids with some propionic acid remaining.

There have been some reports on the influence of volatile fatty acids mixtures on *C. protothecoides* growth (Fei *et al.,* 2015). It was reported by Fei *et al.* (2015) that in modified Bristol medium with 2.0 g/L of volatile fatty acids (ratio 6:1:3) and 0.5 g/L of urea, the C. *protothecoides* UTEX 25 biomass was determined at 0.56 g/L and 47 % of lipids were produced. The authors also studied 6:1:3, 7:2:1, 4:3:3, and 8:1:1 volatile fatty acids ratios (acetic:propionic:butyric acids) to evaluate growth and lipid production by C. *protothecoides* as they did for *C. albidus* (Fei *et al.,* 2011). An 8:1:1 ratio of acetic acid: propionic acid: butyric acid (2.0 g/L in total) was determined as the best for biomass (0.65 g/L) and lipid (48.7 %) production by microalgae. They observed consumption of acetic, propionic and butyric acids: at 96 h of growth the acetic acid was fully consumed, propionic acid at 168 h, and up to 25 % of butyric acid was left in the medium (Fei *et al.,* 2015). In our system at 96 h algae C. *protothecoides* used mainly acetic and propionic acids and to a lesser extent, butyric acids. Similarly to Fei *et al.,* 2015 observation, based on the 1:3 dilution

system and by 168 hours of *C. protothecoides* growth, was observed that acetic acid was totally consumed and ~30 % of the butyric acid left in the media. The difference was detected for propionic acid consumption: ~20 % of the propionic acid was left in the media. This study finding showed time-dependent preference of volatile fatty acid consumption as well as dependence on their initially loaded concentrations in to the cultivation media. The phenomenon of diauxic type of growth could be explained by propionate and butyrate inhibition of cell growth when acetate as a substrate was consumed in first place. The similar trend of diauxic consumption of volatile fatty acids was observed by Turon *et al.* (2015) during heterotrophic growth of *Chlorella sorokiniana* using acetate and butyrate as substrate. Turon *et al.* (2015) also conclude that presence of butyrate as inhibitor caused the diauxic growth of *C. sorokiniana.*

Based on the earlier reports of Hu *et al.* (2008), Mata *et al.* (2010), Fei *et al.* (2011); Christophe *et al.* (2012), Fontanille *et al.* (2012), Cerón-García *et al.* (2013), and Fei *et al.* (2015) the fatty acids composition of the microalgae oil, as well as yeast oil, may be affected by the feedstock used for growth and stress conditions caused by nitrogen depletion applied to the cells during fermentation. Moreover, changes in composition of media used for cultivation of oleaginous microorganisms induced significant changes in fatty acid profiles. The remarkable increases in oleic acid concentration observed using the undiluted and 1:1 diluted volatile fatty acid mixture during fermentation by *C. curvatus* and *C. protothecoides* can be explained by activation of Δ-9 desaturases (Sijtsma *et al.,* 1998). Downregulation of Δ-9 desaturases (Yu *et al.,* 2011; Bellou *et al.,* 2014) in *C. protothecoides* cells during heterotrophic fermentation using volatile fatty acids can be supported by the presence

of only saturated fatty acids in the undiluted media. As shown by Aguilar, de Mendosa, 2006, the expression of genes responsible for downregulation of Δ-9 desaturases are triggered by activation of a signal transduction pathway due to changing the cell membrane physical properties caused by a stressful environment. The fatty acid profiles of *C. curvatus* and *C. protothecoides* grown in glucose were found to be enriched in oleic, linoleic, and palmitic acids in different concentrations that corresponds with previous reports (Fei *et al.,* 2011; Lian *et al.,* 2012; Espinosa-Gonzalez *et al.,* 2014b; Liu et al., 2015). Fatty acid profiles of *C. protothecoides* grown in undiluted and one time diluted volatile fatty acids model media showed enhanced amount of saturated fatty acids, when unsaturated fatty acids were observed during subsequent media dilution. Bressler & Wakil, 1961, showed that palmitic and steric acids are the major products when acetate and butyrate are used. They are also noted that the presence of propionate led to the formation odd chain fatty acids, mainly heptadecanoic. In the fermentation media with 1:3 dilution of the initial acetic, propionic, butyric acids mixture (20:3.3:1), a positive contribution to drop-in-fuel production was observed: increasing levels of oleic and linoleic acid in *C. curvatus* and increasing of the levels of oleic acid in *C. protothecoides*. In this work, the total amount of lipids was quantified. Increasing of the fatty acids level of unsaturation in fatty acid profile of membrane lipids are possible based on observation of the possibility of trafficking lipids stored in lipid droplets into membrane lipids (Printz, 2010).

The carbon to nitrogen ratio in the aqueous phase after hydrolysis of 4 % biosolids was 1.2 and in the aqueous phase after hydrolysis of the biosolids/brown

grease mixture was 8.5. As established in the literature, a carbon to nitrogen ratio greater than 20 is required to induce lipid accumulation by oleaginous microorganisms (Papanikolaou, Aggelis, 2011). The amount of the carbon initially present in the aqueous phase after hydrolysis of 4 % biosolids was very low.

Based on low amounts of volatile fatty acids and amino acids as well as the low carbon to nitrogen ratio present in the hydrolyzed biosolids streams, these streams cannot be used as a suitable carbon substrate to lipid production by oleaginous microorganisms unless supplemented with an carbon source. For our studies, we additionally supplemented the hydrolysis by-product streams with glycerol. We expected to get positive contribution using glycerol to biomass and lipid production because of increasing amount of carbon available for biomass production and adjustment of higher carbon to nitrogen ratio. Results of additional supplementation with glycerol (to adjust carbon to nitrogen ratios to 50/1 or 30/1) along with prolonged cultivation showed a positive contribution to biomass growth and minimal contribution into lipid production. Apparently, carbon to nitrogen ratio was not only parameter that important to achieve sufficient biomass and lipid productivity. Glycerol addition into the media was done in high concentrations that led to increasing the media osmotic pressure. Additionally, the glycerol as a carbon source was not totally consumed. In general, the total lipids produced under supplementation conditions were extremely low: 9 – 10 %. Data published by Chinnasamy *et al.* (2010) supported our observations. They observed (C:N:P ratio was 41.1:4.06:1) less than 9 % of the lipid production by *C. protothecoides* using untreated carpet mill industrial wastewater (Chinnasamy *et al.,* 2010).

In addition, the concentration of essential minerals in the aqueous phase after hydrolysis of 4 % biosolids are lower than needed to promote good growth of oleaginous yeast and microalgae. By addition of the essential minerals, we expected to get high biomass and lipid production as shown by Dou *et al.,* 2013. Our results supported conclusions about significantly increasing biomass production by *C. protothecoides*, but lipid production was low. The low lipid production in this case by *C. protothecoides* can be explained by insufficient carbon supplementation to maintain appropriate carbon to nitrogen ratio needed to produce lipids.

