TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

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Production of Oleaginous Microbial Biomass by Reusing Wastewaters

Julkaisu 1348 • Publication 1348

Tampere 2015

Tampereen teknillinen yliopisto. Julkaisu 1348
Tampere University of Technology. Publication 1348

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Thesis for the degree of Doctor of Science in Technology to be presented with due permission for public examination and criticism in Festia Building, Auditorium Pieni Sali 1, at Tampere University of Technology, on the 28th of November 2015, at 12 noon.

Tampereen teknillinen yliopisto - Tampere University of Technology
Tampere 2015

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Painopaikka:
Juvenes Print
Suomen Yliopistopaino Oy
Tampere 2015

ISBN 978-952-15-3631-1 (printed)
ISBN 978-952-15-3632-8 (PDF)
ISSN 1459-2045

Abstract

Global energy demand continues to increase, which raises the question regarding how to solve the energy crisis caused by diminishing fossil fuels. There is no single alternative energy source that could substitute the fossil fuels, but microbial single cell oils (SCO) could be part of the solution. SCOs can be produced by cultivating microorganisms in wastewater in which nutrients and carbon from the wastewater are used for biomass production. In optimized conditions, microorganisms begin to accumulate lipids, and these lipids can be further refined for the production of biodiesel or renewable diesel. The lipid accumulation of the microorganisms may be enhanced by culturing the microorganisms under stressful conditions. The most commonly used strategy for enhancing lipid accumulation is nitrogen starvation, but it is even more effective when combined with another stress factor, such as moderately increased salinity.

In microbial lipid production, the major cost factor is often the substrate needed for the microorganisms. Therefore, utilizing inexpensive substrates and waste materials for the cultivation of oleaginous microorganisms is very desirable. Various wastewaters from municipalities, agriculture, and industrial sources have been studied, and many of these wastewaters have shown the potential for lipid-rich biomass production. Unfortunately, most of the studies have been conducted using sterilized wastewater. In large-scale applications, the sterilization of the wastewater is not cost-effective; therefore, lipid-accumulating microorganisms able to compete with the indigenous microorganisms of the wastewater need to be further studied.

The aim of this work was to sustainably produce oleaginous biomass by reusing the carbon and nutrients from wastewaters. This work included an evaluation of the suitability of various wastewaters for lipid-lipid rich biomass production (**Paper I**), the isolation of yeasts and fungi, which could possibly accumulate lipids by utilizing wastewater as substrate (**Paper II**), and the determination of the ability of the isolated microorganisms to accumulate lipids by comparing them with known lipid accumulating yeasts (**Paper II**). Unlike yeasts and fungi, microalgae are able to use an inorganic carbon source for their growth. This feature enables the combination of wastewater and flue gas treatment. Therefore, the growth and lipid accumulation of three microalgal species were compared (**Paper III**), and the suitability of the most potential microalgal species for accumulating lipids in sterilized and non-sterilized wastewater was studied (**Paper III & IV**).

Based on the results of this study, palm oil mill effluent (POME) has more potential for lipid production than chemithermomechanical pulp mill effluent (CTMP) or municipal wastewater (MWW) (**Paper I**). The residual lipids and solids of POME obstructed the analyses of the microbial SCOs.

Eukaryotes isolated from POME with agar plates were genetically identified as *Candida silvae* NRRL Y-6725 (with 100% similarity), *Galactomyces geotrichum* LMA-20 (with 99.8% similarity), *Lecythophora hoffmannii* CBS245.38T (with 96.7% similarity), and *Graphium penicillioides*

JCM9300 (with 99.3% similarity) (**Paper II**). The fungus *Graphium penicillioides* had a great potential for lipid accumulation based on the comparison study with well-known oleaginous yeast strains (*Yarrowia lipolytica* DSMZ8212, *Cryptococcus curvatus* DSMZ70022, & *Cryptococcus albidus* DSMZ701097) in a synthetic medium (**Paper II**). The lipid content per dry weight was higher with *G. penicillioides* compared to *C. curvatus* after 15 days of incubation (29.1±3.0 wt% vs 20.2±2.9 wt%, **Paper II**). Unfortunately, the overall lipid concentration was lower due to a lower biomass concentration. *G. penicillioides* contained more than 20% lipids, so it can be called oleaginous.

From the three microalgae isolated from a Taiwanese freshwater area (*Chlorella sorokiniana* CY1, *Chlorella vulgaris* CY5, & *Chlamydomonas* sp. JSC-04), *C. vulgaris* accumulated more lipids when various media, nitrogen sources, and nitrogen concentrations were studied (**Paper III**). The *C. vulgaris* in the BG-11 medium, initially containing 0.38 g $NaNO_3$/L, produced 3.8 g/L biomass and 57.5 wt% lipids after 12 days of incubation. The most suitable wastewater dilution for the lipid accumulation of *C. vulgaris* on sterilized anaerobically treated piggery wastewater was 5x dilution, which resulted in initial chemical oxygen demand and total Kjeldahl nitrogen of 75.4 mg/L and 57.4 mg/L, respectively. *C. vulgaris* was suitable for accumulating lipids on both sterilized and non-sterilized anaerobically treated piggery wastewater (PW) (**Paper IV**). The highest lipid content and productivity with the non-sterilized wastewater were rather promising (32.5±3.2 wt%, 71.2±2.2 g/L/d). However, under the conditions of these experiments, *C. vulgaris* excreted dissolved organic carbon (**Paper III** & **IV**), and the aim in wastewater treatment is the removal of organic carbon.

In summary, this work demonstrates the potential of indigenous eukaryotic microorganisms for lipid-rich biomass production. *G. penicillioides* isolated from POME has the potential for lipid-rich biomass production in a synthetic medium, which has not been previously reported. Similarly, *C. vulgaris* has the potential for lipid-rich biomass production in non-sterilized piggery wastewater, while most of the studies in the literature on *C. vulgaris* and wastewater have been conducted using sterilized wastewater. To enable simultaneous accumulation of lipids and efficient treatment of wastewater, special attention should be focused on the growth conditions.

Tiivistelmä

Maailmanlaajuinen energian tarve jatkaa kasvamistaan samalla kun fossiilisten polttoaineiden tunnetut varannot vähenevät. On hyvin epätodennäköistä, että löytyisi yksi kaikki fossiiliset polttoaineet korvaava energiantuotantomenetelmä. Mikrobiöljyt voivat olla osa tulevaisuuden energiantuotantoa. Mikrobiöljyjä voidaan tuottaa kasvattamalla mikrobeja jätevedessä, jolloin jäteveden ravinteet ja hiili hyödynnetään mikrobibiomassan tuottamiseen. Optimoiduissa eli useimmiten stressaavissa kasvuolosuhteissa mikro-organismit varastoivat rasvaa, jota voidaan hyödyntää biodieselin ja uusiutuvan dieselin tuotannossa. Rasvojen varastointikyvyn parantamisessa yleisin keino on typen saannin rajoittaminen, mutta tehokkaampaa on yhdistää se jonkin muun stressitekijän, kuten suolaisuuden noston, kanssa.

Mikrobiöljyjen tuotannossa eniten kustannusta on aiheuttanut mikro-organismien kasvattamiseen tarvittava substraatti. Siksi halpojen kasvualustojen ja jätemateriaalien hyödyntäminen rasvapitoisten mikrobibiomassan tuottamisessa on erittäin olennainen tutkimuskohde. Useita jätevesiä, niin kunnista, maataloudesta kuin eri teollisuuden aloilta on tutkittu rasvapitoisen mikrobibiomassan tuottamisessa. Valitettavasti useimmissa tutkimuksissa on tutkittu sterilisoituja jätevesiä. Suuremman mittakaavan prosesseissa jätevesien sterilisointi ei kuitenkaan ole kustannustehokasta ja siksi tulisi tutkia enemmän rasvoja kerryttävien mikro-organismien kilpailukykyä jätevesissä joissa on myös eläviä jäteveden mikro-organismeja.

Tämän työn tarkoitus oli tutkia kestävän kehityksen mukaista rasvapitoisen mikrobibiomassan tuottamista hyödyntäen jätevesien hiiltä ja ravinteita. Tässä työssä tutkittiin eri jätevesien potentiaalia rasvapitoisen biomassan tuottamisessa (**Julkaisu I**), mahdollisesti rasvoja kerryttävien hiivojen ja sienien eristämistä jätevedestä (**Julkaisu II**) sekä eristettyjen hiivojen ja sienten potentiaalia varastoida rasvoja vertaamalla niitä tunnettuihin rasvoja varastoiviin hiivoihin (**Julkaisu II**). Mikrolevät eivät tarvitse kasvuunsa orgaanista hiiltä toisin kuin hiivat ja sienet, vaan voivat hyödyntää kasvuunsa hiilidioksidia. Tämän ominaisuuden ansioista mikroleviä voidaan hyödyntää samanaikaisesti jätevesien ja hiilidioksidia sisältävien savukaasujen käsittelyssä. Siksi tässä työssä verrattiin myös kolmen taiwanilaisesta makeasta vedestä eristetyn mikroleväkannan potentiaalia varastoida rasvoja (**Julkaisu III**), potentiaalisimman leväkannan kykyä varastoida rasvoja jätevesialustassa (**Julkaisu III**), sekä jäteveden mikrobien vaikutusta mikrolevän rasvojen varastointikykyyn jätevesialustassa (**Julkaisu IV**).

Kemiallisen koostumuksensa perusteella palmuöljyteollisuuden jätevesi (POME) soveltui paremmin rasvapitoisen biomassan tuottamiseen kuin kemitermomekaanisen selluteollisuuden jätevesi (CTMP) tai kunnallinen jätevesi (MWW) (**Julkaisu I**). POME:n jäännösrasvat ja kiintoaine kuitenkin häiritsivät mikrobien rasvavarastojen tutkimista.

Geneettisen tunnistuksen perusteella POME:sta maljojen avulla eristetyt eukaryootit olivat: *Candida silvae* NRRL Y-6725 (100 % vastaavuus), *Galactomyces geotrichum* LMA-20 (99,8 %

vastaavuus), *Lecythophora hoffmannii* CBS245.38T (96,7 % vastaavuus), ja *Graphium penicillioides* JCM9300 (99,3 % vastaavuus) (**Julkaisu II**). Sieni *G. penicillioides* varastoi rasvoja erittäin hyvin kun eristettyjä kantoja verrattiin tunnettuihin rasvoja varastoiviin hiivakantoihin (*Yarrowia lipolytica* DSMZ8212, *Cryptococcus curvatus* DSMZ70022 & *Cryptococcus albidus* DSMZ701097) keinotekoisella kasvatusalustalla (**Julkaisu II**). *G. penicillioides* -kannan rasvapitoisuus kuivapainosta oli 15 päivän inkuboinnin jälkeen korkeampi kuin tunnetun rasvoja kerryttävän hiivan *C. curvatus* (29,1±3,0 % vs 20,2±2,9 %, **Julkaisu II**). Valitettavasti rasvojen konsentraatio oli matalampi *G. penicillioides* -kannalla kuin *C. curvatus* -kannalla, koska biomassan konsentraation oli alhaisempi. *G. penicillioides* kuitenkin varastoi rasvoja yli 20 % kuivapainostaan, joten sitä voidaan kutsua rasvapitoiseksi (engl. oleaginous) organismiksi.

Tutkituista taiwanilaisista makean veden levistä (*Chlorella sorokiniana* CY1, *Chlorella vulgaris* CY5 & *Chlamydomonas* sp. JSC-04) *C. vulgaris* varastoi eniten rasvoja, kun niiden kasvua verrattiin keskenään eri synteettisissä medioissa, erilaisilla typen lähteillä ja eri typen pitoisuuksilla (**Julkaisu III**). Kun *C. vulgaris* -kantaa kasvatettiin BG-11 mediassa 12 päivän ajan käyttäen 0,38 g $NaNO_3$/L alkukonsentraatiota, saavutettiin biomassan konsentraatio 3,8 g/L ja lipidipitoisuus 57,5 % kuivapainosta. Jätevesilaimennoksista 5x laimennos oli soveliain rasvojen tuottoon *C. vulgaris* -mikrolevällä. Tällöin jäteveden kemiallinen hapenkulutus ja Kjeldahl typpi olivat alussa 75,4 mg/L ja 57,4 mg/L. *C. vulgaris* tuotti rasvaa kasvaessaan sekä steriloidussa ja steriloimattomassa anaerobisesti esikäsitellyssä sikalan jätevedessä (**Julkaisu IV**). Korkeimmat steriloimattomalla jätevedellä saavutetut rasvapitoisuus ja rasvantuotto olivat hyvin lupaavia (32,5±3,2 %, 71,2±2,2 g/L/d). Tämän työn koeolosuhteissa *C. vulgaris* eritti ympäristöönsä liukoista orgaanista hiiltä (**Julkaisu III** & **IV**), kun taas jäteveden käsittelyn tarkoituksena on poistaa hiiltä.

Tämä tutkimus siis havainnollistaa eukaryoottisten mikro-organismien kykyä tuottaa rasvapitoista biomassaa. Jätevedestä eristetty sieni *G. penicillioides* tuotti rasvapitoista biomassaa keinotekoisessa kasvatusalustassa. Kyseisen sienen rasvojen varastointikykyä ei ole raportoitu aiemmin. Levä *C. vulgaris* kerrytti rasvoja varsin tehokkaasti kasvaessaan steriloimattomassa jätevedessä, kun taas kirjallisuudessa on raportoitu pääosin vain levien rasvojen varastointikykyä steriloidussa jätevedessä. Jotta mikro-organismeja voitaisiin hyödyntää samanaikaisesti rasvapitoisen biomassan tuottamiseen ja jäteveden käsittelyyn, kasvuolosuhteiden optimointiin on kuitenkin kiinnitettävä erityistä huomiota.

Preface

The experimental work for this thesis was carried out in the Department of Chemistry and Bioengineering at Tampere University of Technology (TUT, Tampere, Finland) and the Department of Chemical Engineering at National Cheng Kung University (NCKU, Tainan, Taiwan). The studies conducted with various wastewaters with mixed cultures and yeasts were conducted as part of an industrially funded project at TUT, while the studies with microalgae were conducted at NCKU and funded by TUT's Graduate School. In addition, I want to thank TUT Foundations, Industrial Research Fund of TUT, Maj & Tor Nessling foundation, A.R. Winter's memorial fund, and Maa- ja vesitekniikan tuki ry for the grant to support my studies and internationalization.

I am thankful to my teachers, Jaakko Puhakka, Pertti Vuoriranta, and Sakari Halttunen, for introducing the fascinating world of activated sludge to me. I am very grateful to my supervisor, Prof. Jaakko Puhakka, for providing me with the opportunity to do my dissertation related to aerobic biological wastewater treatment, which turned out to involve more biology than technology. For interesting discussions and advices during the project meetings, I want to thank the industrial representative Dr. Perttu Koskinen from Neste Oil Oyj and the other researchers who worked on that project, Aino-Maija Lakaniemi, Elena Efimova, Petteri Laaksonen, and Johanna Haavisto.

For the adventurous and enlightening exchange year in Taiwan, I am grateful to Prof. Jo-Shu Chang. Not only did he take care of me as a worker, but also as a lonely girl far away from home. In addition, I am grateful for the amazing teams at NCKU, including Dr. Chun-Yen Chen, staff of the B1 lab and the 10^{th} floor algae lab.

Against her advice on scientific writing, I will repeat my gratitude to my instructor, Dr. Aino-Maija Lakaniemi. Without her support and high expectations during this dissertation project, I would not have written these words. I want to thank Pertti Vuoriranta, Tarja Karjalainen, and Antti Nuottajärvi for their help with practical issues and my roommates and lunch mates for the psychotherapy. I am grateful for the staff of the Department of Chemistry and Bioengineering for their friendliness and support throughout my studies.

I'm astonished how my parents and friends have had the energy to listen about this process for these four years. I do not know how to express how thankful I am for my parents and for my friends: Aino, Niina, Ansku and Kristiina. I am also deeply indebted to Toni for challenging my thoughts during the last few years. Without the support and questioning of my passion from the wonderful people around me, I would not be who I am today.