The aqueous by-product stream after hydrolysis of the mixed feedstock (biosolids : brown grease at 1:1 (w/w)) initially contained more carbon and extra supplementation with essential minerals and/or with glycerol (carbon to nitrogen ratio was 50/1 and 30/1) was expected to promote oleaginous microorganisms' growth and lipid production. For *C. curvatus,* the produced amount of biomass significantly decreased, when lipid amount increased up to 10 %. For *C. protothecoides,* the produced amount of biomass significantly increased, when lipid amount increased up to 10 %. These results can be explained, first, by the difference in glycerol consumption: for *C. curvatus* it was unexpectedly low amounts. Second, the high concentration of initially loaded glycerol, needed to maintain a high carbon to nitrogen ratio, can itself inhibit cell growth (substrate inhibition) due to increased media osmotic pressure (Sara *et al.,* 2016; Zambanini *et al.,* 2016). Third, the addition of essential minerals perfectly stimulates oleaginous algae growth as activators of cell metabolic reactions (Dou *et al.,* 2013; Miazek, 2015). These experiments have clearly demonstrated the necessity of extra supplementation with a

source of carbon and/or essential minerals of the aqueous phase after hydrolysis of 4 % biosolids or mixtures (hydrolyzed 50/50 (w/w) 4 % biosolids and brown grease).

Received data about proportional distribution of fatty acid due to glycerol supplementation support the observation made by Espinosa-Gonzalez *et al.* (2014 b). Thus, the fatty acid composition of *C. curvatus* grown in the aqueous by-product stream after hydrolysis of the mixed feedstock with supplementation by essential minerals and/or with glycerol was composed of more saturated (palmitic and stearic) acids than unsaturated (oleic, linoleic, and linolenic) acids. The fatty acid composition of C. *protothecoides* was composed of more unsaturated (oleic and linolenic) acids than saturated (palmitic and stearic) acids.

In this thesis research, the two different oleaginous microorganisms yeast *C. curvatus* and microalgae *C. protothecoides* were studied in terms of their ability to accumulate lipids using volatile fatty acid as a major carbon-containing compound thus utilizing aqueous waste feedstock from lipid pyrolysis technology. The most important parameter suitable for further microorganisms application is lipid productivity (Griffiths & Harrison, 2009; Christophe *et al.,* 2012). There was no significant deference in lipid productivity of both studied microorganisms. The improvement of lipid productivity is necessary for *C. curvatus* and *C. protothecoides* application.

5.2. Conclusions and future work recommendations

In this study, a range of different aqueous by-product streams from lipid pyrolysis were characterized. The volatile fatty acids, as carbon-containing compounds, were detected and applied to *C. curvatus* and *C. protothecoides* growth and lipid production. The volatile fatty acids (acetic, propionic and butyric acids in a 20:3.3:1 ratio) are present in the aqueous by-product stream received after the pyrolysis step.

C. curvatus and *C. protothecoides* can grow and tolerate acetic acid, propionic acid and butyric acid, as single and mixed carbon sources at neutral pH, when volatile fatty acid present in dissociated form. The three times dilution of the initial volatile fatty acid mixture in the model media was the best dilution used to grow the highest amount of *C. curvatus* as well as *C. protothecoides* biomass. Based on the observation of the volatile fatty acids consumption during fermentation of *C. curvatus* and C. *protothecoides,* it was noticed that it was time- and concentration-dependent characteristic.

Lipid production in *C. curvatus* and *C. protothecoides* grown on volatile fatty acids was demonstrated. The highest amount of total lipids and lipid yield were received after yeast and microalgae fermentation in the three times dilution of initially loaded model volatile fatty acid mixture. The proportional distribution of fatty acid of *C. curvatus* grown in volatile fatty acids indicated the enhanced amount of unsaturated fatty acids. Fatty acid profiles of *C. protothecoides* grown in undiluted and one time diluted volatile fatty acids model media showed enhanced

amount of saturated fatty acids, when unsaturated fatty acids were observed during subsequent media dilution.

Low amounts of carbon, as well as a low carbon to nitrogen ratio were determined in the aqueous phase after hydrolysis of 4 % biosolids. This stream cannot be used for fermentation and lipid production by *C. curvatus* and *C. protothecoides*. In the aqueous phase after hydrolysis of mixed feedstock (50/50 w/w 4 % biosolids and brown grease) the amount of available carbon was high compared to the aqueous phase after hydrolysis of 4 % biosolids that led to high biomass production. Based on the low carbon to nitrogen ratio, the lipid production was low as well. In general, supplementation with glycerol and essential minerals resulted in a significant rise in biomass production compared to low amounts of total lipids produced. The fatty acid profiles of *C. curvatus* and *C. protothecoides* indicated decreasing levels of unsaturation.

There are several recommendations regarding future work. The aqueous by-product stream after pyrolysis can be applied in flask-, 5L and 10 L scale fermentation using *C. curvatus* and *C. protothecoides* for a lipid production. This will enable recycling of the aqueous waste to grow oleaginous organisms that can be subsequently processed through lipid pyrolysis technology (Figure 5.1). In this case, only recycled aqueous by-product streams would be involved.

It will be necessary to evaluate the lipid productivity of *C. curvatus* and *C. protothecoides* at 96 h, 120 h, and 144 h of growth on volatile fatty acid mix. It will give more valuable estimation of oleaginous microorganisms performance through aqueous by-product waste stream utilization. It will be possible, that shorter

cultivation time is advantage of *C. curvatus* application in valuable aqueous by-product stream utilization.

It is also essential to identify the potential inhibitors of *C. curvatus* and *C. protothecoides* that are present in the aqueous by-product streams after hydrolysis and pyrolysis. Additionally, among the wide range of compounds detected other than volatile fatty acids, possible a new value-added products could be found.

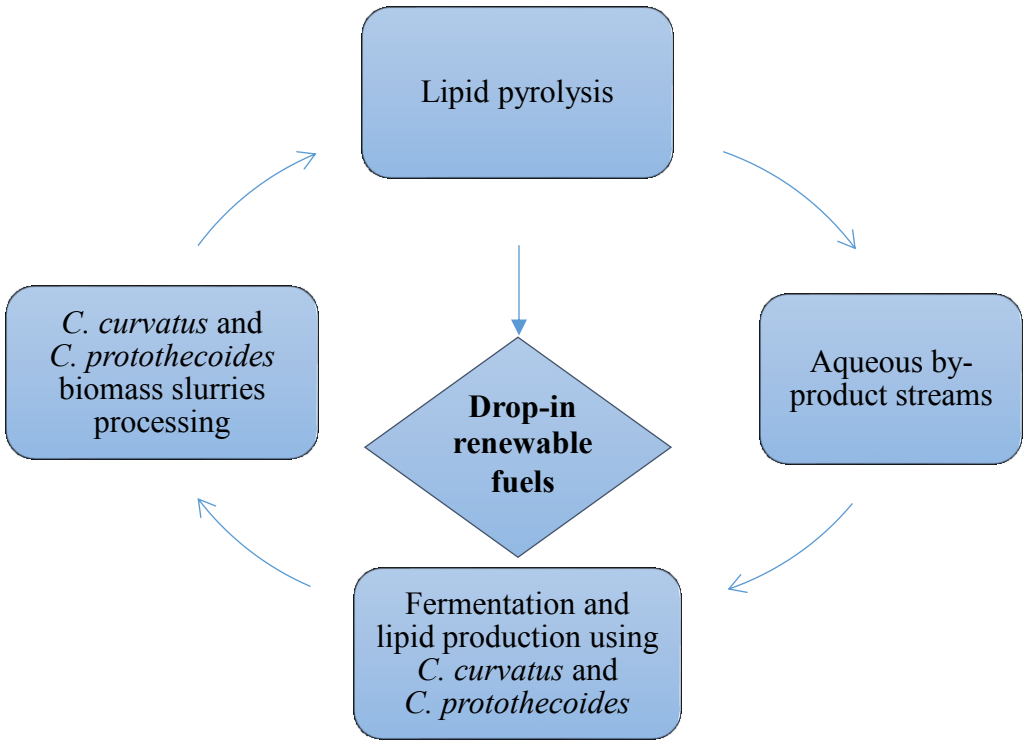

Figure 5.1. Recyclable process scheme: from aqueous by-product streams through lipids to biofuels.

More work needs to be done in terms of the evaluation of biomass and increasing lipid production by *C. curvatus* and *C. protothecoides* in the aqueous by-product stream after hydrolysis of mixed feedstock (50/50 w/w 4 % biosolids and brown grease). It is possible to address different optimization approaches to get

better lipid production. For example, to stimulate the lipid production by oleaginous yeast or algae, media should be diluted first, and, than, supplemented by essential minerals and carbon source (glucose, galactose, and glycerol).

The research presented in this thesis has advanced the search for value-added applications of by-product streams from lipid pyrolysis. Lipids from oleaginous yeast and microalgae biomass slurries grown on the aqueous by-product streams of lipid pyrolysis could be used for a sustainable renewable fuel production. Application of aqueous by-product streams for a lipid production using oleaginous yeast and algae solves waste issues and offers many advantages and potential benefits to the Alberta's resource sector as well as the Canadian economy.

BIBLIOGRAPHY

Abou-Shanab, R.A.I., Ji, M.-K., Kim, H.-C., et al. (2013). Microalgal species growing on piggery wastewater as a valuable candidate for nutrient removal and biodiesel production. *Journal of Environmental Management*. 115: 257 – 264.

Adams, J.M., Gallagher, J.A. & Donnison, I.S. (2009). Fermentation study on *Saccharina latissima* for bioethanol production considering variable pre-treatments. *Journal of Applied Phycology* 21: 569.

Aguilar, P. de Mendosa, D. (2006). Control of fatty acid desaturation: a mechanism conserved from bacteria to humans. *Molecular Microbiology.* 62(6): 1507 – 1514.

Anthony, D.W., Larkum, A.W. et al. (2012). Selection, breeding and engineering of microalgae for bioenergy and biofuel production. *Trends in Biotechnology*. 30 (4): 198 – 205.

Appadoo, R.J. (2011). Price volatility of commodities – impact on biofuel production and supply. AFSI report. 35 p. Retrieved on January 23, 2017 from: http://www.icao.int/Meetings/EnvironmentalWorkshops/Documents/2011-SUSTAF/20_Appadoo.pdf

Asomaning, J., Mussone, P., Bressler, D. C. (2014a). Two-stage thermal conversion of inedible lipid feedstocks to renewable chemicals and fuels. *Bioresource Technology*. 158: 55-62.

Asomaning, J., Mussone, P., Bressler, D. C. (2014b). Pyrolysis of polyunsaturated fatty acids. *Fuel Processing Technology*. 120: 89 – 95.

Asomaning J., Mussone P. G., Bressler D. C. (2014c). Thermal deoxygenation and pyrolysis of oleic acid. *Journal of Analytical and Applied Pyrolysis*. 105: 1 – 7.

ASTM D3792-05 Standard test method for water content of coatings by direct injection into gas chromatograph. ASTM International. West Conshohocken, PA, 2005.

BAR, (2013). Biofuels Annual Report. Canada. GAIN Report. 40 p. Retrieved on January 23, 2017 from: https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Biofuels%20Annual_Ottawa_Canada_6-28-2013.pdf

BAR, (2016). Biofuels Annual Report. Canada. GAIN Report. 40 p. Retrieved on April 27, 2017 from: https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Biofuels%20Annual_Ottawa_Canada_8-9-2016.pdf

Baroukh, C., Turon, V., & Bernard, O. (2017). Dynamic metabolic modeling of heterotrophic and mixotrophic microalgal growth on fermentative wastes. *PLoS Computational Biology*, *13*(6), e1005590.

Barclay, W., Weaver, C., Metz, J., Hansen, J. (2010). Development of a docosahexaenoic acid production technology using *Schizochytrium*: historical perspective and update, In: Cohen Z., Ratledge C., eds. *Single Cell Oils*. 2nd ed. Champaign, IL: AOCS Press, 75–96.

Behera, S., Singh, R., Arora, R., *et al.* (2014). Scope of algae as third generation biofuels. *Frontiers in Bioengineering and Biotechnology*. *2*: 90

Bellou, S., *et al.* (2014). Microalgal lipid biochemistry and biotechnology perspectives. *Biotechnology advances*. 32, 8: 1476 – 1493.

Beopoulos, A., Mrozova, Z., Thevenieau, F., *et al.* (2008). Control of lipid accumulation in the yeast *Yarrowia lipolytica*. *Applied Environmental Microbiology*. 74: 7779–7789.

Black, B. *et al*. (2016). Aqueous stream characterization from biomass fast pyrolysis and catalytic fast pyrolysis. *ACS Sustainable Chemistry and Engineering*. 4: 6815 – 6827.

BM, (2016). Biosolids management. City of Edmonton. Retrieved on January 24, 2017 from: http://www.edmonton.ca/environmental/wastewater_sewers/biosolids-management.aspx

Borines, M., de Leon, R.L., Cuello, J.L. (2013). Bioethanol production from the macroalgae *Sargassum* spp. *Bioresource Technology* 138: 22 – 29.

Borowitzka, M.A. (2010). Algae oils for biofuels: chemistry, physiology, and production. Chapter 13. AOS Press. 272 – 289. Retrieved on February 8, 2017 from: www.knovel.com

Bradburn, K. (2014). CanBio report on the status of bioenergy in Canada. *Renewed Energies*. 54 p. Retrieved on January 23, 2017 from: http://www.fpac.ca/wp-content/uploads/2014_CanBio_Report.pdf

Braunwald, T. et al. (2013). Effect of different carbon to nitrogen ratios on carotenoid and lipid production by *Rhodotorula glutinis*. *Applied Microbiology and Biotechnology*. 97: 6581 – 6588.

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

Bressler, D. (2011). Methods for producing fuels and solvents (from lipids and fats utilizing pyrolysis technology). Lipid to Hydrocarbon Pyrolysis. Patent US8067653B2.230.

Bressler, R., Wakil, S. (1961). Studies of the mechanism of fatty acid synthesis. IX The conversion of malonyl coenzyme A to long chain fatty acids. *The Journal of Biological Chemistry*. 236, 6: 1643 – 1651.

Bridgwater, A. (2012). Review of fast pyrolysis of biomass and product upgrading. *Biomass and Bioenergy*. 38: 68 – 94.

Bröker, D., Elbahloul, Y., Steinbuchel, A. (2010). Production of lipids for biofuels using bacteria. In: Cohen Z, Ratledge C, eds. *Single Cell Oils*, 2nd ed. Champaign, IL: AOCS Press, 291–314.

Brown, R. (2003). Biorenewable resources. Engineering new products from agriculture. Iowa State Press. A Blackwell Publishing Company. 1st edition. 286 p.

Bruant, G., Lévesque, M.J., Peter, C. et al. (2010). Genomic analysis of carbon monoxide utilization and butanol production by *Clostridium carboxidivorans* strain P7. *PLoS ONE* 5(9): e13033.

Callebaut, V. (2010). What are – and who's making - 2G, 3G and 4G biofuels. *Biofuels Digest.* Retrieved on January 23, 2017 from: http://www.biofuelsdigest.com/bdigest/2010/05/18/3g-4g-a-taxonomy-for-far-out-—-but-not-far-away-—-biofuels/

Cao, Y., Pawłowski, A. (2012). Sewage sludge-to-energy approaches based on anaerobic digestion and pyrolysis: brief overview and energy efficiency assessment. *Renewable and Sustainable Energy Reviews*. 16 (3): 1657 – 1665.