Tampere, November 2015

Jatta Marjakangas

Contents

Abbreviations

ACC	Acetyl coenzyme A carboxylase
ACL	ATP: citrate lyase
ACS	Acyl coenzyme A synthase
ACP	Acyl carrier protein
Acyl-CoA	Acyl coenzyme A
AMP	Adenosine monophosphate
AS	Activated sludge
ATP	Adenosine triphosphate
BOD_7	Biological oxygen demand
C	Carbon
COD_s/COD_{tot}	Soluble - / Total chemical oxygen demand
CTMP	Chemithermomechanical pulp mill wastewater
DAG	Diacylglycerol
DGAT	Acyl coenzyme A: diacylglycerol acyl-transferase
DIC	Dissolved inorganic carbon
DO	Dissolved oxygen
DOC	Dissolved organic carbon
ER	Endoplasmic reticulum
FA	Fatty acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FAS	Fatty acid synthesis
ID	Isocitrate dehydrogenase
LED	Light-emitting diode
LP	Lipid particle
LPAT	Lysophosphatidate acyl-transferase
MAG	Monoacylglycerol
ME	Malic enzyme

MWW	Municipal wastewater
N / TN / N_s	Nitrogen / Total nitrogen / Soluble nitrogen
NADPH	Nicotinamine dinucleotide phosphate
NREL	National Renewable Energy laboratory in the USA
P / P / P_s	Phosphorus / Total phosphorus / Soluble phosphorus
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
OD	Optical density
POME	Palm oil mill effluent
PtdOH	Phosphatidic acid
PW	Piggery wastewater
SCO	Single cell oil
SE	Steryl ester
TAG	Triacylglycerol
TDC	Total dissolved carbon
TKN	Total Kjehldahl nitrogen
TLC	Thin layer chromatography
VFA	Volatile fatty acid
WE	Wax ester
WS/DGAT	Wax ester synthase/acyl coenzyme A: diacylglycerol acyltransferase
wt%	Weight-% from dry weight
YM	Universal yeast medium

List of Publications

I. Efimova, E., Marjakangas, J.M., Lakaniemi, A.-M., Koskinen, P.E.P. & Puhakka J.A. 2013. Lipid profile characterization of wastewaters from different origins. *Water Science & Technology*, 68(11), 2505−14, doi:10.2166/wst.2013.538.

II. Marjakangas, J.M., Efimova, E., Lakaniemi A.-M., Koskinen, P.E.P., Chang, J.S., Puhakka J.A. 2015. Lipid production by eukaryotic microorganisms isolated from palm oil mill effluent. *Biochemical Engineering Journal*, 99, 48−54, doi:10.1016/j.bej.2015.03.006.

III. Marjakangas, J.M., Chen, C.-Y., Lakaniemi, A.-M., Puhakka, J.A., Whang, L.-M., Chang, J.-S. 2015. Selecting an Indigenous Microalgal Strain for Lipid Production in Anaerobically Treated Piggery Wastewater. *Bioresource Technology*, 191, 369−376, doi:10.1016/j.biortech.2015.02.075.

IV. Marjakangas, J.M., Chen, C.-Y., Lakaniemi, A.-M., Puhakka, J.A., Whang, L.-M., Chang, J.-S. 2015. Simultaneous nutrient removal and lipid production with *Chlorella vulgaris* on sterilized and non-sterilized anaerobically pretreated piggery wastewater. *Biochemical Engineering Journal*, 103, 177−184, doi:10.1016/j.bej.2015.07.011.

Author's Contribution

Paper I: Marjakangas conducted part of the analyses, while Ph.D. Efimova conducted and interpreted the lipid analyses. Marjakangas presented the results in a poster session of the 10th IWA Leading Edge Conference on Water and Wastewater Technologies (2-6.6.2013, Bordeaux, France). Marjakangas prepared the paper with Ph.D. Efimova and revised it with Dr. Lakaniemi. Prof. Puhakka and Dr. Koskinen worked as advisors. Marjakangas is the corresponding author of the paper.

Paper II: Marjakangas designed the experiments with Prof. Puhakka and Dr. Lakaniemi. Marjakangas conducted the majority of the experiments. Dr. Lakaniemi conducted the isolation of the microorganisms, and Dr. Efimova conducted part of the lipid analyses. Marjakangas interpreted the results, wrote the paper, and is the corresponding author of the paper.

Paper III: Marjakangas designed the experiments with Prof. Chang, Dr. Chen, and Dr. Lakaniemi. Prof. Whang provided the wastewater for the experiments, and Marjakangas conducted the experimental work. Marjakangas interpreted the results and presented them in the poster session of the 3rd Asia-Oceania Algal Innovation Summit 2014 (17-20.11.2014, Daejeon, Korea). She also wrote the paper and is the corresponding author of the paper.

Paper IV: Marjakangas designed the experiments with Prof. Chang, Dr. Chen, and Dr. Lakaniemi. Prof. Whang provided the wastewater for the experiments, and Marjakangas conducted the experimental work. Marjakangas interpreted the results and wrote the paper with Dr. Lakaniemi, while Prof. Chang and Prof. Puhakka reviewed the manuscript. Marjakangas is the corresponding author of the paper.

The experimental work was performed under the supervision of Prof. Jaakko Puhakka (**Papers I-IV**), Dr. Aino-Maija Lakaniemi (**Papers I-IV**), Prof. Jo-Shu Chang (**Papers II-IV**), and Dr. Chun-Yen Chen (**Papers III-IV**).

1 Introduction

A global energy crisis is a highly likely future scenario. As Demain and Báez-Vásquez (2013) stated: "*The fossil based economy is getting closer and closer to the end of its life cycle.*" Diminishing fossil fuel reserves have been a hot topic already for decades and there have been conflicting views on when we will run out of fossil fuels. Former Saudi Arabian oil minister Sheikh Zaki Yamani has irrronically said that "*The Stone Age did not end for lack of stone, and the Oil Age will end long before the world runs out of oil*." (The Economist, 2003). The development of exploration and extraction technologies has meant that known and utilizable fossil fuel reservers have not diminished as fast as has been estimated since the oil crisis. Based on the latest approximations by Demain and Báez-Vásquez (2013), the petroleum reserves will last for 40 years, natural gas for 60 years, and coal for 130 years. Although new strategies to utilize the unrenewable natural energy resources have been developed, it is extremely important that every nation promotes new renewable energy alternatives. One of these alternatives is microbial based bioenergy. Microorganisms (combined with some processing technologies) can be used to produce transport fuels, such as alcohols (bioethanol, biobuthanol, & biomethanol), hydrocarbons (biomethane), or the alcohol esters of fatty acids (Wackett, 2008). This thesis focuses on microbial oils, which can be used for biodiesel and renewable diesel production.

The production volumes of biodiesel have been rising sharply during the last few years (Atabani et al., 2012), but the first demonstration to use peanut oil derived biodiesel in compression ignition engine was actually conducted already on 1898 at the World Exhibition in Paris (Crew, 1963). Biodiesels can be divided into three generations based on the raw material used for its production: the first generation biodiesel is produced from edible plants oils, the second generation biodiesel from unedible plant oils, and the third gereration biodiesel from microbial oils (Sitepu et al., 2014). Traditionally biodiesel is produced by the esterification of vegetable oils, while renewable diesel is a high-quality fuel produced by the hydrogenation or the thermal conversion of vegetable oils and waste fats (Prince, 2010). Biodiesel is usually mixed with petroleum diesel, while renewable diesel is fully compatible with existing fuel logistics, distribution, and vehicle engines and results in lower air emissions than fossil diesel (Demirbas, 2009; Knothe, 2010; Prince, 2010). However, the use of vegetable oils or sugars from food crops for transportation fuel production has been criticized,

because they compete with food production. Production of food crops requires herbicides, insecticides and fertilizers as well as large areas of fertile land, and oil extraction from oil crop is very energy intensive. For example, the energy input for producing sunflower oil has been shown to be higher than the energy content of the produced sunflower biodiesel (Pimentel & Patzek, 2005). Disadvantage of microbial oils is that at least 5 tons of sugar would be needed to produce 1 ton of microbial oil (Ratledge & Cohen 2008). Therefore, waste and residue materials are more attractive raw materials for biofuel production. Microbial single cell oil (SCO) technology allows for oil production from waste and side streams. SCOs are microbial oils, which can be produced with oleaginous microorganisms, including bacteria, yeasts, and microalgae (Yousuf, 2012). The composition of SCOs is similar to vegetable oils such as palm oil and cocoa butter (Hassan et al. 1994b; Hassan et al., 1995, Ratledge & Wynn, 2002); thus, SCOs could be used to replace vegetable oils in the production of biodiesel and renewable diesel.

There are several advantages of using SCOs instead of plant oils (Li et al., 2008). First, the duplication time for microorganisms is short. Second, their growth is less affected by seasonal variations and climates than plant cultivation. Third, the production of microorganisms is often easy to scale-up. Finally, the production process generally requires less labor than plant cultivation. (Li et al., 2008.) In addition, photoautotrophic microorganisms can fix CO_2 similarly as plants, which excludes the need for an organic carbon source and possibly even provides a way to mitigate CO_2 emissions (Mata et al. 2010). Some oleaginous microorganisms are able to accumulate lipids up to 80%, and lipid composition can be manipulated by changing the cultivation conditions, such as the type and concentration of the carbon source (Ageitos et al., 2011; Chisti, 2007). A high C/N ratio is the most commonly used method for enhancing the microbial lipid production (Ratledge, 2002).

The potential to use oleaginous yeasts and microalgae as microbial oil producers has been known for decades (Woodbine, 1959). The studies on microbial oil production for fuel purposes using yeasts have been conducted in Germany already during the World War I and II (Lundin, 1950; Stanier, 1946). These studies already demonstrated that decrease in the nitrogen concentration can increase the lipid production (Lundin, 1950). Even a factory producing yeast derived oil by using straw and sawdust as raw materials was constructed during the World War II in Germany (Lundin, 1950). In addition to Germany, high lipid content yeasts were also studied in Sweden before the 1950s (Nilsson et al, 1943). The Oil crisis in the 1970s increased again the interest for the development of microbial based renewable transportation fuels, and US Department of energy started the Aquatic Species Program on 1978 (Sheehan et al., 1998). The program focused on algal biodiesel production from waste CO_2. Unfortunately, the low cost of crude oil on 1990s terminated the Aquatic Species program on 1996 (Gallagher, 2011). The uncertainties around the availability of fossil fuels and the price of crude oil have promoted the development of microbial oils again. Although, the history of eukaryotic microbial oils is more concentrated on the production of high value nutritional oils, such us polyunsaturated fatty acids (for a review, see Sitepu et al., 2014), the commercial production of the high value oils with eukaryotic microorganisms (Cohen & Ratledge, 2005; Weisman et al., 2012) demonstrates their potential to produce microbial biodiesel

economically in the future. For example, California-based company Solazyme has several patents on the use of yeast oils for production of fuels and other chemicals (Franklin et al., 2011; Trimbur et al., 2011; Trimbur et al., 2012).

Several carbon sources for heterotrophic SCO production have been studied (Li et al., 2008; Ageitos et al. 2011; Bialy et al., 2011; Ratledge, 2004). The most desirable feedstocks for diesel production are different waste streams, as their use minimizes the costs and enables for combining waste treatment with energy production. For example, municipal sewage sludge and pulp and paper mill effluents are possible feedstocks for biodiesel production (Kargbo, 2010; Kouhia et al., 2015). This also applies for many industrial effluents, such as chemithermomechanical pulp mill (CTMP) wastewater, palm oil mill effluent (POME), distillery wastewater, and piggery wastewater (PW) (Abou-Shanab et al., 2013; Du et al., 2011; Gonzalez-Garcia et al., 2013; Hadiyanto & Nur, 2014).

The microbial production of biodiesel and renewable diesel from wastewater can be roughly divided into three parts: 1) biomass production, 2) lipid extraction, and 3) processing the lipids into biodiesel or renewable diesel (Devi et al., 2012). This thesis focuses on the first part by selecting suitable wastewaters for oleaginous biomass production with eukaryotic microorganisms, including yeasts and microalgae. The background of this work includes the theory of lipid accumulation in microorganisms and a review of the factors affecting microbial lipid accumulation. The original aim was to optimize the conditions of a wastewater treatment plant for oleaginous biomass production. Unfortunately, the mixed cultures of the activated sludge (AS) and the palm oil mill effluent (POME) were not efficient lipid producers. Therefore, eukaryotes were isolated from the POME and cultivated in a synthetic medium to accumulate lipids. The lipid accumulation potential of the isolated microorganisms was compared with known oleaginous yeasts. The yeasts always require organic carbon, while microalgae can also grow with an inorganic carbon source. Using inorganic carbon from flue gas in addition to organic carbon and the nutrients from the wastewater(s) would combine CO_2 fixation with simultaneous wastewater treatment and oleaginous biomass cultivation for biodiesel or renewable diesel production. Therefore, the potential of various microalgae for lipid production and then for wastewater treatment was studied. The aim of this study was to enhance the microbial lipid productivity of the yeasts and microalgae using wastewater as the nutrient and carbon source.

2 Background

2.1 Microbial Lipids

Almost all living organisms from bacteria to mammals are able to store energy as lipids (Murphy, 2001); however, bacteria, yeasts, fungi, and microalgae usually store energy as lipid inclusions only under specific growth conditions (Li et al., 2008). In most microorganisms, the cell membranes consist of phospholipids, and energy can be stored as specific ester compounds. According to Alvarez and Steinbüchel (2002), the occurrence of triacylglycerols (TAGs), fatty acid triesters of glycerol, is widespread in eukaryotic microorganisms, but these compounds can also be found in some prokaryotes. The typical lipid-based storage products in prokaryotes are polyhydroxyalkanoates (PHAs), including poly(3-hydroxybutyric acid) (PHB), but lipids can also be stored as TAGs and wax esters (WEs), and in some cases even as steryl esters (SEs) (Alvarez, 2010; Garay et al., 2014). Alvarez (2010) has reviewed the bacteria capable of storing lipids as TAGs or WEs, as shown in **Table 2.1**.

***Table 2.1**. Bacteria able to accumulate triacylglycerols (TAGs) and/or wax esters (WEs) (Alvarez, 2010).*

Gram-positive Bacteria	Type of stored lipids	Gram-negative Bacteria	Type of stored lipids
Rhodococcus opacus	TAG/WE	*Acinetobacter baylyi*	TAG/WE
Rhodococcus erythropolis	TAG	*Acinetobacter lwoffi*	TAG/WE
Rhodococcus fascians	TAG	*Alcanivorax borkumenis*	TAG/WE
Rhodococcus rubber	TAG	*Alcanivorax jardensis*	WE
Rhodococcus jostii	TAG/WE	*Marinobacter hydrocarbonoclasticus*	WE
Nocardia asteroides	TAG		
Nocardia coralline	TAG		
Nocardia globerula	TAG		
Nocardia restricta	TAG		
Mycobacterium tuberculosis	TAG		
Mycobacterium smegmatis	TAG		
Mycobacterium ratisbonense	TAG/WE		
Dietzia maris	TAG		
Gordonia amarae	TAG		
Streptomyces coelicolor	TAG		

Similarly, in eukaryotes, a major part of the neutral lipids consists of TAGs, but instead of WEs, lipids may also be stored as SE (Athenstaedt, 2010). The structures of these storage lipids are shown in ***Figure 2.1***. TAGs, WEs, and SEs are called neutral lipids because they are hydrophobic molecules lacking charged groups. Because of their neutrality, they are water-insoluble and osmotically inert, and they are also less toxic than free fatty acids or hydroxyl fatty acids. Therefore, they are ideal compounds for energy storage (Wältermann & Steinbüchel, 2006).