CBA (2004). Canadian Bioenergy Association. Biomass sources for bioenergy use in Canada. Retrieved on January 23, 2017 http://www.canbio.ca

CBR (2014). CanBio report on the status of bioenergy in Canada. Renewed Energies on behalf of CanBio. Retrieved on January 23, 2017 from: ttp://www.fpac.ca/wp-content/uploads/2014_CanBio_Report.pdf

CCME, (2014). Canadian Council of Ministers of the Environment. Resources: biosolids. Retrieved electronically on Retrieved on January 24, 2017 from: http://www.ccme.ca/en/resources/waste/biosolids.html.

Cerón-García, M.C., *et al.* (2013). A process for biodiesel production involving the heterotrophic fermentation of *Chlorella* glycerol with glycerol as the carbon source. *Applied Energy*. 103: 341 – 349.

Chang, H.N. *et al.* (2010). Biomass-derived volatile fatty acid platform for fuels and chemicals *Biotechnology and Bioprocess Engineering*. 15(1): 1-10.

Chen, T., Liu, J., Guo, B. *et al.* (2015). Light attenuates lipid accumulation while enhancing cell proliferation and starch synthesis in the glucose-fed oleaginous microalga *Chlorella zofingiensis*. *Scientific Reports*, *5*, 14936.

Chen, Y., Xiao, B., Chang, J. *et al.* (2009). Synthesis of biodiesel from waste cooking oil using immobilized lipase in fixed bed reactor. *Energy Conversion and Management.* 50: 668-673.

Chen, Y.-H., Walker, T.H. (2011). Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel-derived crude glycerol. *Biotechnology Letters.* 33 (10): 1973 – 1983.

Chi, Z., *et al*. (2011). Lipid production by culturing oleaginous yeast and algae with food waste and municipal wastewater in an integrated process. *Applied Biochemistry Biotechnology*. 165: 442 – 453.

Chinnasamy, S., Bhatnagar, A., Hunt, R., Das, K. (2010). Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. *Bioresource Technology*. 101 (9): 3097 – 3105.

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances*. 25: 294 – 306.

Choia, H., Woob, H., and Suha, D. (2014). Pyrolysis of seaweeds for bio-oil and bio-char production. *Chemical engineering transactions*. 37: 121 – 126.

Christophe, G. et al. (2012). Production of oils from acetic acid by the oleaginous yeast *Cryptococcus curvatus. Applied Biochemistry and Biotechnology.* 167:1270 – 1279.

Cohen, B. (1990). The nuclear energy option. Chapter 3. Published by Plenum Press. Retrieved on January 23, 2017 from: http://www.phyast.pitt.edu/~blc/book/index.html

Crocker, M. (2010). Thermochemical conversion of biomass to liquid fuels and chemicals. Cambridge: Royal Society of Chemistry. RSC Publishing RSC Energy and Environment Series. No. 1. 532 p.

Dalitz, F., et al. (2012). ^{1}H NMR techniques for characterization of water content and viscosity of fast pyrolysis oils. *Energy Fuels.* 26(8): 5274 – 5380.

Dalrymple, O. K., Halfhide, T., Udom, I., *et al.* (2013). Wastewater use in algae production for generation of renewable resources: a review and preliminary results. *Aquatic Biosystems*. *9*: 2.

De la Hoz, H. (2012). Optimization of biomass and lipid production in heterotrophic microalgal cultures, in Department of Chemical and Materials Engineering. *PhD thesis*, University of Alberta.

Dean, E.W., Stark, D.D. (1920 in print, 2002 online). A convenient method for the determination of water in petroleum and other organic emulsions. *The Journal of Industrial and Engineering Chemistry*. 12(5): 468 – 490.

Delgenes, J.P., Moletta, R., Navarro, J.M. (1996). Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, *Zymomonas mobilis, Pichia stipitis*, and *Candida shehatae*. *Enzyme Microbiology and Technology.* 19: 220 – 225.

Demirbas, A. (2003). Biodiesel fuels from vegetable oils via catalytic and non-catalytic supercritical alcohol transesterifications and other methods: a survey. *Energy Conversion & Management. 44:* 2093 – 2109.

Dien, B., et al. (2016). Identification of superior lipid producing *Lipomyces* and *Myxozyma* yeasts. *AIMS Environmental Science.* 3(1): 1 – 20.

Dizge, N., Aydiner C., Imer D.Y., *et al.* (2009). Biodiesel production from sunflower, soybean, and waste cooking oils by transesterification using immobilized lipase. *Bioresource Technology,* 100: 1983-1991.

Dong, T., Knoshaug, E.P., Pienkos, P.T., Laurens, L. (2016). Lipid recovery from wet oleaginous microbial biomass for biofuel production: a critical review. *Applied Energy*. 177: 879 – 895.

Dou, X., Lu, X.-H., Lu, M.-Z., *et al.* (2013). The effects of trace elements on the lipid productivity and fatty acid composition of *Nannochloropis oculata*. *Journal of Renewable Energy.* Article ID 671545. 6 p.

Dry, M.E. (2004). Present and future applications of the Fischer-Tropsch process. *Applied Catalysis A: General*, 276: 1 – 3.

Du, Y. *et al.* (2011). Reduce COD and BOD in CTMP effluent. *BioResources*. 6(3): 3505–3514.

Duncan, S. H., Barcenilla, A., Stewart, C. S., Pryde, S. E., & Flint, H. J. (2002). Acetate utilization and butyryl Coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. *Applied and Environmental Microbiology*, *68*(10): 5186 – 5190.

Echim, C., Verhe, R., De Greyt, W., Stevens, C. (2009). Production of biodiesel from side-stream refining products. *The Royal Society of Chemistry: Energy Environmental Science*. 2: 1131 – 1141.

Elliot, D., *et al.* (2015). Hydrothermal liquefaction of biomass: developments from batch to continuous process. *Bioresource technology*. 178: 147 – 156.

Ellis, J., Frahm, J., Li, L., Coleman, R. (2010). Acyl-coenzyme A synthetases in metabolic control. *Current Opinion Lipidology*. 21(3): 212 – 217.

Espinosa-Gonzalez, I. (2014b). Development of technological alternatives to produce renewable fuels from oleaginous microorganisms. *PhD thesis*, University of Alberta.

Espinosa-Gonzalez, I., Asomaning, J., Mussone, P., Bressler, D.C. (2014a). Two-step thermal conversion of oleaginous microalgae into renewable hydrocarbons. *Bioresource Technology*. 158: 91 – 97.

Espinosa-Gonzalez, I., Parashar A., Chae, M., and Bressler, D.C. (2014c). Cultivation of oleaginous yeast using aqueous fractions derived from hydrothermal pre-treatments of biomass. *Bioresource Technology*. 170: 413 – 420.

Evanylo, G. Land application of biosolids. Chapter 10. p. 226 – 252. Retrieved on January 24, 2017 from: https://extension.umd.edu/sites/default/files/_docs/programs/anmp/MANMChapter10.pdf

Fay, D., and Gerow, K. (2013). A biologist's guide to statistical thinking and analysis. WormBook, ed. Retrieved on February 05, 2017 from: http://www.wormbook.org/chapters/www_statisticalanalysis/statisticalanalysis.pdf

Fei, W., *et al.* (2011). Fld1p, a functional homologue of human seipin, regulates the size of lipid droplets in yeast. *The Journal of Cell Biology*. 180,3: 473 – 482.

Fei, Q., Chang, H., Shang, L., *et al.* (2011). The effect of volatile fatty acids as a sole carbon source on lipid accumulation by *Cryptococcus albidus* for biodiesel production. *Bioresource Technology*. 102: 2695 – 2701.

Fei, Q., Fu, R., Shang, L., *et al.* (2015). Lipid production by microalgae *Chlorella protothecoides* with volatile fatty acids (VFA) as carbon sources in heterotrophic cultivation and its economic assessment. *Bioprocess Biosystems Engineering*. 38(4): 691 – 700.

Feng, P., Deng, P., Fan, L., Hu, Z. (2012). Lipid accumulation and growth characteristics of *Chlorella zofingiensis* under different nitrate and phosphate concentrations. *Journal of Bioscience and Bioengineering.* 114: 405 – 410.

Feron, G., et al. (2005). Metabolism of fatty acid in yeast: characterization of ß-oxidation and ultrastructural changes in the genus *Sporidiobolus sp.* cultivated on ricinoleic acid methyl ester. *FEMS Microbiology Letters.* 250: 63 – 69.

Fontanille, P., Kumar, V., Christophe, G., *et al.* (2012). Bioconversion of volatile fatty acids into lipids by the oleaginous yeast *Yarrowia lipolytica*. *Bioresource Technology*. 114: 443 – 449.

Fonts, I., Gea, G., Azuara, M., *et al.* (2012). Sewage sludge pyrolysis for liquid production: a review. *Renewable* and *Sustainable Energy* Reviews. 16: 2781 – 2805.

Gabriel, F., Accoceberry I., Bessoule, J-J., Salin B., Lucas-Guérin, M., Manon, S., *et al.* (2014). A Fox2-dependent fatty acid ß-oxidation pathway coexists both in peroxisomes and mitochondria of the ascomycete yeast *Candida lusitaniae*. *PLoS ONE* 9(12): e11453

Garay, L., Boundy-Mills, K., & German, J. (2014). Accumulation of high-value lipids in single-cell microorganisms: a mechanistic approach and future perspectives. *Journal of Agricultural and Food Chemistry*. *62* (13): 2709 – 2727.

Garcia-Perez, M., Shen, J., Wang, X.-S., Li, C.-Z. (2010). Production and fuel properties of fast pyrolysis oil/bio-diesel blends. *Fuel Processing Technology*. 91: 296 – 305.

Gong Z. *et al.* (2013). Efficient conversion of biomass into lipids by using the simultaneous saccharification and enhanced lipid production process. *Biotechnology for biofuels*. 6: 36.

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

Griffiths, M., Harrison, S. (2009). Lipid productivity as a key characteristic of choosing algal species for biodiesel production. Journal of applied Phycology. 21(5): 493 – 507.

Guadalupe-Medina, V., *et al.* (2013). Carbon dioxide fixation by Calvin-Cycle enzymes improves ethanol yield in yeast. *Biotechnology for Biofuels.* 6: 125.

Guo, Y., *et al.* (2009). Lipid droplets at a glance *Journal of Cell Science* 122: 749 – 752.

Gupta, V., Tuohy, M. (2013). Biofuel technologies. Recent developments. Springer-Verlag Berlin Heidelberg, 1st edition. 534 p.

Gupta, S. (2016). Breeding oilseed crops for sustainable production: opportunities and constraints. Book. Elsevier Inc. 551p.

Hadiyanto & Nur, M. (2014). Lipid extraction of microalga *Chlorella sp*. cultivated in palm oil mill effluent (POME) medium. *World Applied Science Journal.* 31(5): 959 – 967.

Hassan, M. *et al*. (1993). Lipid production by an unsaturated fatty-acid auxotroph of the oleaginous yeast *Apiotrichum curvatum* grown in single-stage continuous-culture. *Applied Biochemistry Biotechnology*. 40: 483 – 488.

Hassan, M., Kalam, M. (2013). An overview of biofuel as a renewable energy source: development and challenges. *Procedia Engineering*. 56: 39 – 53.

Heo, H.S., Kim, S.G., Jeong, K.-E. *et al.* (2011). Catalytic upgrading of oil fractions separated from food waste leachate *Bioresource Technology*. 102: 3952 – 3957.

Heredia-Arroyo, T., Wei, W., Hu, B. (2010). Oil accumulation via heterotrophic/mixotrophic *Chlorella protothecoides. Applied Biochemistry and Biotechnology.* 162: 1978 – 1995.

Higgins, M.J. *et al.* (2006). Cycling of volatile organic sulfur compounds in anaerobically digested biosolids and it its implications for odors. *Water Environmental Research*. 78: 243 – 252.

Higgins, M.J. *et al.* (2008). Role of protein, amino acids, and enzyme activity on odor production from anaerobically digested and dewatered biosolids. *Water Environmental Research*. 80(2): 127 – 135.

Ho, D.P., Ngo, H.H., Guo, W. (2014). A mini review on renewable sources for biofuel. *Bioresource Technology*. 169: 742 – 749.

Van der Hoeven, D. (2016). Cellulosic ethanol: feedstock costs predominant, sugar cane cheapest. Bio Based Press. Retrieved on April 24, 2017 from: http://www.biobasedpress.eu/2016/02/cellulosic-ethanol-feedstock-costs-predominant-sugar-cane-cheapest/

Holt-Gimenez, E. (2008). The world food crisis: what is behind it and what we can do. Retrieved on January 23, 2017 from: http://www.worldhunger.org/articles/09/editorials/holt-gimenez.htm

Howard, T.P., Middelhaufe, S., Moore, K., *et al*. (2013). Synthesis of customized petroleum-replica fuel molecules by targeted modification of free fatty acid pools in Escherichia coli. *PNAS.* 110,19: 7636 – 7641.

Hu Q., *et al.* (2008). Microalgal triacylglycerols as feedstock for biofuel production: perspectives and advances. *The Plant Journal.* 54: 621 – 639.

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

IEA, (2017). Renewables. Biomass definition. Retrieved on January 23, 2017 from: https://www.iea.org/topics/renewables/subtopics/bioenergy/

Jacquier N. *et al.* (2011). Lipid droplets are functionally connected to the endoplasmic reticulum in *S. cerevisiae*. *Journal of Cell Science*. 124: 2424 – 2437.

Jena, U. et al. (2015). Oleaginous yeast platform for producing biofuels via co-solvent hydrothermal liquefaction. *Biotechnology for biofuels* 8: 167.

Jenab, E., Mussone, P., Bressler, D. (2014). Production of renewable hydrocarbons from thermal conversion of abietic acid and tall oil fatty acids. *Energy & Fuels*. 28: 6988 – 6994.

Jin, M. *et al.* (2015). Microbial lipid-based cellulosic biorefinery: feasibility and challenges. *Trends in Biotechnology*. 33(1): 43 – 54.

Jones, K.J., Moore, K., Sambles, C., *et al.* (2016). Draft genome sequences of *Achromobacter piechaudii* GCS2, *Agrobacterium sp*. strain SUL3, *Microbacterium sp.* strain GCS4, *Shinella sp.* strain GWS1, and *Shinella sp*. strain SUS2 isolated from consortium with the hydrocarbon-producing alga *Botryococcus braunii. Genome Announcements*, 4(1): e01527–15

Jönsson, L. J., Alriksson, B., & Nilvebrant, N.O. (2013). Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnology for Biofuels*, *6*, 16.

Jouzani, G., Taherzadeh, M. (2015). Advances in consolidated bioprocessing systems for bioethanol and butanol production from biomass: a comprehensive review. *Biofuel Research Journal*. 5: 125 – 195.