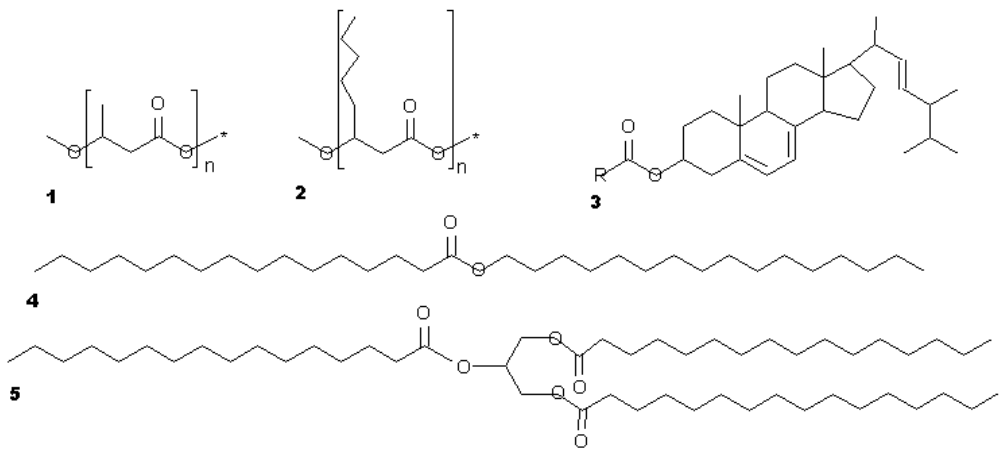

Figure 2.1. *Chemical structures of selected storage lipids: 1: Poly(3-hydroxybutyric acid) (PHB); 2: poly(3-hydroxyoctanoate), medium-chain polyhydroxyalkanoate (PHA); 3: Ergosterol ester, one of the yeast steryl esters (SE); 4: cetylpalmitate, one of the of the bacterial wax esters (WEs); 5: tripalmitoylglycerol, one of the triacylglyserols (TAGs). (Modified from Athenstaedt, 2010; Wältermann & Steinbüchel, 2006)*

Some microorganisms are able to accumulate lipids more efficiently than others. The microorganisms that accumulate lipids of more than 20% of their dry weight are called oleaginous (Ratledge & Wynn, 2002). The lipid contents and FA profiles of some yeast and microalga have been summarized in **Table 2.2**. The most common oleaginous yeasts belong to the genera of *Candida*, *Cryptococcus*, *Lipomyces*, *Rhodotorula*, *Rhodosporidium*, *Rhizopus*, *Trichosporon,* and *Yarrowia* (Ageitos et al., 2011; Beopoulos et al., 2009b). The most common oleaginous microalgae belong to the genera of *Chlorella*, *Crypthecodinium*, *Cylindrotheca*, *Dunaliella*, *Isochrysis*, *Nannochloris*, *Nannochloropsis*, *Neochloris*, *Nitzschia*, *Phaeodactylum*, *Porphyridium*, *Schizochytrium*, and *Tetraselmis* (Mata et al., 2010). The FA composition of microbial oils is similar to vegetable oils (**Table 2.2**), which demonstrates that oleaginous microorganisms can be used for the production of new generation biofuels in a similar manner as vegetable oils. The values presented in **Table 2.2** are not universal values, because the growth conditions affect the lipid content and FA composition of microorganisms.

***Table 2.2**. Lipid contents and fatty acid profiles for some oleaginous yeasts (Beopoulos et al., 2009b) and microalgae (Nascimento et al., 2012) compared with the fatty acid profiles of selected vegetable oils (Cristophe et al., 2012, Karmakar et al., 2010; Van Gerpen et al., 2004).*

Species	Lipid content (wt%)	Major fatty acid residues (relative % w/w)					
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Yeast							
Cryptococcus curvatus	58	25	n.d.*	10	57	7	n.d.*
Cryptococcus albidus	65	12	1	3	73	12	n.d.*
Candida sp. 107	42	44	5	8	31	9	1
Lipomyces starkeyi	63	34	6	5	51	3	n.d.*
Rhodotorula glutinis	72	37	1	3	47	8	n.d.*
Rhodotorula graminis	36	30	2	12	36	15	4
Rhizopus arrhizus	57	18	n.d.*	6	22	10	12
Trichosporon pullulans	65	15	n.d.*	2	57	24	1
Yarrowia lipolytica	36	11	6	1	28	51	1
Algae		**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
Ankistrodesmus falcatus	16.5	30	1	3	25	2	27
Ankistrodesmus fusiformis	20.7	27	traces	2	20	12	27
Kirchneriella lunaris	17.3	25	n.d.*	2	21	5	40
Chlamydomonas sp.	15.1	51	traces	12	14	3.9	3
Chlamydocapsa bacillus	13.5	25	1	3	18	13	26
Coelastrum microporum	20.6	26	1	3	45	9	11
Desmodesmu brasiliensis	18.0	28	n.d.*	3	42	12	9
Scenedesmus obliquus	16.7	52	n.d.*	8	22	5	3
Pseudokirchneriella subcapit ta	28.4	28	n.d.*	3	47	8	10
Chlorella vulgaris	28.1	40	3	8	30	9	2
Botryococcus braunii	45.0	7	n.d.*	2	77	5	5
Botryococcus terribilis	49.0	35	n.d.*	3	40	5	7
Vegetable oils							
Coconut	63-65	8-11	n.d.*	1-3	5-8	<1	n.d.*
Corn (Germ)	48	8-12	n.d.*	2-5	19-49	34-62	traces
Jatropha	30-40	12-17	n.d.*	5-10	37-63	19-41	n.d.*
Palm oil	30-60	32-59	n.d.*	1-8	27-52	5-14	n.d.*
Peanut oil	45-55	6-13	n.d.*	3-6	37-61	5-41	n.d.*
Rapseed	38-46	5	n.d.*	2	33	20	7
Rice bran	15-23	12-18	n.d.*	1-3	40-50	29-42	0.5-1
Soyabean	15-20	7-14	n.d.*	1-6	19-34	43-62	4-11
Sunflower	25-35	3-10	n.d.*	1-10	14-65	20-75	<1.5

**n.d.: not detected*

2.2 Lipid Accumulation in Microorganisms

Lipid accumulation may occur through *ex novo* or *de novo* accumulation (Beopoulos et al., 2009a). In an *ex novo* synthesis, FAs for biosynthesis are taken up from the environment. An *ex novo* synthesis requires the hydrolysis of the uptaken hydrophobic compounds before they can be directed to metabolism to produce lipid-based storage compounds. In addition to the FAs, hydrophobic substrates for *ex novo* lipid synthesis may also be triglycerides or alkenes (Thevenieau et al., 2010). In a *de novo* synthesis, FAs are produced by the cells, and then the produced FAs are stored within the cells through biosynthetic pathways. According to Athenstaedt (2010), "*lipid metabolism is well conserved across the different kingdoms of life*", while the biosynthesis of storage compounds slightly varies between different domains, as will be demonstrated in chapter 2.2. Biochemical reactions in different domains are very similar, but the genes and encoded enzymes are somewhat different. The *de novo* lipid accumulation consists of four steps: 1) the production of acetyl coenzyme A (acetyl-CoA) and reducing power as nicotinamide adenine dinucleotide phosphate (NADPH), 2) the biosynthesis of the fatty acyl chain,

3) the allocation of acyl moieties to either polar or neutral lipid pools, and 4) the lipid droplet biosynthesis (Garay et al., 2014). The steps of the *de novo* lipid accumulation are explained in more detail in the following subchapters and summarized for yeasts and microalgae in **Figure 2.5**.

2.2.1 Biosynthesis of Lipids

Acetyl-CoA and NADPH are produced prior to the biosynthesis of FAs, and the produced NADPH pool is an important source of reducing power for fatty acid synthesis (FAS) (Garay et al., 2014). FAS may occur through different pathways. The type one FAS (FASI) is a multienzyme process consisting of discrete functional domains and normally occurs in eukaryotic cytoplasm, but it also occurs in some bacteria. In bacteria, type two FAS (FASII) is more common than FASI. FASII can also occur in other organelles, such as plastids or mitochondria. **Figure 2.2** illustrates the FASII pathway (López-Lara & Geiger, 2010).

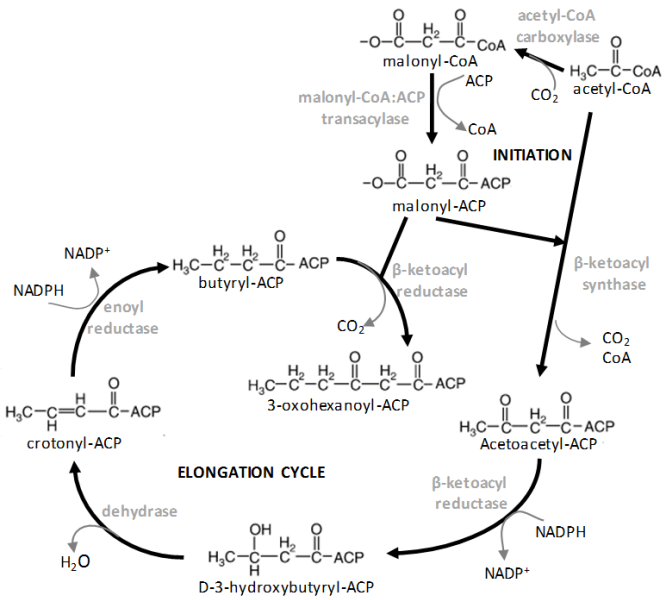

***Figure 2.2**. The FASII biosynthesis pathway. FASII consists of two stages: initiation and cyclic elongation. The first step of FASII is catalyzed by acetyl-CoA carboxylase, forming malonyl-CoA, which forms malonyl-acyl carrier protein (malony-ACP). The reaction is catalyzed by malonyl-CoA:ACP transacylase. In initiation, β-ketoacyl synthase condensates malonyl-ACP with acetyl-CoA, and acetoacetyl-ACP and CO_2 are produced. The cyclic elongation begins with condensing; thus, the condensing already occurs for the first time in the initiation by an elongation condensing enzyme β-ketoacyl synthase. Second, acetoacetyl-ACP is reduced to D-3-hydroxybutyryl-ACP by β-ketoacyl reductase. Third, D-3-hydroxybutyryl-ACP is dehydrated into crotonyl-ACP by dehydrase. In the last step, in the second reduction of the cyclic elongation, crotonyl-ACP is reduced into butyryl-ACP by enoyl reductase. Thereafter, elongation continues, and 2 carbons are added to the chain during each cycle. The steps in the elongation cycle are also called condensation, first reduction, dehydration, and second reduction. (Modified from López-Lara & Geiger, 2010; Rangan & Smith, 2002; Tehlivets et al., 2007.)*

In FASII (**Figure 2.2**), the synthesis of FAs begins with acyl carrier protein (ACP) and two carbon atoms. The carbon atoms are added pairwise to the carbon chain until the full length has been achieved. A malonate compound with ACP (malonyl-ACP) acts as a donor of carbon atoms, and the process releases CO_2 in each step (Gunstone, 1996).

Synthesized FAs are often used to form more complex lipids for cell structures and energy storage compounds, but FAs may also be used as signal molecules. The focus of this work was in storage lipids, more specifically in TAGs because they are preferred in biodiesel or renewable diesel production. In TAG biosynthesis, fatty acyl-compounds are produced, glycerol intermediates are formed, and glycerol is further esterified with fatty acyl residues (Alvarez & Steinbüchel, 2002). This biosynthesis occurs through the Kennedy pathway, which contains a series of the esterification of glycerol-3-phospate backbone (Garay et al., 2014). The four steps of the esterification of TAG from m-glycerol-3-phosphate and the three enzymatic step reactions for the biosynthesis of WE in bacteria is well-illustrated and explained by Kalscheuer (2010) and Lehner & Kuksis (1996).

The synthetization of TAGs in eukaryotes may occur through several different pathways, as shown in **Figure 2.3**. The first step of the TAG synthesis is the formation of phosphatidic acid (PtdOH). It may occur through the glycerol-3-phosphate pathway or the dihydroxyacetone pathway. The glycerol-3-phosphate pathway consists of two acylation reactions. The dihydroxyacetone pathway consists of three steps: acylation, the reduction of 1-acyl-dihydroxyacetone phosphate into 1-acyl-glycerol-3-phosphate (lyso-PtdOH), and another acylation. PtdOH may also be produced from glycerolphospholipids with phospholipase D. Diacylglycerol (DAG) is formed from the produced PtdOH through dephosphorylation. Other possible pathways for DAG formation are the degradation of glycerolphospholipids with phospholipase C and reverse TAG synthesis, also called the deacylation of TAG. The final step in TAG formation may be acyl-CoA dependent or acyl-CoA independent. In the acyl-CoA dependent pathway, acyl-CoA provides the acyl-group, but in acyl-CoA, the independent pathway acyl-group is provided by glycerolphospholipids. In eukaryotes, the final step may also occur through a transacylase reaction in which two DAGs are converted into TAG and monoacylglycerol (MAG); however, the enzymes for the transacylase reaction have only been found in higher eukaryotes and not from yeasts (Athenstaedt, 2010).

The synthetization of SE from the sterols in eukaryotes may occur through acyl-CoA dependent or independent pathways. In the acyl-CoA independent pathway, the phospholipid acts as an acyl-donor; however, the enzymes for this reaction have only been found in higher eukaryotes. In yeasts, SE formation has always been acyl-CoA dependent (Athenstaedt, 2010).

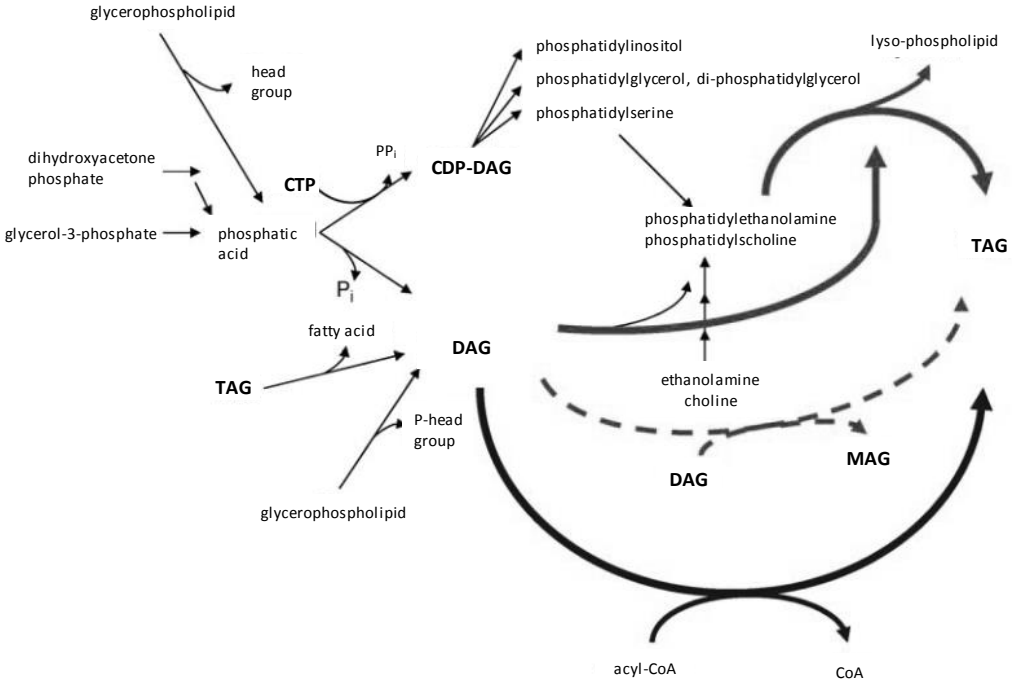

***Figure 2.3**. Possible pathways of triacylglyceride (TAG) synthesis in eukaryotes. The de novo synthesis consists of phosphatidic acid (PtdOH) formation and the dephosphorylation of PtdOH into diacylglycerol (DAG), which acts as a precursor for further TAG synthesis. Acyl-CoA dependent or independent* TAG synthesis are shown as the black and dashed gray line, respectively*. The acyl-CoA independent pathway may get the fatty acid (FA) from the phospholipid (gray line) or from a DAG molecule (dashed gray line). CDP-DAG: cytidindiphosphate-diacylglycerol.CTP: cytuduntriphosphate; CDP-DAG: cytidindiphosphate-diacylglycerol; MAG: monoacylglycerol; P-head group: phosphorylated head group of a phospholipid (Athenstaedt, 2010).*

2.2.2 Lipid Droplet Formation

In eukaryotic microorganisms, storage lipids are formed in the endoplasmic reticulum (ER), but bacteria do not have an ER. Therefore, the formation location of lipid particles (LP) varies (Wältermann & Steinbüchel, 2006). In bacteria, lipid inclusions are found in the cytoplasm, and the accumulation is initiated at peripheral lipid domains; however, the mechanism behind the transformation of lipid prebodies into lipid inclusions in bacteria is still unknown. The average diameter of lipid prebodies is 300 nm before detaching from the oleaginous layer and migrating into the cytoplasm. Based on phase contrast microscopy, at the beginning of the lipid accumulation, lipid inclusions have been small and visible, while at the later stage, the inclusions have been larger and brighter, filling almost the entire cytoplasm. Both spherical and nonspherical lipid inclusions have been found in bacteria (Wältermann & Steinbüchel, 2006).