Kagan, J. (2010). Third and fourth generation biofuels: technologies, markets and economics through 2015. Retrieved on January 23, 2017 from: https://www.greentechmedia.com/research/report/third-and-fourth-generation-biofuels

Kaliaperumal, N. *et al.* (2002). Seasonal changes in growth, biochemical constituents and phycocolloid of some marine algae of mandapam coast. *Seaweed Research Utilization.* 24 (1):73 – 7. Retrieved on January 25, 2017. Retrieved on February 28, 2017 from: http://eprints.cmfri.org.in/5948/1/Article_03.pdf

Karatzos, S., McMillan, J., Saddler, J. (2014). The potential and challenges if the drop-in-fuels. IEA Bioenergy Task 39 report. 21p. Retrieved on August 11, 2017 from http://task39.sites.olt.ubc.ca/files/2014/01/Task-39-drop-in-biofuels-report-summary-FINAL-14-July-2014-ecopy.pdf

Kargbo, D. (2010). Biodiesel production from municipal sewage sludges. *Energy & Fuels. 24*: *2791*–2794.

Kemp, W.H. (2006). Biodiesel: basic and beyond - a comprehensive guide to production and use for home and farm. Aztext Press, Ontario, Canada. 588 p.

Khosla, V. (2017). Where will biofuels and biomass feedstocks come from? Retrieved on January 23, 2017 http://www.khoslaventures.com/wp-content/uploads/Where-will-Biofuels-and-Biomass-Feedstocks-Come-from-.pdf

Kim, S.R. *et al.* (2013). Strain engineering of *Saccharomyces cerevisiae* for enhanced xylose metabolism, *Biotechnology Advances.* 31: 851- 861.

Kouhia, M., Holmberg, H., & Ahtila, P. (2015). Microalgae-utilizing biorefinery concept for pulp and paper industry: Converting secondary streams into value-added products. *Algal Research*. 10: 41–47.

Krochta, J.M. *et al.* (1984). Alkaline thermochemical degradation of cellulose to organic acids. Biotechnology and Bioengineering Symposium, Sixth Symposium on Biotechnology for Fuels and Chemicals; Gatlinburg, TN, USA. 14: 37 – 54.

Kulkarni, M.G., Dalai, A.K. (2006). Waste cooking oil - an economical source for biodiesel: a review. *Ind. Eng. Chem. Res*., 45: 2901 – 2913.

Lam, M.K., Lee, K.T. (2012). Microalgae biofuels: a critical review of issues, problems and the way forward. *Biotechnology Advances*. 30: 673–690.

Latif, H., Zeidan, A.A., Nielsen, A.T., Zengler K. (2014). Trash to treasure: production of biofuels and commodity chemicals via syngas fermenting microorganisms. *Current Opinion in Biotechnology,* 27: 79 – 87.

Ledesma-Amaro, R., Dulermo, T., & Nicaud, J. M. (2015). Engineering *Yarrowia lipolytica* to produce biodiesel from raw starch. *Biotechnology for Biofuels*. *8*: 148.

Lee, R.A., Lavoie, J.M. (2013). From first to third generation biofuels - challenges of producing a commodity from a biomass of increasing complexity. *Animal Frontiers* - The review magazine of animal agriculture. 3 (2): 6 – 11.

Leiva-Candia, D.E. et al., (2014). The potential for agro-industrial waste utilization using oleaginous yeast for the production of biodiesel. *Fuel*. 123: 33 – 42.

Lian, J., *et al.* (2012). Yeast fermentation of carboxylic acids obtained from pyrolytic aqueous phases for lipid production. *Bioresource technology*. 118: 177 – 186.

Liang, Y., Sarkany, N. and Cui, Y. (2009). Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnology Letters*. 31: 1043 – 1049.

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

Liu, J., *et al.* (2016). Bioconversion of volatile fatty acids derived from waste activated sludge into lipids by *Cryptococcus curvatus. Bioresource Technology*. 211: 548 – 555.

Lu, Q., He, Z., and Stoffella, P.J. (2012). "Land Application of Biosolids in the USA: A Review," Applied and Environmental Soil Science, vol. 2012, Article ID 201462, 11 pages.

Luque-Moreno, L.C. (2015). Pyrolysis Based Biorefineries for the Production of Fermentable Substrates. *Electronic Thesis and Dissertation Repository*. 3357. Retrieved on March 15 from: http://ir.lib.uwo.ca/etd/3357

Maggio-Hall, L. & Keller, N. (2004). Mitochondrial ß-oxidation in *Aspergillus nidulans. Molecular Microbiology.* 54(5): 1173 – 1185.

Magrini, K. (2015). Reforming pyrolysis aqueous waste streams to process hydrogen and hydrocarbons. DOE Bioenergy Technologies Office (BETO) Project Peer Review. Retrieved on February 13, 2017 from: https://www.energy.gov/sites/prod/files/2015/04/f22/thermochemical_conversion_magrini_231311.pdf

Maher, K.D., Bressler, D.C. (2007). Pyrolysis of triglycerides for the production of renewable fuels and chemicals. *Bioresource Technology*. 98: 2351-2368.

Maher, K.D. *et al.* (2008). Pyrolytic decarboxylation and cracking of stearic acid. *Industrial & Engineering Chemistry Research.* 47: 5328 – 5336.

Mangory, A. *et al.* The Edmonton region biosolids strategy Retrieved on January 24, 2017 from: http://www.ewmce.com/resources/documents/alan_mangory_-_edmonton_region_biosolids_strategy.pdf

Mata, T., Martins, A., Caetano, N. (2010). Microalgae for biodiesel production and other applications: a review *Renewable and Sustainable Energy Reviews.* 14 (1): 217 – 232.

Matsushika, A., Inoue, H., Kodaki, T. *et al.* (2009). Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives. *Applied Microbiology and Biotechnology*. 84: 37.

Meesters, P.A.E.P. *et al.* (1996). Cultivation of the oleaginous yeast *Cryptococcus curvatus* in a new reactor with improved mixing and mass transfer characteristics (Surer). *Biotechnology Technics.* 10: 277 – 282.

Mekonnen, T. *et al.* (2015). Subcritical hydrolysis and characterization of waste proteinaceous biomass for value added applications. *Journal of Chemical Technology and Biotechnology*. 90: 476 – 483.

Meng X., et al. (2009). Biodiesel production from oleaginous microorganisms. *Renewable energy*. 34: 1 – 5.

Miao, X., Wu, Q. (2004). High yield bio-oil production from fast pyrolysis by metabolic controlling of *Chlorella protothecoides*. *Journal of Biotechnology.* 110 (1): 85 – 93.

Miazek, K. *et al.* (2015). Effect of metals, metalloids and metallic nanoparticles on microalgae growth and industrial product biosynthesis: a review. *International Journal of Molecular Sciences*. 16 (10): 23929 – 23969.

Mittelbach, M., Remschmidt C. (2004). Biodiesel – a comprehensive handbook. Second edition, 9 p. Retrieved on January 23, 2017 from: http://www.globalbioenergy.org/uploads/media/0711_Coyle_-_The_Future_of_Biofuels.pdf

Moller, R.M. (2007). California Research Bureau, "A brief on biosolids: options for biosolids management" (2007). California Agencies. Paper 308. Retrieved on January 24, 2017 from: http://digitalcommons.law.ggu.edu/caldocs_agencies/308

Muller, E., Sheik, A., Wilmes, P. (2014). Lipid-based biofuel production from wastewater. *Current Opinion in Biotechnology*. 30: 9 – 16.

Murphy, D.J. (2012). The dynamic roles of intracellular lipid droplets: from archaea to mammals. *Protoplasma.* 249: 541 – 558.

Naghdi, F., Gonzàlez L., Chan, W., Scheenk, P. (2016). Progress on lipid extraction from wet algal biomass for biodiesel production. *Microbial biotechnology*. 9 (6): 718 – 726.

Naik, S.N., Goud, V.V., Rout, P.K., Dalai, A.K (2009). Production of first and second generation biofuels: A comprehensive review. *Renewable and Sustainable Energy Reviews*. 14 (2): 578 – 597.

Nollet, L. (2000). Handbook of water analysis. 1st edition. Marcel Dekker. Inc., New York. 944 p.

Nollet, L., Toldra, F. (2013). Food analysis by HPLC. Third edition. CRC Press. Taylor & Francis Group, LLC. 1062 p.

NRC, (2000). National Research Council (US) Committee on biobased industrial products. Biobased industrial products: priorities for research and commercialization. Washington (DC): National Academies Press (US); Raw Material Resource Base. Retrieved on January 23, 2017 from: https://www.ncbi.nlm.nih.gov/books/NBK232951/

NRES, (2017). Nonrenewable and Renewable Energy Sources. Retrieved on January 23, 2017 from: http://www.eia.gov/energyexplained/?page=nonrenewable_home

Ochsenreither, K. *et al.* (2016). Production strategies and applications of microbial single cell oils. *Frontiers in Microbiology*. *7*: 1539.

Olofsson, K., Runquist, D., Hahn-Hägerdal, B., & Lidén, G. (2011). A mutated xylose reductase increases bioethanol production more than a glucose/xylose facilitator in simultaneous fermentation and co-fermentation of wheat straw. *AMB Express*, 1, 4.

Paasikkallio, V. *et al.* (2014). Product quality and catalyst deactivation in a four-day catalytic fast pyrolysis production run. *The Royal Society of Chemistry: Green Chemistry*. 16: 3549 – 3559.

Pagnanelli, F., Altimari, P., Trabucco, F. and Toro, L. (2014). Mixotrophic growth of *Chlorella vulgaris* and *Nannochloropsis oculata*: interaction between glucose and nitrate. *Journal of Chemical Technology and Biotechnology.* 89: 652 – 661.

Palmqvist, E., Hahn-Hägaerdal, B. (2000). Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresource Technology.* 74: 25 – 33.

Pandey, A. *et al.* (2011). Biofuels: alternative feedstocks and conversion processes / edited by Ashok Pandey. Elsevier. Imprint: Academic Press.1st ed. 26 Chapters, 608 p.

Papanikolaou, S., Aggelis, G. (2011). Lipids of oleaginous yeast. Part II: Technology and potential applications. *European Journal of Lipid Science Technology 113*: 1052 – 1073.

Park, H.J., Heo, H.S., Park, Y.-K. *et al.* (2010). Clean bio-oil production from fast pyrolysis of sewage sludge: effects of reaction conditions and metal oxide catalysts *Bioresource Technology.* 101: 83 – 85.

Pearson, D., Dale, A. (2013). Sustainable waste management: the value of recycling and composting programs. *Sustainable Community Development.* Retrieved on January 23, 2017 from: https://crcresearch.org/community-research-connections/crc-case-studies/sustainable-waste-management-value-recycling-and-com

Pittman, J.K., Dean. A.P., Osundeko. O. (2011). The potential of sustainable algal biofuel production using wastewater resources. *Bioresource Technology.* 102(1): 17-25.

Pol, A., Gross, S., Patron, R. (2014). Biogenesis of the multifunctional lipid droplet: lipids, proteins, and sites. *Journal of Cell Biology*. 204 (5): 635 – 646.

Prinz, W. (2010). Lipid trafficking sans Vesicles: where, why, how? *Cell*. *143*(6): 870–874.

Pronk, J. *et al.* (1994). Propionate metabolism in *Saccharomyces cerevisiae*: implications for the metabolon hypothesis. *Microbiology*. 140: 717 – 722.

Quintana, N., Van der Kooy, F., Van de Rhee, M. D., *et al.* (2011). Renewable energy from *Cyanobacteria*: energy production optimization by metabolic pathway engineering. *Applied Microbiology and Biotechnology*, 91(3), 471–490.

Raimondi, S. *et al.* (2014). Getting lipids from glycerol: new perspectives on biotechnological exploitation of *Candida freyschussii. Microbial cell factories.* 13:83

Ramos, M.J. *et al.* (2009). Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresource Technology*, 100: 261 – 268.

Rasrendra, C. B. *et al.* (2011). Recovery of acetic acid from an aqueous pyrolysis oil phase by reactive extraction using tri-n-octylamine. *Chemical Engineering Journal*. *176*(1): 244 – 252.

Ratledge, C. (1974). Microbial production of oils and fats. In: Birch GG, Parker KJ, Worgan JT, eds. *Food from Waste*. UK: Applied Science Publishers, 98–113.

Ratledge, C. (1991). Microorganisms for lipids. *Acta Biotechnologica*. 11:429 – 438.

Ratledge, C. (2004). Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie* 86: 807–815.

Ratledge, C., Cohen, Z. (2008). Microbial and algal oils: do they have a future for biodiesel or as commodity oils? *Lipid Technology*. 20: 155–160.

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

Rattray, J., Schibeci A., Kidby, D. (1975). Lipids of yeast. *Bacteriological Reviews*. 39(3): 197 – 231.

Reddy, H.K., Muppaneni, T., Patil, P.D. *et al.* (2014). Direct conversion of wet algae to crude biodiesel under supercritical ethanol conditions. *Fuel.* 115: 720 – 726.

RFA (2017). Renewable Fuels Association. Industry statistics. Retrieved on April 20, 2017 from: http://www.ethanolrfa.org/resources/industry/statistics/#1460745352774-cd978516-814c

RHB (2016). Renewable Hydrocarbons Biofuel, 2016. Retrieved on January 23, 2017 from: http://www.afdc.energy.gov/fuels/emerging_hydrocarbon.html

Robles-Medina, A. *et al.* (2009). Biocatalysis: towards even greener biodiesel production. *Biotechnology Advances.* 27 (4): 398 – 408.

Rutz, D., Janssen, R. (2007). Biofuel technology handbook. Retrieved on January 23, 2017 http://www.academia.edu/17992510/Biofuel_technology_handbook

Ryckebosch, E., Drouillon, M., Vervaeren, H. (2011). Techniques for transformation of biogas to biomethane. *Biomass and Bioenergy*. 35(5): 1633 – 1645.

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

Sanchez, O., Cardona, C. (2008). Trends of biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology* 99(13): 5270 – 5295.

Santamauro, F., Whiffin, F. M., Scott, R. J., & Chuck, C. J. (2014). Low-cost lipid production by an oleaginous yeast cultured in non-sterile conditions using model waste resources. *Biotechnology for Biofuels*, *7*, 34.

Sara, M., Brar, S., Blais, J. (2016). Lipid production by *Yarrowia lipolytica* grown on biodiesel-derived crude glycerol: optimization of growth parameters and their effects on the fermentation efficiency. *Royal Society of Chemistry Advances*. 6, 90547.

Schenk, P., *et al.* (2008). Second generation biofuels: high-efficiency microalgae for biodiesel production. *Bioenergy Research*. 1:20 – 43.

Schöffski, K., Strohm, D. (2011). Karl Fisher moisture determination. Encyclopedia of analytical chemistry: applications, theory and instrumentation. R.A. Meyers (Ed.) John Wiley & Sons Ltd. 14289 – 14301.