The most probable mechanism for eukaryotic LP formation is the budding of LP from the ER, as shown in **Figure 2.4**. First, a micro-droplet is formed, which acts as a precursor for LP formation. Second, the droplet grows between the phospholipid layers of the ER by forming a TAG core surrounded by layers of SE. When the droplet is large enough, it begins to bud from the ER, and finally, the LP is detached. In the final LPs, hydrophobic neutral lipids (TAG & SE) are surrounded by a phospholipid monolayer. There are small amounts of proteins in the phospholipid monolayer, which play a key role in lipid metabolism, such as the activation of fatty acids (Johnson et al., 1994), synthesis, the degradation of TAG (Athenstaedt & Daum, 2005), and changes in the amount of stored lipids (Leber et al., 1998). The proteins associated with LP are well-conserved through the different organisms (Athenstaedt, 2010; Czabany et al., 2007).

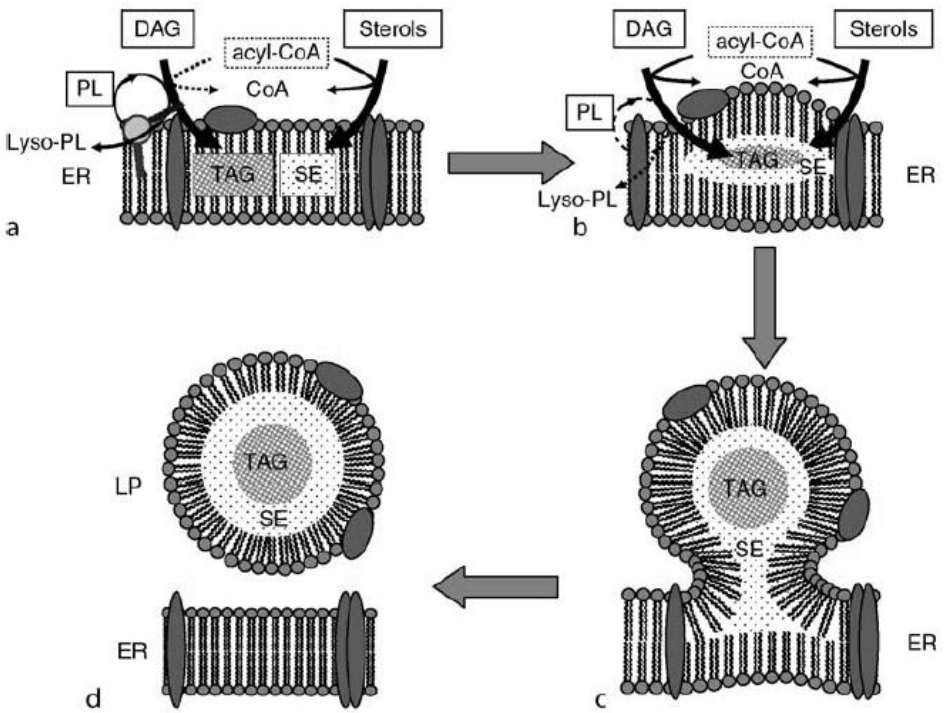

Figure 2.4. *Formation of lipid particle (LP) in eukaryotes. (a) Triacylglycerols (TAG) are acylated from diacylglycerol (DAG) and fatty acid originating from the phospholipid (PL) or acyl-CoA. Steryl ester formation in yeasts is always acyl-CoA dependent. (b) Due to the limited space in the phospholipid bilayer, neutral lipids are deposited between the bilayer. (c) Ongoing synthesis leads to the budding of an LP from the endoplasmic reticulum (ER). (d) After obtaining the critical size, the mature LP buds off from the ER. Figure by Athenstaedt (2010), published with permission from Springer through RightLink®.*

2.2.3 Key Enzymes in Lipid Accumulation

In stressful growth conditions with excess carbon, the normal growth of the cells may cease, and the accumulation of lipids can begin. According to the review by Ratledge (2002), the main enzymes in the metabolism of storage lipids are ATP:citrate lyase (ACL) and malic enzyme (ME). In the review by Beopoulos et al. (2009b), acyl-CoA carboxylase (ACC) is also stated to be one of the main enzymes. Lipid accumulation in oleaginous microorganisms normally begins with the activation of adenosine monophosphate (AMP) deaminase, and this reaction is often caused by a deficiency of nitrogen. The activation of AMP deaminase produces ammonia, as shown in **Equation (2.1)** (Beopoulos et al., 2009b; Ratledge, 2002).

$$AMP \rightarrow IMP + NH_4^+ \qquad \textbf{(2.1)}$$

A decrease in AMP concentration leads to a sudden change in oxygen and CO_2 concentrations and slows down the activity of isocitrate dehydrogenase (ID). The decrease in the activity of this enzyme leads to the formation of unmetabolized isocitrate, which is converted back to a citrate and transported into cytosol via a malate/citrate translocase system. Cytosolic citrate is cleaved by ATP:citrate lyase (ACL), forming acetyl-CoA and oxaloacetate, as shown in **Equation (2.2)** (Beopoulos et al., 2009b; Ratledge, 2002; Garay et al., 2014).

$$citrate + CoA + ATP \rightarrow acetyl - CoA + oxaloacetate + ADP + P_i \qquad \textbf{(2.2)}$$

Providing a continuous supply of malonyl-CoA is also important for lipid accumulation (Beopoulos et al., 2009b). Malonyl-CoA is generated from acetyl-CoA, which is catalyzed by acetyl-CoA carboxylase, as shown in **Equation (2.*3*)** (Beopoulos et al., 2009b).

$$acetyl - CoA + HCO_3^- + ATP \rightarrow malonyl - CoA + ADP + P_i \qquad \textbf{(2.3)}$$

ACL activity correlates positively with the microorganism's ability to accumulate lipids. All organisms that can accumulate lipids more than 20% of their dry weight have demonstrated ACL activity (Ratledge & Wynn 2002); however, ACL activity does not correlate with the extent of lipid accumulation. Thus, it is not the sole explanation for lipid accumulation. The extent of lipid accumulation is assumed to also be regulated by malic enzyme (ME) (Ratledge, 2002). Wynn et al. (1997) demonstrated that the inhibition of ME decreased the lipid accumulation of *Mucor circinelloides* from a normal 25% to 2%. Similarly lipid accumulation of *Aspergillus nidulans* decreased from 25% to 12% when the strain was genetically modified to lack ME activity (Wynn & Ratledge, 1997). For a high rate of lipid accumulation, a continuous supply of acetyl-CoA as well as a constant supply of NADPH is needed. For lipid accumulation, the function of ME activity is to

provide NADPH from malate, as shown in **Equation (2.4)** (Beopoulos et al., 2009b; Ratledge, 2002).

$$malate + NADP^{+} \rightarrow pyruvate + NADPH \qquad \textbf{(2.4)}$$

In FAS, 2 mol of NADPH is needed for the growth of the fatty acyl chain with one acetyl group, and each reductive step (3-ketoacyl reductase & 2,3-enoyl reductase) requires 1 mol of NADPH. Although ME provides only a part of NADPH production, it has a crucial effect on lipid accumulation by providing NADPH for FAS (Ratledge, 2002).

2.2.4 Lipid Accumulation in Yeasts and Microalgae

Yeasts can grow heterotrophically by metabolizing sugars and other simple compounds, such as glycerol (Garay et al., 2014). **Figure 2.5a** summarizes the *de novo* storage lipid synthesis in yeasts. Microalgae can grow photoautotrophically, heterotrophically, or mixotrophically. There are nine divisions of eukaryotic microalgae (Garay et al., 2014), but in this study, the focus is on green algae because green algae have been shown to contain a significant level of storage lipids under stress conditions (Thompson, 1996). Lipid accumulation in microalgae is rather similar to yeasts (**Figure 2.5a** vs. **Figure 2.5b**); however, microalgae can obtain their energy phototrophically with a chloroplast, which yeasts do not have, and therefore the various steps of lipid formation may occur in different locations than in heterotrophic yeasts. For example, in yeast, the acetyl-CoA pool is mainly cytosolic, while in microalgae, the acetyl-CoA pool can be found in plastids and/or cytosol. In microalgae, acetyl-CoA can also be found in mitochondria; however, the acetyl-CoA pool for mitochondrial FASII do not produce storage lipids (Hiltunen et al., 2010). Plastids play a key role in the *de novo* lipid biosynthesis of microalgae (**Figure 2.5b**), as photosynthesis provides most of the acetyl-CoA. In mixotrophic conditions, acetate can be directly incorporated into lipid biosynthesis, or it can first be converted to glucose (Liu & Benning, 2013). Due to the partitioning of carbon to starch synthesis and lipid synthesis, the starch synthesis may inhibit the lipid accumulation (Athenstaedt & Daum, 2006). The biosynthesis of the fatty acyl chains for storage lipids in microalgae occurs in plastidial FASII, while cytosolic FASI can also occur in heterotrophic microalgae. The formation of TAG from FAs may occur in plastids or in the ER. In the ER, the formation of lipid bodies occurs through the Kennedy pathway, which is similar to yeasts, and in plastids, the pathway also resembles the Kennedy pathway. (Garay et al., 2014.) **Figure 2.5** summarizes the lipid accumulation in yeasts and microalgae. Lipid accumulation can be affected by various nutritional and environmental factors, as explained in chapter 2.3.

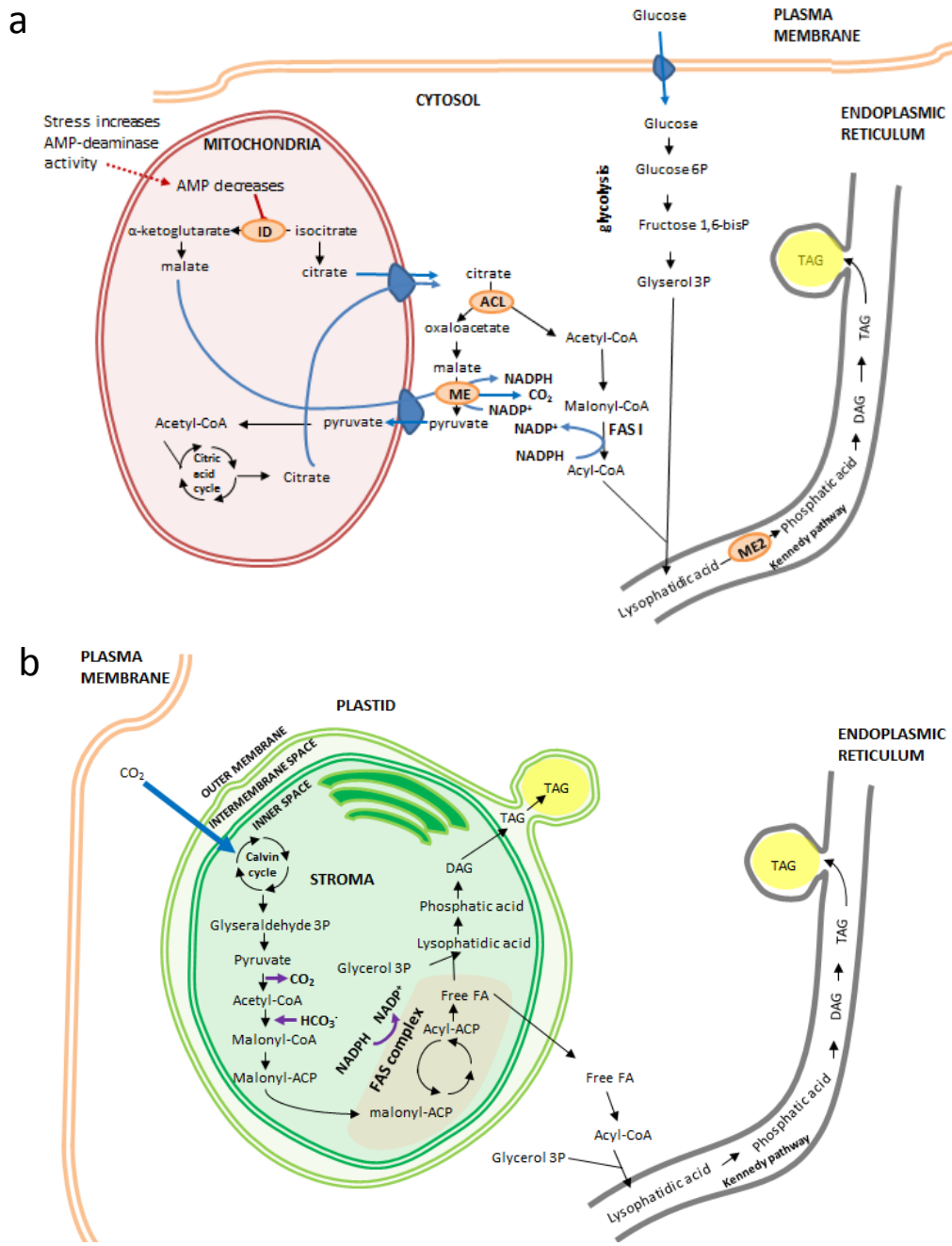

***Figure 2.5**. De novo storage lipid synthesis in yeasts (a) and microalgae (b). ID = Isocitrate dehydrogenase, ACL = ATP:citrate lyase, ME = malic enzyme (modified from Bellou et al., 2014; Cristophe et al., 2012; Dey & Maiti, 2013; Garay et al., 2014; Liu & Benning 2013; Ratledge & Wynn, 2002).*

2.3 Factors Affecting Lipid Accumulation

It has been shown that several factors can be used to enhance lipid accumulation in microorganisms. The main approaches have been changing the nutrient concentrations, controlling the growth conditions (e.g., pH, salinity, and temperature), and modifying the genome of the microorganisms (Li et al., 2008; Sharma et al., 2012). Stressful conditions generally lead to enhanced lipid accumulation. However, with microalgae, stressful conditions may also lead to the excretion of carbohydrates, as for example microalgae *C. vulgaris* and *Scenedesmus quadricauda* have shown to excrete polysaccharides as a response to oxidative stress caused by the toxin microcystin (Mohamed, 2008).