Sedgwick, G.W., Fenton, T.F., Thompson, J.R. (1991). Effect of protein precipitating agents on the recovery of plasma free amino acids. *Canadian Journal of Animal Science*. 71: 953-957.

Seo, Y., Lee, I., Han, J. (2013). Cultivation and lipid production of yeast *Cryptococcus curvatus* using pretreated waste active sludge supernatant. *Bioresource Technology.* 135: 304 – 308.

Stemmler, K., Massimi, R., & Kirkwood, A. E. (2016). Growth and fatty acid characterization of microalgae isolated from municipal waste-treatment systems and the potential role of algal-associated bacteria in feedstock production. *PeerJ*, *4*, e1780.

Stiefelmeyer, K., *et al.* (2006). The economic impact of Canadian biodiesel production on Canadian grains, oilseeds and livestock producers. Final report. Retrieved on January 28 2017 from: http://www.georgemorris.org/publications/Final_Report-Canola_Biodiesel_May_15-06.pdf

Sijtsma, L. *et al.* (1998). Recent advances in fatty acid synthesis in oleaginous yeast and microalgae. *Recent Research Development in Microbiology.* 2: 219 – 232.

Suganya, T., Varman M., Masjuki, H.H., Renganathan S. (2016). Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach. *Renewable and Sustainable Energy Reviews.* 55: 909 – 941.

Sutton, S. (2011). Measurement of microbial cells by optical density. Journal of Validation Technology. Winter 2011: 46 – 49. Retrieved on February 28 2017 from http://www.microbiologynetwork.com/content/JVT_2011_v17n1_Measurement-of-Microbial-Cells-by-Optical-Density.pdf

Toledo-Cervantes, A., Morales, M., Novelo, E., Revah, S. (2013). Carbon dioxide fixation and lipid storage by *Scenedesmus obtusiusculus*. *Bioresource Technology*. 130: 652–658.

Trinh, N. T., Jensen, P. A., Sørensen, H. R., Dam-Johansen, K., & Hvilsted, S. (2012). Flash pyrolysis properties of algae and lignin residue. *Abstract from European Biomass Conference and Exhibition*, Milan, Italy. Retrieved on January 25, 2017 from: http://orbit.dtu.dk/files/10198080/prod21343995681272.Flash_pyrolysis_properties_of_algae_and_lignin_residue_1201.pdf

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

Turon, V., Trably, E., Fouilland, E., Steyer J.-P. (2015). Growth of *Chlorella sorokiniana* on a mixture of volatile fatty acids: the effects of light and temperature. Bioresource technology. 198: 852 – 860.

UNEP, (2017). United Nations Environment Programme. Biosolids management: an environmentally sound approach for managing sewage treatment plant sludge. An

introductory guide to decision makers. Retrieved on January 24, 2017 from: http://www.unep.or.jp/ietc/publications/freshwater/fms1/fig2_600px.html

UNFCCC, (2006). Greenhouse Gas Data, 2006 United Nations Framework Convention on Climate Change. Retrieved on January 24, 2017 https://unfccc.int/resource/docs/publications/handbook.pdf

Vital, M., Howe, A., Tiedje, J. (2014). Revealing the bacterial butyrate synthesis pathways by analyzing (meta) genomic data. *American Society for Microbiology*. 5, 2: e00889-14.

Vitasari, C., Meindersma, G., de Haan, A. (2015). Conceptual process design of an integrated bio-based acetic acid, glycolaldehyde and acetol production in a pyrolysis oil-based biorefinery. *Chemical engineering Research and Design.* 95: 133 – 143.

Wang, C. *et al.* (2013). Bio-oil based biorefinery strategy for the production of succinic acid. *Biotechnology for Biofuels.* 6: 74.

Wang, CW. (2015). Lipid droplet dynamics in budding yeast. *Cellular and Molecular Life Sciences.* 72,14: 2677 – 2695.

Wen, Q., Chen, Z., Li, P., *et al*. (2013a). Lipid production for biofuels from hydrolyzate of waste activated sludge by heterotrophic *Chlorella protothecoides*. *Bioresource Technology*. 143: 695 – 698.

Wen, Q., *et al.* (2013b). Lipid production for biofuels from effluent-based culture by heterotrophic *Chlorella protothecoides. BioEnergy Research*. 6 (3): 877 – 882.

WEO (2016). World Energy Outlook 2016. Executive summary. Retrieved on January 23, 2017 from: http://www.iea.org/publications/freepublications/publication/WorldEnergyOutlook2016ExecutiveSummaryEnglish.pdf

Wheeler, D. (2007). Commission to study methods and costs of sewage, sludge and septage disposal (HB 699, Chapter 253, Laws of 2007), final report, 41 p.

Willits, C.O. (**1951 in print, 2002 online).** Methods of determination of moisture-oven drying. *Analytical Chemistry.* 23(8): 1058 – 1062.

Wilson, C., Gorgan, J., Katehis, D. (assessed in March 22, 2017). Odors associated with stabilized biosolids. Greeley and Hansen presentation. Retrieved on March 22, 2017 from: http://www.mi-ea.org/docs/Wilson_Odors_associated_with_stabiilized_biosolids.pdf

Wood, S.M., Layzell. D.B.A. (2003). Canadian biomass inventory: feedstocks for a bio-based economy, BIOCAP Canada Foundation. Retrieved on January 23, 2017 http://www.biocap.ca

WWI, (2011). Biofuels make a comeback despite tough economy". Worldwatch Institute. Retrieved on January 23, 2017 http://www.worldwatch.org/biofuels-make-comeback-despite-tough-economy

Xu, J., Jiang, J., Zhao, J. (2016). Thermochemical conversion of triglycerides for production of drop-in fuels. *Renewable and Sustainable Energy Reviews*. 58: 331 – 340.

Xue, Z.X., Sharpe, P.L., Hong, S.P., *et al.* (2013). Sustainable source of omega-3 eicopentaenoic acid by metabolic engineering of *Yarrowia lipolytica*. *Nature Biotechnology*. 31: 734–740.

Yilancioglu, K., *et al.* (2014). Oxidative stress is a mediator for increased lipid accumulation in a newly isolated *Dunaliella salina* strain. PLoS ONE 9(3): e91957

Yu, W.-L., Ansari, W., Schoepp, N. *et al.* (2011). Modifications of the metabolic pathways of lipid and triacylglycerol production in microalgae. *Microbial Cell Factories*. 10: 91.

Yu, X., Zeng, J., Zheng Y., Chen, S. (2014). Effect of lignocellulose degradation products on microbial biomass and lipid production by the oleaginous yeast *Cryptococcus curvatus*. *Process Biochemistry. 49 (3): 457 – 465.*

Zambanini, T., Kleineberg, W., Sarikaya, E. *et al.* (2016). Enhanced malic acid production from glycerol with high-cell density *Ustilago trichophora* TZ1 cultivations. *Biotechnology for Biofuels*. 9: 135.

Zhang, H., Zhang, L., Chen, H., *et al.* (2013). Regulatory properties of malic enzyme in the oleaginous yeast, *Yarrowia lipolytica*, and its non-involvement in lipid accumulation. *Biotechnology Letters*. 35 (12): 2091–2098.

Zhou, L., Liu, X., Bai, H. (2011). A simple method for determining water content in organic solvents based on cobalt (II) complexes. *Chinese Chemical Letters.* 22(2): 189 – 192.

Zhu, J., and Chertow, M. (2016). Greening industrial production through waste recovery: "Comprehensive utilization of resources" in China. *Environmental Science and Technology*. 50: 2175 – 2182.