2.3.1 Macronutrients

Carbon. Various carbon sources for the production of lipid-rich microbial biomass have been studied. These include glucose, xylose, glycerol, starch, cellulose hydrolysate, and organic wastes (Subramaniam et al., 2010). When Dai et al. (2007) screened 250 yeast strains for lipid production and xylose assimilation, the yeast identified as *Rhodotorula glutinis* appeared to have the most potential for biodiesel generation. It accumulated a 49.3% lipids of its dry weight in flask studies and 60.7% in a 5 L bioreactor experiment (Dai et al., 2007). Of course, the suitability of a carbon source depends on the microorganism, and results with various yeast species on different carbon sources are summarized in **Table 2.3**. Yeast *Candida tropicalis* produced more lipids with glucose than with xylose (Dey & Maiti, 2013), and similarly, yeast *Trichosporon fermentans* produced the highest biomass concentration with glucose, followed by fructose, sucrose, xylose, and lactose (Zhu et al., 2008). On the contrary, with the unidentified yeast isolated from Chinese soil, the highest biomass production (16.1±0.44 g/L) and lipid yield (8.3±0.68 g/L) were obtained with sucrose compared to glucose, maltose, and lactose, while the yeast was not able to utilize dextrin, starch, or xylose at all (Liu et al., 2010). In some cases, several sugars can be used simultaneously, for example, the yeast *Trichosporon cutaneum* was able to use glucose and xylose at the same time (Hu et al. 2011). *C. curvatus* did not use xylose or cellobiose when glucose was available, but without glucose, xylose and cellobiose were co-utilized at the same rate (Yu et al., 2014). The oleaginous yeast *Rhodotorula garminis* may also grow on various sugars and glycerol (Galfassi et al., 2012). The most suitable sugar for biomass production with *R. garminis* was glucose, followed by mannose, glycerol galactose, cellobiose, and xylose. The highest lipid content was also obtained with glucose, followed by mannose, galactose, glycerol, xylose, and cellobiose (Galfassi et al., 2012). In addition to biomass concentration and lipid content, the carbon source was shown to influence the lipid composition. From the accumulated lipids, roughly 20-30% was saturated, but the proportion of monounsaturated fatty acids was much higher with mannose than with the other sugars or glycerol (75% vs. 40-60%) (Galafassi et al., 2012).

The advantage of photoautotrophic microorganisms is their ability to grow on inorganic carbon sources. Thus, CO_2 can be used as a carbon source instead of other, sometimes expensive organic carbon sources; however, the use of organic wastes for microalgal biomass production opens possibilities to recycle wastes. For example, the growth of *C. vulgaris* has been compared using CO_2, sodium acetate, acetic acid, oxalic acid, and citric acid as the carbon sources, and the highest biomass production was obtained with sodium acetate (Battah et al., 2013). Glucose, sucrose, and acetate have also been demonstrated to enhance the algal biomass production in the mixotrophic cultivation of *Chlamydomonas globosa*, *Chlorella minutissima,* and *Scenedesmus bijuga* (Bhatnagar, 2011). Silaban et al. (2014) studied the effect of organic carbon (sodium acetate, dextrose) on the biomass and lipid production of the co-culture of microalga *C. vulgaris* and cyanobacterium *Leotilyngbya* sp. No growth was detected in dark conditions without organic carbon. The maximum specific growth rate was obtained with a mixotrophic culture with dextrose as the carbon source, and the highest biomass production was with a mixotrophic culture with acetate (Silaban et al., 2014). According to Silaban et al. (2014), acetate enhanced the lipid productivity more than dextrose. On one hand, acetate may activate acetyl-CoA with a one-step reaction catalyzed by acetyl-CoA synthase, while more steps are required for dextrose and glucose (Perez-Garcia et al., 2011; Heifetz et al., 2000). On the other hand, dextrose may have been converted to sucrose and polysaccharides instead of lipids (Tanner, 2000). It is important to study the growth of the microorganisms with sugars, but for biodiesel production, pure sugars are too expensive. Therefore, the suitability of wastewaters as a carbon source for microorganisms to accumulate lipids for biodiesel production is discussed in chapter 2.4.

Table 2.3. *Lipid and biomass production of various yeasts cultivated on different substrates. In addition to the type of carbon source, its concentration and the C/N ratio affect lipid accumulation.*

Yeast strain	Carbon source	C conc. (g/L)	C/N ratio (g/g)	Cultivation mode	Biomass (g/L)	Lipid content (wt%)	Reference
Cryptococcus curvatus	Glucose	20-35	20-75	Fed-batch	70	53	Hassan et al., 1996
Cryptococcus curvatus	Glucose	30	40	Continuous	10.8	32	Hassan et al., 1993
Lipomyces starkeyi	Glucose	40	60	Batch	14.6	40	Angerbauer et al., 2008
Lipomyces starkeyi	Glucose	100	150	Batch	9.5	68	Angerbauer et al., 2008
Yarrowia lipolytica	Glucose	30	57	Batch	9.2	25	Aggelis & Komaitis, 1999
Yarrowia lipolytica	Glucose	30	85	Batch	5.5	14	Papanikolaou et al., 2009
Cryptococcus curvatus	Glycerol	3-10	-	Fed-batch	69	48	Thiru et al., 2011
Cryptococcus curvatus	Glycerol	8-256	80	Fed-batch	118	25	Meester et al., 1996a
Cryptococcus curvatus	Glycerol	16	-	Fed-batch	91	33	Meester et al., 1996b
Yarrowia lipolytica	Glycerol	28	70	Batch	4.7	22	Makri et al., 2010
Yarrowia lipolytica	Glycerol	105	250	Batch	6.7	20	Makri et al., 2010
Cryptococcus curvatus	Lactose	50	-	Batch	15.7	42	Iassonova et al., 2008
Cryptococcus curvatus	Whey	64	25-70	Batch	19.7-23.2	18-58	Ykema et al., 1988
Cryptococcus curvatus	Whey	64	40	Fed-batch	85	35	Ykema et al., 1988
Cryptococcus curvatus	Whey	64	20-40	Contiuous	20	36	Ykema et al., 1988
Cryptococcus curvatus	Whey	64	40	Partial recycling	91.4	33	Ykema et al., 1988

CO_2 concentration. The concentration of inorganic carbon is an important factor affecting microalgal growth under photosynthetic and mixotrophic growth conditions, although some can also grow heterotrophically using organic carbon. Similarly to organic carbon sources for yeasts, increasing the inorganic carbon concentration in microalgal cultivation increases biomass production (Ip et al., 1982); however, very high CO_2 concentrations begin to inhibit the photosynthetic growth of microalgae (Lee & Tay, 1991). Despite the enhancement in growth with a higher CO_2 content, there is a limit for CO_2. As Nakanishi et al. (2014) reported, the optimal CO_2 content for the lipid productivity of *Chlamydomonas* sp. JSC4 was 4% rather than 8%. For the growth and lipid accumulation of *C. vulgaris,* the optimal CO_2 concentration was 5% and the optimal aeration rate 0.5 vvm when Zheng et al. (2012a) studied various CO_2 concentrations (0.03, 1, 5, 10, 15%) and aeration rates (0.1, 0.5, 1.0, 1.5, 2.0 vvm). On the contrary, Lv et al. (2010) did not detect any significant difference in the biomass production of *C. vulgaris* at different CO_2 injection concentrations (0.5, 1.0, 6.0, 12%), but the highest lipid content was obtained at 1% CO_2. Studies with various microalgal strains for determining the optimal CO_2-% for lipid production are also summarized in **Table 2.4**.

Widjaja et al. (2009) studied the effect of CO_2 content (0.03-3.33% v/v at 6 L/min aeration rate) on the lipid production of *C. vulgaris*. Increasing the CO_2 concentration decreased the culture pH, as CO_2 is a weak acid. Reasons for pH change may have been the conversion of unused CO_2 to H_2CO_3 at high CO_2 injection concentrations, and the microalgal use of a carbonate for growth at low CO_2 injection concentrations. At low and moderate CO_2 concentrations, the highest lipid productivity was achieved during N depletion, and at a high CO_2 content, the highest lipid productivity was achieved at the end of the linear growth during normal nutrition. Overall, increasing the CO_2 content increased the growth, and it therefore had an important role for increasing lipid productivity (Widjaja et al., 2009). When Chiu et al. (2009) cultivated *Nannochloropsis oculate* with various CO_2 concentrations (2, 5, 10, 15% CO_2 at 0.2 L/min aeration rate), the most suitable concentration for biomass and lipid production was 2% CO_2. They also reported that the lipid accumulation of *N. oculata* increased remarkably when the growth phase changed from logarithmic to stationary (Chiu et al., 2009).

Table 2.4. *Optimal CO_2-% for lipid accumulation with various microalgal strains on different substrates.*

Microalgal strain	Studied CO_2 injections	Optimal CO_2 for lipid production	Reference
Chlamydomonas sp.	0.04-8%	4%	Nakanishi et al. 2014
Chlorella vulgaris	0.03-15%	5%	Zheng et al. 2012a
Chlorella vulgaris	0.5-12%	1%	Lv et al. 2010
Nannochloropsis oculate	2-15%	2%	Chiu et al. 2009

In general, the aim is to use flue gases and not pure CO_2. By using flue gases, a cultivation system can also be used for carbon capture; however, flue gases have possible disadvantages, such as high temperatures and varying chemical compositions. For example, the concentration of sulphur

oxides and nitrogen oxides may vary in flue gases, and these oxides may inhibit algal growth (Negoro et al., 1991). Yoo et al. (2010) studied the lipid production of *Botryococcus braunii*, *C. vulgaris,* and *Scenedesmus* sp. with ambient air containing 10% CO_2 and with flue gas from a heat generator burning liquefied petroleum gas containing 5.5% CO_2. They proposed *B. braunii* to be the most suitable for the production of biodiesel, as its lipid productivity increased 3.7-fold when flue gas was used and the total lipid content was 2-4 times higher that of the other strains.

Nitrogen. The effect of nitrogen on microbial lipid accumulation and the inhibiting effect of free ammonia on microbial growth have been studied for a very long time (Evans & Ratledge, 1984; Azov & Goldman, 1982; Přibyl et al., 2013). As early as 1949, Spoech & Miller reported that nitrogen deficiency increases the lipid accumulation in microalga *Chlorella pyrenoidosa* (see Přibyl et al., 2013). As stated in chapter 2.2.3, nitrogen deficiency is generally believed to increase lipid accumulation due to the activation of AMP deaminase (Beopoulos et al. 2009b; Ratledge 2002). Jakobsen et al. (2008) have suggested that the activation of AMP deaminase may be a general stress or starvation response in oleaginous microorganisms rather than a consequence of nitrogen limitation. Despite this recent finding, nitrogen limitation and the optimization of the C/N ratio are the most studied and the most successful methods to enhance lipid accumulation in microorganisms. It is generally known that microorganisms begin to accumulate lipids in the stationary growth phase when there is no nitrogen but sufficient carbon. The nitrogen limitation prevents the formation of new cells as it decelerates protein synthesis and activates nitrogen recycling, which leads to the utilization of the existing carbon for the production of compounds that do not contain nitrogen, such as lipids or hydrocarbons (Jakobsen et al., 2008; Liu et al., 2009).

Several researchers have studied the effect of the C/N ratio on the biomass and lipid production of yeasts. According to Zhu et al. (2008), the optimal molar C/N ratio for maximum lipid productivity and for maximum biomass productivity with yeast *T. fermentans* were 140 (≈120 g/g) and 163 (≈140 g/g), respectively. With *Sporobolomyces carnicolor,* the highest total lipid content was achieved with the molar C/N ratio of 90 (≈77 g/g) (Matsui et al., 2011). For *Lipomyces starkeyi,* the optimal ratio was 60−150 g/g (Angerbauer et al., 2008). The optimal C/N ratio also depends on the carbon source, as the optimal molar C/N ratio for *Yarrowia lipolytica* grown on glucose was 35 (≈30 g/g) (Papanikolaou et al., 2001) and 180 (≈155 g/g) on glycerol (Papanikolaou & Aggelis, 2002). Karatay and Dönmez (2010) studied the effect of the C/N ratio with *Candida lipolytica*, *C. tropicalis,* and *Rhodotorula mucilaginosa* by using various concentrations of molasses (6%, 8%, 10%) and $(NH_4)_2SO_4$ (0.5, 1.0, 1.5 g/L), and the optimal conditions for lipid accumulation were achieved with 8% molasses and 1.0 g/L $(NH_4)_2SO_4$. To enhance lipid accumulation, the C/N ratio should be high but not extremely high. For example, *Y. lipolytica* produced more citric acid and less lipids when the molar C/N ratio was as high as 80-120 (≈70-100 g/g) (Beopoulos et al., 2009b). Similarly, the optimal C/N ratio for lipid and biomass production with the yeast *Cryptococcus curvatus* was 20−50 g/g, while a ratio over 50 g/g already decreased both lipid and biomass production (Hassan et al., 1996). The critical nitrogen concentration for lipid production according to Cescut (2009) is 10^{-3} mol/L, and at higher nitrogen concentrations, more secondary metabolites are produced, which

decreases lipid accumulation. Thus, based on the literature review, high C/N ratios are efficient for lipid accumulation, but the optimal C/N ratio is case specific and should be optimized for each combination of microorganisms and substrates. Studies with various yeast strains for determining the optimal C/N ratio for lipid production are also summarized in **Table 2.5**. As mentioned above, carbon and nitrogen sources also have an effect on lipid accumulation.

Table 2.5. *Optimal C/N ratios for lipid accumulation with various yeast strains on different substrates. In addition to the type of carbon and nitrogen sources, their concentrations affect lipid accumulation.*

Yeast strain	Optimal C/N ratio for lipid production	Reference
Cryptococcus curvatus	20-50	Hassan et al. 1996
Lipomyces starkeyi	60-150	Angerbauer et al. 2008
Sporobolomyces cornicolor	70	Matsui et al. 2011
Trichosporon. fermentans	140	Zhu et al. 2008
Yarrowia lipolytica	30	Papanikolaou et al. 2001
Yarrowia lipolytica	155	Papanikolaou & Aggelis 2002

Liu et al. (2010) studied various nitrogen sources (yeast extract, peptone, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, and KNO_3) for the unidentified yeast strain SCIM 2.012, which was isolated from Chinese soil. The highest lipid (8.6±0.19 g/L) and biomass yields (16.7±0.29 g/L) were achieved with NH_4NO_3. Battah et al. (2013) found urea to be a better nitrogen source than nitrate because urea provides extra carbon for algal growth. Similarly, Zhang et al. (2011) stated that an organic nitrogen source (e.g., yeast extract and peptone) is a better option for lipid production than an inorganic nitrogen source (e.g., NH_4Cl) despite the higher price. They reported that the organic nitrogen source affects both the biomass production and lipid accumulation of *C. curvatus*, while inorganic nitrogen affects only the biomass production (Zhang et al., 2011). Of the inorganic nitrogen sources, nitrate seems to be a better nitrogen source for biomass and lipid production with *C. curvatus* than ammonium (Zheng et al., 2012b). Increasing the initial ammonium concentration (131-3140 g/m^3) inhibited the growth of *C. curvatus* on acetate and other volatile fatty acids, but it did not inhibit growth on the medium containing pyruvate generating substances, such as glucose and glycerol. These results indicate that ammonium may affect the lipid accumulation with acyl-CoA synthase inhibition (Zheng et al., 2012b).

Nitrogen limitation is also one of the most commonly used methods for enhancing the lipid production of microalgae. Griffiths et al. (2012) studied 11 microalgal species and reported that decreasing the nitrate concentration increased the lipid yield of all microalgae except prokaryotic *Spirulina platensis*. The most promising freshwater species for lipid production were *C. vulgaris* and *Scenedesmus* sp., whereas the most promising marine species were *Clavulinopsis fusiformis* and *Nannochloropsis* sp. Nitrogen deficiency has also been shown to enhance the lipid accumulation of *Chlorella zofingiensis* (Feng et al., 2012). Similarly, with *Chlamydomonas* sp., the lower nitrate concentration increased the lipid content and productivity, while a higher lipid content and productivity were obtained with urea as a nitrogen source rather than nitrate or ammonium

(Wu et al., 2012a). Nitrogen limitation also increased the lipid content of *Scenedesmus* sp. (Xin et al. 2010b), while for biomass production and the removal of nitrogen and phosphorus, nitrate and urea were better nitrogen sources than ammonium due to the inhibitory effect of acidic pH caused by NH_4^+ utilization (Xin et al., 2010a).

Generally, nitrogen limitation decreases the biomass production and increases the cellular lipid content, which therefore maximizes the lipid productivity, as g per L requires the optimization of the nitrogen concentration. For example, with *C. vulgaris,* an increase in the KNO_3 concentration (0.02-0.50 g/L) increased the biomass concentration but decreased the lipid content of individual cells, and the maximum lipid productivity was obtained with 0.1 mM KNO_3 concentration (Lv et al., 2010). Similarly, with *Chlorella* sp., an increase in the urea concentration (0.025-0.200 g/L) increased the biomass production and decreased the lipid content, and the highest lipid productivity was obtained at 0.1 g/L urea (Hsieh & Wu, 2009).

Nitrogen starvation is a very efficient way to enhance lipid accumulation, but even better results have been achieved by combining nitrogen limitation with some other stressor, such as oxygen limitation (Jakobsen et al., 2008). The cultivation of *C. vulgaris* in the presence of the microalgae growth-promoting bacterium *Azospirillum brasilense* with varying ammonium and nitrate concentrations demonstrated the population density effect on the take up of nitrogen (de-Bashan et al., 2005). The availability of nitrogen was not only affected by the soluble nitrogen concentration but also by the presence of other microorganisms competing for the available nitrogen. The higher the population density, the less nitrogen each cell could take up. This may be one of the reasons for a higher lipid content of *C. vulgaris* grown with the bacteria (de-Bashan et al., 2002). To maximize the lipid productivity, combining nitrogen starvation and some other stressor may lead to better results than nitrogen starvation alone.

Phosphorus. Most of the nutrient limitation studies to enhance lipid accumulation have been done under nitrogen limited growth conditions, but some studies have been conducted to reveal the effects of phosphorus limited growth conditions. Wu et al. (2010) reported phosphorus limitation to be equally effective as nitrogen limitation for enhancing the lipid accumulation of the yeast *Rhodosporidium toruloides*. They studied the effect of the C/P ratio at high nitrogen concentrations. They emphasized the importance of applying phosphorus limited lipid accumulation with nitrogen-rich effluents, and the ease of phosphorus removal compared to nitrogen removal. Phosphorus limited conditions can be achieved more economically than nitrogen limited conditions due to the easy chemical precipitation of phosphorus (Wu et al., 2010).

Phosphorus limitation has also been shown to increase the lipid content of the microalga *Scenedesmus* sp. from 23-38% to 53%, but the lipid productivity did not increase due to a lower biomass production (Xin et al., 2010b). Studies with *Scenedesmus* sp. have demonstrated that phosphorous limitation is an effective way to increase the biomass yield per phosphorus (Wu et al., 2012b); however, *C. zofingiensis* accumulated more lipids in a nitrogen deficient medium than in a

phosphorus deficient medium (Feng et al., 2012). In addition to phosphorus limitation, increasing the phosphorus concentration in the nitrogen limited condition may enhance the lipid accumulation, as has been demonstrated with *C. vulgaris* (Chu et al., 2013). With *C. pyrenoidosa*, phosphorus limitation severely inhibited the growth of the microalga and only modestly increased the lipid content (Fan et al., 2014). Fan et al. (2014) suggested that the depletion of phosphorus severely impacted the metabolism of the alga by inhibiting production of phosphorus-rich ribosomes, which are important for growth (Ågren, 2004). Chu et al. (2013) hypothesized that in nitrogen limited conditions, phosphorus was used for producing energy and other sufficient materials for the algae to continue lipid accumulation.

Phosphorus limitation may not be the best way to enhance lipid productivity, as the yeast *Y. lipolytica*, and several other yeasts and microalgae, can store phosphate as polyphosphate granules (Biryukova et al., 2011; Rhee, 1973). It has even been shown that the polyphosphate granules can be formed as a stress response for example in *Y. lipolytica* (Biryukova et al., 2011). Also, Jakobsen et al. (2008) reported that the cells of heterotrophic protist thraustochystrid *Aurantiochytrium* sp. are able to store phosphorus, as biomass containing only a low level of lipids was still produced after extracellular phosphorus was consumed. These storage compounds may be one of the reasons why phosphorus limitation is not suitable for enhancing the lipid accumulation in all microorganisms. Choosing to increase or decrease the phosphorus concentration should be evaluated on a case by case basis.

2.3.2 Micronutrients

Among the trace metals, the effect of iron on lipid production has been most widely studied, but the effect of other trace metals, such as cadmium, copper, magnesium, manganese, and zinc, have been studied as well (Li et al., 2008). According to Hassan et al. (1996), iron limitation did not affect growth, lipid production, or lipid composition of the yeast *C. curvatus* in a batch culture; however, in a fed-batch culture when both iron and nitrogen were limited, the stearic acid (C18:0) concentration increased, and the highest biomass concentration (70 g/L) was achieved after 172 h, resulting in lipid content of 53% (w/w). Hassan et al. (1996) hypothesized that iron stimulated lipid accumulation after complete carbon exhaustion. Fan et al. (2014) did not report any enhancement in lipid accumulation of the microalga *C. pyrenoidosa* with iron limitation; however, iron deficiency has been reported to increase the lipid content of *C. vulgaris* and an unidentified microalga isolated from Thailand (Liu et al., 2008; Yeesang & Cheirsilp, 2011). The effects of other trace metals on lipid accumulation have been studied at least with the microalga *Micractinium pusillum* (Abou-Shanab et al., 2012). Adding four times more manganese and copper increased the biomass and lipid productivity 1.5 times, but no increase was detected with zinc or cobalt. Similarly, increasing the copper concentration increased the lipid content of the microalga *Euglena gracilis* grown in autotrophic, heterotrophic, and mixotrophic conditions, but increasing the cadmium and zinc concentrations also increased the total lipid content (Einicker-Lamas et al., 2002).

2.3.3 Environmental Factors

Temperature. Temperature can be used as a stressor for lipid accumulation, but it also affects the cellular lipid composition of the microorganisms by the natural adaption of FAs. Biryukova et al. (2011) detected numerous unknown globular structures on the cell wall surface and polyphosphate granules and lipid particles in the cytoplasm of the yeast *Y. lipolytica* under heat stress at 37 °C. Suutari et al. (1990) studied the effect of temperature on the FA composition of various yeasts and showed that the effect was strain specific. With *Saccharomyces cerevisiae,* the mean FA chain length changed based on temperature. With *Candida utilis* cultivated at temperatures below 20−26 ºC, the C16/C18 ratio and FA content decreased with increasing temperatures, while at temperatures above 20−26 ºC, the degree of saturation and FA content increased with increasing temperatures. With *L. starkeyi,* temperature adaption was biphasic, which is similar to *C. utilis*, but the FA content significantly increased at temperatures below 20 ºC. With *Candida oleophila,* the FA content decreased with increasing temperatures, but the degree of saturation increased significantly at temperatures above 20 ºC. With *Rhodosporium toruloides,* the degree of saturation and FA content increased with increasing temperatures. According to Zhu et al. (2008), the optimal temperature for both lipid content and for the biomass production of the yeast *T. fermentans* was 25 ºC when temperatures between 20 and 35 ºC were studied. On the contrary, with the obligate psychrophilic yeast *Rhodotorula glacialis* DBVPG 4785, a decreasing temperature increased the lipid yield (Rossi et al., 2009). Further studies with *R. glacialis* showed that a temperature decrease increased the growth rate and did not affect the yield coefficient of biomass and lipid production. Thus, a temperature decrease only increased the volumetric lipid productivity (Amaretti et al., 2010).

According to Ip et al. (1982), a decrease in temperature favored the biomass production of green algae due to the increased solubility of CO_2; however, an increase in the cultivation temperature has been more often used to enhance microalgal biomass production. For example, Sayegh & Montagnes (2011) reported higher microalgal biomass and lipid productions with higher growth temperatures (25-30 ºC vs.15 ºC). In addition, thermotolerant microalgae, such as *Desmodesmus* sp., can accumulate lipids at rather high temperatures, such as 35 ºC (Ho et al., 2014). In outdoor cultivations in tropical climates, temperatures may increase to a rather high level during the day, so it is important to use microorganisms, which are able to grow and accumulate lipids at high temperatures; however, the effect of temperature on lipid production appears to be species dependent.

Illumination. The photoperiod and light intensity have been known to affect the biomass production of the photoautotrophic microorganisms for a long time (Ip et al., 1982). Su et al. (2012) studied the nutrient removal efficiency of an algal mixture (*Chlamydomonas reinhardtii*, *Scenedesmus rubescens* and *Chlorella vulgaris*) on wastewater with continuous illumination, altering illumination and no illumination. For biomass production, continuous illumination was the best option, while without illumination, the nutrient removal was poor and the biomass production

low. No change in nutrient removal efficiency was observed when comparing continuous and altering illumination, but a higher biomass concentration was achieved with continuous illumination. With illumination, the dissolved oxygen (DO) concentration was always high, while without illumination, the DO concentration decreased in the middle of the cultivation due to low algal autotrophic growth and intensive nitrification. With continuous and altering illumination, the pH increased from 8 to almost 10 due to the photosynthetic consumption of inorganic carbon, while without illumination, the pH remained at 8-9. (Su et al., 2012.) With *Pavlova lutheri,* the highest biomass and lipid production was also achieved with continuous illumination when compared with altering illumination and no illumination (Shah et al. 2014).

Light intensity also had an effect on palmitoleic acid (C16:1) content, as its content was significantly higher at the higher light intensity (Shah et al. 2014). When Olguín et al. (2001) studied the growth of *Spirulina* sp. in a complex medium containing sea-water and digested pig waste with low light intensity (66 & 144 µmol photons/m^2/s), they found the lipid content to be higher at the lower light intensity; however, due to a higher biomass concentration, the lipid productivity was higher at the higher light intensity (Olguín et al., 2001). Similarly, Olguín et al. (2003) reported higher biomass productivity with *Spirulina* in an outdoor raceway in Mexico during the summer when the light intensity was higher compared to the autumn or winter. In outdoor conditions, other environmental conditions, such as temperature and rain, also affect the results. Ho et al. (2012) studied the effect of light intensity (60-540 µmol photons/m^2/s) on *Scenedesmus obliquus* CNW-N. The optimal light intensity for biomass and lipid productivity was 420 µmol photons/m^2/s, and the biomass production mainly increased with increasing light intensity; however, the highest light intensity (540 µmol photons/m^2/s) already began to remarkably decrease CO_2 fixation efficiency, biomass production, and lipid production due to the photo-inhibition effect (Ho et al., 2012).

In addition to the light intensity and the illumination cycle, various light wavelengths have been studied. For example, the maximum specific growth rate of *Nannochloropsis* sp. decreased when light was changed from blue to white, green, and red (Das et al., 2011). The highest lipid content was obtained with green light, while the highest lipid concertation was obtained with blue light due to the better biomass production. On the contrary, the most suitable LED light wavelength for the biomass production of *C vulgaris* was red, followed by white, yellow, purple, blue, and green (Yan et al., 2013). Similarly, another study with *C. vulgaris* in high-strength wastewater reported the most suitable LED for biomass production and nutrient removal to be red, followed by white, yellow, and blue (Ge et al., 2013). The optimal light intensity for nutrient removal from high-strength wastewater with *C. vulgaris* was 2000-2500 µmol photons/m^2/s and 1000-2000 µmol photons/m^2/s for economic efficiency (Ge et al., 2013); however, the use of artificial lights is considered as an uneconomical option for large-scale algal cultivation for the production of low-value commodities, such as energy and fuels. Therefore, the only possible light source for large-scale cultivations is natural sunlight, which makes it difficult to predict or control the light intensity or the illumination

cycle. It is always possible to geographically select the optimal place for an outdoor algal cultivation system, but otherwise, controlling the light conditions in nature is impossible.

Oxygen concentration and oxidative stress. Oxygen limitation is a possible stress factor for the growth of yeasts, and the effects of oxygen limitation on growth and lipid accumulation have been studied. Jakobsen et al. (2008) reported that the limitation of N, P, and O_2 enhanced the lipid accumulation of *Aurantiochytrium* sp. strain T66, but oxygen limitation (below 1% of saturation) in nutrient deficient conditions had the greatest effect by increasing the lipid content to 60% of the dry weight. On the contrary, in the experiment by Aguedo et al. (2005), increasing the air pressure from the atmospheric pressure to 5 bars stimulated the cell growth of *Y. lipolytica*. With yeast *R. glutinis*, a low DO concentration decelerated growth and enhanced lipid accumulation, but on a g per L basis, a low DO favored lipid production (Yen & Zhang, 2011). Under oxidative stress (developed with H_2O_2), Biryukova et al. (2011) detected numerous unknown globular structures on the cell wall surface and polyphosphate granules and lipid particles in the cytoplasm of *Y. lipolytica,* which is similar to their findings under heat stress. Another study by Biryukova et al. (2008) demonstrated a decrease in the respiratory activity of *Y. lipolytica* after heat shock or treatment with an oxidant (H_2O_2).

In the case of microalgae, Chi et al. (2009) reported a low oxygen concentration to be obligatory for lipid accumulation. In their study with *Schizochytrium limacinum* SR 21 in a 5 L fermentor with DO control, 50% of the DO concentration from saturation lead to an almost two times higher cell density than a 10% DO concentration, but the DO concentrations above 50% decreased the culture pH and reduced lipid accumulation (Chi et al., 2009). The effect of oxygen concentration is different for yeasts and microalgae because microalgae can produce oxygen photosynthetically. Still, a low concentration of oxygen appeared to be obligatory for the lipid accumulation of yeasts and microalgae.

Culture pH. The culture pH is important for controlling the growth of the microorganisms and can also be used for controlling lipid accumulation. The optimal pH to maximize cellular lipid content (wt%) of *L. starkeyi* was 5.0, while the highest lipid concentration (g/L) was obtained at pH 6.5 (Angerbauer et al., 2008). For *T. fermentans,* the optimal pH was 6.5 for both lipid content and biomass production (Zhu et al., 2008). Karatay and Dönmez (2010) studied the lipid accumulation properties of *C. lipolytica*, *C. tropicalis,* and *R. mucilaginosa* using molasses at various pH values (4−7) and obtained the highest lipid contents for all of the species at pH 5 after a 4-day incubation. With *C. lipolytica,* lipid production was sharply reduced at pH values below and above 5, while with *C. tropicalis* and *R. mucilaginosa,* the reduction was not as sharp as for *C. lipolytica* (Karatay & Dönmez, 2010). Shah et al. (2014) studied pH values from 5 to 10 for the cell growth and lipid content of the microalga *P. lutheri*. They reported an increasing biomass concentration and lipid content with increasing pH until pH 8, and with the pH value 10, the biomass concentrations and lipid content decreased remarkably (Shah et al., 2014). Chiu et al. (2009) studied various CO_2 concentrations, which also affected the culture pH, and they suggested that the pH increase from

7.0 to 7.8 is the main reason for the increasing lipid content of *N. oculata*. The optimal pH depends on the microorganism used for lipid production, but Angerbauer et al. (2008) reported that the choice of substrate also affects the optimal pH of lipid accumulation. In general, normal or slightly acidic pH values have been efficient to maximize the lipid accumulation of yeasts. For microalgae, neutral or slightly basic pH values appear to enhance lipid accumulation in microalgae.

Salinity. Salinity has been used to enhance microbial lipid production, but the effect of salinity depends on the microorganism and its tolerance for salinity. Some thraustochytrid species, such as *Aurantiochytrium mangrovei* and *S. limacinum*, have demonstrated a decrease in biomass and lipid production at very low salinities (Chaung et al., 2012; Zhu et al., 2007). When Shah et al. (2014) studied the effect of salt concentration (15-40 g NaCl/L) on the biomass and lipid content of the microalga *P. lutheri*, the optimal concentration for both was 35 g/L, while a remarkable decrease in the biomass concentration was detected at a 40 g/L NaCl concentration. When Campenni et al. (2013) studied the effect of different salt concentrations (10-30 g NaCl/L) on *Chlorella protothecoides*, the highest carotenoid and FA production in the nitrogen depleted medium was achieved at an NaCl concentration of 20 g/L. On the contrary, with the freshwater algae *Chlamydomonas mexicana* and *S. obliquus,* the highest biomass concentration and lipid content were already obtained at 1.9 g/L of NaCl, while higher concentrations (3.5 and 6.2 g/L) inhibited the growth (Salama et al., 2013). Similarly, with a mixed microalgal culture isolated from a still, freshwater body receiving domestic wastewater effluents, the lipid productivity increased with an increasing NaCl concentration up to1 g/L, but 2 g/L inhibited the growth (Mohan & Devi, 2014). In addition, using 20% deep-sea water with a synthetic medium enhanced the biomass growth and lipid accumulation of the freshwater microalga *C. sorokiniana* CY1 (Chen et al., 2013). Based on the reviewed literature, it is evident that increased salinity (to a certain threshold level) can be used as a stress factor for enhancing lipid accumulation, but it should also be taken into account that salts may interfere with the further processing of microbial lipids into biodiesel.

Culture mode. Due to the photosynthetic activity of microalgae, they can be cultivated phototrophically (nutrients from inorganic compounds, and energy from light), heterotrophically (nutrients and energy from organic compounds, without light), photoheterotrophically (nutrients from organic compounds, and energy from light), or mixotrophically (nutrients from organic and inorganic compounds, and energy from organic compounds and light) (Grobbelaar, 2004). The biomass yields and lipid productivities obtained with the same organism at these different culture modes can be very different. For example, in studies with the *Chlorella* species in autotrophic, heterotrophic, photoheterotrophic, and mixotrophic conditions, the highest lipid productivities (g/L/d) have been obtained at mixotrophic conditions and the highest lipid contents (wt%) at autotrophic conditions, while heterotrophic conditions have enabled the lowest lipid productivity (g/L/d) (Li et al., 2014; Liang et al., 2009; Yeh & Chang, 2012). The growth rate of *Nannochloropsis* sp. was also slightly faster in a mixotrophic cultivation with glycerol than in phototrophic cultivation (Das et al., 2011). With the mixture of microalgae in a biphasic cultivation (in which the algae were subjected to different culture modes), the mixotrophic condition was the most suitable for the first phase of

maximal biomass production, while the autotrophic condition followed by the heterotrophic condition were more suitable for the second phase (stress induced starvation) for maximal lipid accumulation (Devi et al., 2013).

The optimization of cultivation conditions is difficult because the optimal conditions for maximum biomass production compared to the highest possible lipid content of cells are different (Lv. et al. 2010). Therefore, two-stage cultivation systems are often a better option for optimizing biomass and lipid production. Su et al. (2011) obtained a 2.8-times higher final lipid yield of *Nannochloropsis oculata* with an optimized two-step system compared to a traditional single-stage batch cultivation system.

2.3.4 Genetic Engineering

Several studies on microbial lipid metabolism have been conducted over the last few years. Defining the metabolic maps of specific microorganisms has revealed several interesting options for genetic engineering to enhance microbial lipid production. Metabolic engineering has been used to enhance the TAG production in bacteria, yeasts, and microalgae (Santala et al., 2011; Kalscheuer et al., 2006; Beopoulos et al., 2008; Guarnieri et al., 2013). Genetic engineering can be applied by over expressing the enzymes relevant for lipid accumulation or blocking the competing pathways. These strategies are discussed in the review by Courchesne et al. (2009). In summary, the main enzymes related to the TAG biosynthesis pathway are Acetyl-CoA carboxylase (ACC), Fatty acid synthese (FAS), Lysophosphatidate acyl-transferase (LPAT), and acyl-CoA:diacylglycerol acyl-transferase (DGAT), while the enzymes directly involved in lipid biosynthesis are acyl-CoA synthase (ACS), malic enzyme (ME), and ATP:citrate lyase (ACL). For example, the National Renewable Energy Laboratory (NREL) in the USA has studied the genetic modification of microalgae, and by transferring additional copies of the ACC gene into microalgae, they have been able increase the lipid content from 5-20% to 60% in laboratory conditions and 40% in outdoor conditions (see Huang et al., 2010). Genetic engineering is thought to be an important part of the future of biofuel production (Colin et al., 2011), but genetically modified microorganisms also have many restrictions and disadvantages. In combined lipid accumulation and wastewater treatment, it is highly possible that genetically modified microorganisms would not survive in the competitive environment of wastewater. Therefore, it could be more feasible to find indigenous oleaginous microorganisms from the wastewater for lipid production rather than use genetically modified microorganisms.

2.4 Simultaneous Production of Microbial Oils and Waste Treatment

The scope of this thesis is to use wastewater as a nutrient and a carbon source for the cultivation of lipid-rich biomass to be used in biodiesel and renewable diesel production. Various municipal and industrial wastewaters have been studied for the production of lipid-rich microbial biomass

(**Table 2.6** & **Table 2.7**). The lipid production of various yeast strains has been studied in prickly-pear juice, molasses from the sugar cane industry, sorghum and rice hull hydrolysate, fish processing wastewater, monosodium glutamate wastewater, crude glycerol, food waste, and municipal wastewater (Cheirsilp et al., 2011; Chi et al. 2011; Economou et al., 2010; Economou et al., 2011; Hassan et al., 1994a; Iassonova et al., 2008; Makri et al., 2010; Tsigie et al., 2012a; Xue et al., 2008). Similarly, several microalgal species have been cultivated for lipid production in alcohol distillery wastewater, pig and poultry manure, palm oil mill effluent, and textile wastewater (Fenton & hUllacháin, 2012; Ji et al., 2013; Kamarudin et al., 2013; Lim et al., 2010; Solovchenko et al., 2014; Whang et al., 2009). In addition, biodiesel production has been studied in municipal wastewater using the mixed microbial community originating from that municipal wastewater treatment plant (Dufreche et al., 2007) and in chemithermomechanical pulp mill (CTMP) effluent using genetically modified bacterium *Rhodococcus* sp. (Du et al., 2011).

The use of waste materials for renewable energy production makes it possible to manage the continuously increasing volumes of waste streams and to simultaneously produce energy sustainably. Biofuels are a very important option because they diminish the use of fossil fuels and also enable the utilization of waste streams, which are available at little or no cost (Tyagi & Lo, 2003). The factors affecting the suitability of the effluents for the cultivation of lipid-rich microbial biomass are the available quantity, the composition of the effluent (such as biodegradability, presence of possible inhibitory compounds), and the possibility to combine various effluents and waste streams. Combining various effluents and waste streams to create an optimal medium for microbial growth may be very desirable; however, the locations and possibilities to transfer the feedstocks efficiently may complicate this option. A key characteristic of the potential wastewater sources is a high content of easily biodegradable organic matter or residual oils. Food processing produces easily biodegradable wastewater with a high organic content. Arboreal based industries produce wastewater with a high organic content, but paper mill effluents may contain chemicals that inhibit microbial growth. Olive oil and palm oil industries produce wastewaters with a high residual oil content, while glycerol, generated as a side product in the biodiesel production process, is also a possible feedstock for SCO production (Papanikolaou & Aggelis, 2002).

2.4.1 Waste As a Nitrogen and Carbon Source for Oleaginous Yeasts

The use of inexpensive substrates and waste materials for the cultivation of oleaginous yeasts has been studied using various yeasts and wastewaters, as shown in **Table 2.6**. Various methods to improve the suitability of wastewater for lipid-rich microbial cultivation have also been studied. For example, Liang et al. (2012b) studied the effect of lime on the pretreatment of sweet sorghum bagasse. When the solids for enzymatic hydrolysis were pretreated with a microwave and lime, the biomass concentration was lower, and the lipid content was higher than without lime. With and without lime, they respectively obtained a 42.5% and 37.7% lipid content for the dry weight. The true cellular lipid content was as high as 73.3% and 64.0%, respectively (Liang et al., 2012b). The effect of bioaugmentation has also been studied. Whang et al. (2013) studied biodiesel production

by bioaugmentating waste sludge hydrolysate with yeasts. Bioaugmentation increased the FAME yield compared to the similarly transesterified hydrolysate of waste sludge without the addition of the yeasts. In the transesterified and bioaugmented hydrolysate of waste sludge, the VFA content was low, and the final FAME yield was 9.24 wt% (Whang et al., 2013).

Thiru et al. (2011) studied the entire biodiesel production process, including biomass production, oil extraction, and trans-esterification of biodiesel, using *C. curvatus*. They were able to reduce the cost of the overall process with *C. curvatus* using a low cost substrate crude glycerol and by substituting the malt extract and baker's yeast extract with deoiled *Cryptococcus* lyasate. With 134 h of incubation in a 26 L fermentor, they obtained 50.4 g/L of biomass (on dry weight basis) and 45 wt% oil content. Based on their results, yeasts are strong contenders in the field of sustainable biofuels (Thiru et al., 2011).

Y. lipolytica has potential in various industrial processes (Groenewald et al., 2014), and it is one of the most studied yeasts for biodiesel production as well (**Table 2.6**). Tsigie et al. (2012b) have successfully enhanced the extraction of neutral lipids from *Y. lipolytica* with sub-critical water pretreatment. Katre et al. (2012) have demonstrated the properties of biodiesel produced with *Y. lipolytica* cultivated on glucose to fulfill the standards for biodiesel. Leiva-Candia et al. (2015) evaluated the biodiesel characteristics of biodiesel produced from lipids of yeast cultivated on sun flower meal, and they found a cold filter plugging point to be better for yeast derived biodiesel than palm oil derived biodiesel.

According to the review by Sitepu et al. (2014), the production of yeast lipids for oleochemical and nutritional uses has been studied on a pilot scale and a commercial scale since the 1950s, while few studies on yeast biodiesel have been reported; however, recently, Santomauro et al. (2014) cultivated the yeast *Metschnikowia pulcherrima* in 500 L raceway ponds with an optimized synthetic medium. The cultivation system and culture medium were non-sterilized, the temperature was roughly 21 °C, the culture was pH 3-4, and the incubation time was 15 days. The final biomass concentration was 2.06 g/L, and the lipid content was 34 wt% (Santomauro et al., 2014). Additional studies with different oleaginous yeasts and real wastewaters are definitely necessary before yeast lipid based diesel can be commercialized.

Table 2.6. *Lipid productions of various oleaginous yeasts using different wastewaters.*

Strain	Substrate	Cultivation	T (°C)	pH	t (h)	Biomass (g/L)	Lipid content (wt%)	Reference
Cryptococcus sp.	corncob hydrolysate	flaks	25	6	96	12.6	60	Chang et al., 2015
Cryptococcus curvatus	distillery wastewater	flask	30	-	144	5.2	25	Gonzalez-Garcia et al., 2013
Cryptococcus curvatus	cheesem whey	flask	30	-	36	7.2	65	Seo et al., 2014
Cryptococcus curvatus	crude glycerol	one-stage fed-batch	30	5.5	288	31.2	44	Liang et al., 2010
Cryptococcus curvatus	crude glycerol	one-stage fed-batch	30	5.5	288	32.9	52	Liang et al., 2010
Cryptococcus curvatus	lactose/fish oil	flaks	30	5.4	24+72	18.4±1.3	50	Iassonova et al., 2008
Cryptococcus curvatus	lactose/clarinol	flask	30	5.4	24+72	16.1±1.0	69	Iassonova et al., 2008
Cryptococcus curvatus	organic brewery waste	flaks	28	-	36	50.4	38	Ryu et al., 2013
Cryptococcus curvatus	pretreated sun flower meal	3.6L bioreactor	28	6	142	34.6	53	Leiva-Candia et al., 2015
Cryptococcus curvatus	prickly-pear juice	fermentor	30	5.5	35	11	46	Hassan et al., 1994a
Cryptococcus curvatus	sorghum bagasse hydrolysate	flask	30	-	72	5.6	-	Liang et al., 2012a
Cryptococcus curvatus	sweet sorghum bagasse with lime	flask	30	-	120	10.8	43	Liang et al., 2012b
Cryptococcus curvatus	sweet sorghum bagasse without lime	flask	30	-	120	15.5	38	Liang et al., 2012b
Cryptococcus curvatus with algae	food waste/municipal wastewater	-	25	6	144	7.5	29	Chi et al., 2011
Lipomyces starkeyi	dried sweet sorghum	flask	30	6	192	6.4	30	Matsakas et al. 2014
Lipomyces starkeyi	fishmeal wastewater	-	-	-	144	17.6	-	Huang et al., 2013
Lipomyces starkeyi	potato starch wastewater	flask	30	-	96	2.6	8.9	Liu et al., 2013
Lipomyces starkeyi	sewage sludge	-	-	5.0	240	-	51-72	Angerbauer et al., 2008
Rhodosporidium diobovatum	waste glycerol	7L bioreactor	30	5.5	168	13.6	51	Munch et al., 2015
Rhodotorula glutinis	distillery wastewater	flask	30	-	144	6.1	27	Gonzalez-Garcia et al., 2013
Rhodotorula glutinis	molasses/glucose/sucrose	fermentor	30	4	120	17.2	39	Johnson et al., 1995
Rhodotorula glutinis	molasses/glucose/sucrose	fermentor	30	4	120	25	42	Johnson et al., 1995
Rhodotorula glutinis	monosodium glutamate wastewater	flask, fermentor	30	5.5	72	25	20	Xue et al., 2008
Rhodotorula glutinis	thin stillage+crude glycerol	5L fermentor	24	5.1	96	14.8	37	Yen et al., 2012
Rhodotorula glutinis with algae	food waste/municipal wastewater	-	25	6	144	5.2	20	Chi et al., 2011
Rhodotorula mucilaginosa	cassava starch	fed-batch	28	6	120	21.8	53	Li et al., 2010
Rhodosporidium toruloides	bioethanol wastewater + glucose	flask	30	-	120	3.8	35	Zhou et al., 2013
Rhodosporidium toruloides	distillery wastewater	flask	25-35	3.8-8.3	72	8.1	44	Ling et al., 2013
Rhodosporidium toruloides	pretreated sun flower meal	3.6L bioreactor	28	6	142	37.4	51	Leiva-Candia et al., 2015
Trichosporon dermatis	buthanol fermentation wastewater	flask	28	-	120	7.4	13.5	Peng et al., 2013
Yarrowia lipolytica	defatted rice bran hydrolysate	-	-	-	-	10.75	48	Tsigie et al., 2012a
Yarrowia lipolytica	Industrial derivative of animal fat	fermentor	28	6	120	15	44	Papanikolaou et al., 2002
Yarrowia lipolytica	Industrial glycerol	flask	-	-	-	8.1	43	Papanikolaou & Aggelis, 2002
Yarrowia lipolytica	Industrial lipids/Glycerol	fermentor	28	6	240	8.7	40	Papanikolaou & Aggelis, 2002
Yarrowia lipolytica	rice bran	-	26	6.5	72	10.75	48	Tsigie et al., 2012a
Yarrowia lipolytica	sugarcane bagasse hydrolysate	-	-	-	-	11.42	59	Tsigie et al., 2011

2.4.2 Waste As a Nitrogen and Carbon Source for Oleaginous Microalgae

The high-rate algal ponds to treat municipal wastewater have been studied around the world since the 1950s (Golueke et al., 1957; Dodd, 1979). Dodd (1979) studied the cultivation of algae in a high-rate pond to treat animal wastewater and to produce a protein-rich microalgal biomass. Thus, using wastewater for microalgal biomass production is not a new invention, but the cost efficiency of these systems has so far not been high enough to produce low-value products, such as fuels and energy (Li et al., 2009). Several companies and institutions around the world have been working with the commercialization of microalgal biodiesel production. For example, these include Neste Oil, Ingrepo, and Biofuel Systems from Europe; Algae Tec and Aquaflow Binomics from Oceania; Pond Biofuels from Canada; and several companies from the US (Algenol Biofuels, Aurora Algae, BioProcess Algae, Cellana, Green Fuel Technology Corp with Arizona Public Service Company, LiveFuels, Imperium Renewables, PetroSun, Sapphire Energy, Shell Corp, Solazyme, and Solix) (Li et al., 2011; Singh & Gu, 2010; Sitepu et al., 2014).

The aquatic species program of the US Department of Energy reported autotrophic open ponds with cultures of *Spirulina* and *Chlorella* to be the most promising option for commercial biofuel production (Sheehan et al., 1998). Aquatic Energy, LLC has a patent for microalgal biomass production, harvesting, and processing (Demaris et al., 2011). Biomass was produced in clay-lined raceway ponds and lipid accumulation of microalgal cells induced in nitrogen limited ponds. In addition, Chinnasamy et al. (2010) have cultivated an algal consortium with pretreated carpet mill effluent and 250 ppm of additional nitrogen in four 950 L raceway ponds with a 10 L/min aeration containing 6% of CO_2. The 10-12 day cultivation average and the maximum biomass productivities were 2.64 g/m^2/d and 4.9 g/m^2/d, respectively. The algal consortium was rich in protein, but the lipid content was only 6.8 wt%. The consortium was rich in *Scenedesmus* sp. when a higher lipid content could be achieved by modifying the consortium to contain lipid-rich species and not protein-rich species. Their results demonstrate the suitability of using a native algae consortium in a carpet mill effluent to produce biodiesel. Genifuels Corporation has also been optimizing the microalgal growth conditions for efficient oil production (Oyler, 2008a). The company has also patented a system consisting of sequential photoautotrophic and heterotrophic algal growth stages for oil production (Oyler et al., 2008b). In the first stage of this system, algal biomass is produced in autotrophic conditions, and in the second stage, lipids are accumulated in heterotrophic conditions by adding sugar; however, the use of pure sugars to gain heterotrophic conditions instead of adding waste materials reduces the sustainability of the production process (Oyler, 2008a). Mulbry et al. (2008a/b) studied pilot-scale 30 m^3 algal turf scrubber raceways and compared the use of swine effluent and dairy effluent as a nutrient source for the benthic alga *Rhizoclonium hieroglyphicum*. A higher lipid content was obtained by using swine effluent (9.3 wt%), while the lipid productivity was higher with dairy effluent compared to swine effluent (21.3 g/m^2/d vs. 10.7 g/m^2/d). Their further studies have also demonstrated effective lipid extraction from the harvested algal biomass after drying (Mulbry et al., 2009). Although microalgae have been studied more than yeasts, microalgal SCO production still requires further studies before commercialization.

Table 2.7. *Lipid productions of various oleaginous microalgae in different wastewaters.*

Strain	Substrate	Cultivation	Time (d)	Biomass (g/L)	Lipid content (wt%)	Reference
Botryococcus braunii	carpet industry wastewater	1L flask	10	0.34-0.37	9.5-13.2	Chinnasamy et al., 2010
Chlamydomonas sp.	industrial wastewater (electronics)	6L photobioreactor	10	1.34	25.3	Wu et al. 2012a
Chlorella sp.	artificial seawater	1 L photobioreactor	6-7	0.5-2.0	66.1-32.6	Hsieh & Wu, 2009
Chlorella sp.	diluted digested dairy manure	250mL flask	21	1.5-1.7	9.0-13.7	Whang et al., 2010
Chlorella sp.	fermented liquid swine manure	250mL flask	7	0.5-1.6	27-30	Hu et al., 2012
Chlorella sp.	municipal wastewater	1L flask	9	0.4	15	Cho et al., 2011
Chlorella sp.	palm oil mill effluent	-	7	0.6-0.7	6.9-7.6	Hadiyanto & Nur, 2014
Chlorella minutissima	pretreated wastewater	5L plastic tray	12	0.8	28	Malla et al., 2015
Chlorella protothecoides	whey permeate (dairy by-product)	flask	9-10	7.3-17.2	20.5-49.9	Espinosa-Gonzalez et al., 2014
Chlorella pyrenoidosa	diluted primary piggery wastewater	1L flask	10	0.1-0.3	12-22	Whang et al., 2012
Chlorella saccharophila	carpet industry wastewater	1L flask	10	0.2	17-18.1	Chinnasamy et al., 2010
Chlorella sorokiniana	alcohol distillery wastewater	photobioreactor	4-5	10-12	20	Solovchenko et al., 2014
Chlorella vulgaris	artificial wastewater medium	2 L photobioreactor		0.7	42	Feng et al., 2011
Chlorella vulgaris	dairy wastewater (UV treated)	-		0.9-1.2	11.4-14.4	Qin et al., 2014
Chlorella vulgaris	dairy wastewater (chlorine treated)	-		1.3-1.9	10.3-11	Qin et al., 2014
Chlorella vulgaris	diluted/filtrated piggery wastewater	500 mL flask	30	1	28	Ji et al., 2013
Chlorella vulgaris	domestic wastewater	11L bioreactor	14	1.5	7.7	Sydney et al., 2011
Chlorella vulgaris	ethanol thin stillage	6 L reactor	4	9.8	43	Mitra et al., 2012
Chlorella vulgaris	industrial tofu wastewater	18L photobioreactor	9	0.8	23.2	Dianursanti et al., 2014
Chlorella vulgaris	monosodium glutamate wastewater	1L flasks	16	1.0	13.5-25.4	Ji et al., 2014
Chlorella vulgaris	piggery wastewater	-	20	0.5	29	Abou-Shanab et al., 2013
Chlorella vulgaris	soy whey	6 L reactor	4	6.3	11	Mitra et al., 2012
Chlorella vulgaris	synthetic medium with Glycerol	-	3	1.2	40.1	Heredia-Arroyo et al., 2011
Chlorella zofingiensis	autoclaved piggery wastewater	1L photobioreactor	10	3.0	33.3	u et al., 2013b
Chlorella zofingiensis	filtrated and autoclaved piggery wastewater	-	10	1.0-3.0	33.9-45.8	Zhu et al., 2013a
Chlorella zofingiensis	NaClO-pretreated piggery wastewater	1L photobioreactor	10	2.9	33.0	Zhu et al., 2013b
Chlorococcum sp.	dairy effluent + waste glycerol	500 mL flask	15	1.9	42	Beevi & Sukumaran, 2014
Desmodesmus sp.	industrial wastewater (electronics)	6L photobioreactor	10d	0.93	19.7	Wu et al., 2012a
Dunaliella tertiolecta	carpet industry wastewater	1L flask	10	0.28-0.38	12.2-15.2	Chinnasamy et al., 2010
Micractinium pusillum	different modification with BBM	250mL flask	17	0.61-1.28	31-41	Abou-Shanab et al., 2012
Micractinium reisseri	municipal wastewater + 3% Mine wastewater	250mL flask	15d	0.8	17	Ji et al., 2014
Nannochloropsis oculata	biologically treated swine wastewater	reactor	5	0.9-3.2	28-48.9	Wu et al., 2013
Pleurochrysis carterae	carpet industry wastewater	1L flask	10	0.33-0.37	11.8-12	Chinnasamy et al., 2010
Scenedesmus obliquus	brewery effluent	-	9	0.9	n.a.	Mata et al., 2012
Scenedesmus obliquus	BBM + 25 mM NaCl	-	20	0.65	34	Salama et al., 2013
Spirulina sp.	effluent from pig waste digestion & seawater	20 L bench-raceway	12	0.77	30	Olguín et al., 2001
Mixture of algae	carpet industry wastewater	950 L raceway pond	10	0.8-1.4	12	Chinnasamy et al., 2010

2.4.3 Co-Culture of Yeasts and Microalgae

Co-cultures of yeasts and microalgae appear to have even better potential for simultaneous wastewater treatment and biomass production than monocultures of yeasts or microalgae, and co-cultures are a novel way to enhance the biofixation of CO_2 and oil formation (Shu et al., 2013). Co-cultures have several synergistic advantages compared to cultures containing only one of these organism types. For example, microalgae can generate O_2 for yeasts, while yeasts provide CO_2 for the algae. The synergistic advantages of co-cultures also include the exchange of substrates and the adjustment of pH (Zhang et al., 2014).

Chi et al. (2011) first cultivated the yeasts *C. curvatus* and *R. glutinis* separately in the mixture of food waste and municipal wastewater, and in a later stage of cultivation, alga *C. sorokiniana* was added to both of the cultures. The final lipid content of the culture with *C. curvatus* and *C. sorokiniana* was 18.7% and 28.6% with *R. glutinis* and *C. sorokiniana* (Chi et al., 2011). Ling et al. (2014) studied a co-culture of the yeast *R. toruloides* and the microalga *C. pyrenoidosa* in flask experiments with a mixture of rice wine distillery wastewater and municipal wastewater. They did not adjust the pH or sterilize the wastewater mixture. After 5 days of incubation, the removal efficiencies of COD_s, phosphorus, and nitrogen were 95%, 51%, and 89%, respectively. They obtained a 63% lipid content and a 4.6 g/L lipid yield. (Ling et al., 2014.) The co-culture of the yeast *R. glutinis* and the microalga *C. vulgaris* improved the biomass yield almost 20% and the lipid yield over 70% compared to monocultures (Zhang et al., 2014). A 5 L photobioreactor co-culture of the yeast *R. glutinis* and the microalga *S. obliquus* increased the biomass production over 40% and the lipid production over almost 70% compared to single cultures (Yen et al., 2015). Similarly, the biomass and lipid productions improved with the co-culture of the yeast *R. glutinis* and the alga *S. platensis* (Xue et al., 2010). In addition to improved oil accumulation, the co-culture of the yeast *S. cerevisiae* and the alga *C. vulgaris* enabled a higher CO_2 fixation and a better oxidative stability of the final biodiesel product (Shu et al., 2013).

Another option to combine the microbial oil production of yeasts and microalgae is using the residual material after lipid extraction from the algae as a carbon and nutrient source for the yeasts. For example, Seo et al. (2015) found residual suspension from the acid-hydrolyzed hot-water extraction of biomass of the microalga *Nannochloropsis salina* to be suitable for growing the oleaginous yeast *Cryptococcus* sp. *Cryptococcus* sp. grew well in the suspension, and the final cell density was 2.25 g/L and the lipid content 23 wt% (Seo et al., 2015).

Several studies have demonstrated the potential of yeasts and microalgae for biodiesel production, but there is still a need to solve several issues before full-scale applications for commercial production can be constructed. For combined microbial oil production and wastewater treatment, the issues are related to the pretreatment of the waste material, the efficiency of lipid extraction, the efficiency of biodiesel production, maintaining the product quality, and the cost efficiency of biodiesel production (Kargbo, 2010; Siddiquee & Rohanim, 2011).

3 Aim of the Present Work

Microorganisms can accumulate lipids and utilize carbon and nutrients from wastewater, while phototrophic microorganisms may also use inorganic carbon from flue gases. Unfortunately, biodiesel and renewable diesel are not high-value products like cosmetics or pharmaceuticals; thus, microbial based transportation fuels still cannot economically compete with conventional fuels. Combining waste treatment and oleaginous biomass production could be an efficient solution for decreasing the production costs if oleaginous microorganisms can survive in the competitive wastewater environment. Therefore, this work attempts to answer the research question: "*Can indigenous microorganisms be used to produce oleaginous biomass for biofuel production by reusing carbon and nutrients from waste streams?"* The aim of this work was to sustainably produce oleaginous biomass by reusing carbon and nutrients from waste streams.

The main objectives of this work were:

- To find suitable wastewaters for oleaginous biomass production (**Papers I-IV**)
- To characterize the composition of different wastewaters focusing on their lipid composition and their possible use in the cultivation of oleaginous microorganisms (**Paper I**)
- To study the suitability of the indigenous microbial community to produce oleaginous biomass using wastewater as the source of carbon and nutrients (**Paper II**)
- To isolate oleaginous eukaryotes from wastewater and to compare their lipid production capacity with well-known oleaginous microorganisms (**Paper II**)
- To select a suitable microalgal strain for oleaginous biomass production (**Paper III**)
- To study the suitability of wastewater as a nutrient and carbon source for the cultivation of the selected oleaginous microalgae (**Paper III**)
- To study the effect of wastewater sterilization on oleaginous microalgal biomass production (**Paper IV**)
- To compare the lipid production of photosynthetic and heterotrophic microorganisms (**Papers II-IV**)

4 Materials and Methods

4.1 Wastewaters

Municipal wastewater and three different industrial wastewaters were studied as potential nutrient and carbon sources for microbial lipid production. The municipal wastewater (MWW) and the chemithermomechanical pulp mill wastewater (CTMP) originated from Finnish wastewater treatment plants, the palm oil mill effluent (POME) originated from Malaysia, and the piggery wastewater (PW) originated from Taiwan.

4.2 Microorganisms, Their Isolation, and Stock Culture Maintenance

A mixed culture of microorganisms was enriched from POME by a series of shake flask incubations (**Paper II**), and four eukaryotic strains (Strain 1-4) were isolated from this enrichment using four types of agar plates: a potato dextrose (PD), a universal medium for yeast (YM), a YM with 50 mg/Lchloramphenicol and a modified YM with xylan as a carbon source instead of glucose (**Paper II**). The compositions of the PD and the YM were as described in **Table 4.1**. In addition to the isolated yeast and fungi species, three yeast strains (*Yarrowia lipolytica* DSMZ8212, *Cryptococcus curvatus* DSMZ70022, & *Cryptococcus albidus* DSMZ70197) were ordered from the culture collection to compare their biomass and lipid production capacity to those of the isolated strains (**Paper II**). Three microalgal species isolated from a Southern Taiwanese freshwater area (*Chlorella sorokiniana* CY1, *Chlorella vulgaris* CY5, and *Chlamydomonas* sp. JSC-04) were also studied (**Paper III**).

The POME enrichment culture was maintained by transferring an aliquot of it into fresh non-sterilized POME once a week (**Paper II**). The strains isolated from POME and ordered from the culture collection were maintained in the YM, but their growth and lipid production ability was studied in a synthetic GA medium (**Table 4.1**). The microalgal species were maintained in BG-11

agar plates, the compositions of which were as described in **Paper III** and **Table 4.2**, which also describes the composition of BBM used for microalgal experiments. Prior to the cultivation experiments, the microalgal strains were pre-incubated in a liquid BG-11 medium for several days to obtain a high enough initial biomass concentration for the experiments.

Table 4.1. *Composition of the various growth media used in the cultivation of the yeasts and fungi.*

Component	YM (g/L)	PD (g/L)	GA (g/L)
agar*	15	15	
dextrose		2	
glucose	10		40
malt extract	3		
peptone (soybeans)	5		
potato infusion		200	
$(NH_4)SO_4$			1
$MgCl_2 \cdot 6H_2O$			0.83
K_2HPO_4			1
KH_2PO_4			0.5
$CaCl_2 \cdot 2H_2O$			0.2
yeast extract	3		2.5

*added only for solid medium

Table 4.2. *Microalgal growth media compostions.*

Component	BBM (g/L)	BG-11 (g/L)
agar*	15	15
K_2HPO_4	0.075	0.04
KH_2PO_4	0.175	
$NaNO_3$	0.25	1.5
NaCl	0.025	
$C_6H_8O_7$		0.006
$NaCO_3$		0.02
$MgSO_4 \cdot 7H_2O$	0.075	0.075
$CaCl_2 \cdot 2H_2O$	0.025	0.036
EDTA	0.05	0.001
KOH	0.031	
$C_6H_8FeNO_7$		0.006
$FeSO_4 \cdot 7H2O$	0.00498	
H_2SO_4	10 mL	
H_3BO_3	0.01142	2.86
$ZnSO_4 \cdot 7H_2O$	0.001412	0.222
$MnCl_2 \cdot 4H_2O$	0.000232	1.81
$CuSO_4 \cdot 5H_2O$	0.000252	0.079
$Ca(NO_3)_2 \cdot 6H_2O$	0.00008	
$Co(NO_3)_2 * 6H_2O$		0.049
$Na_2MoO_4 \cdot 2H_2O$	0.000192	0.39

*added only for solid medium

4.3 Cultivation Experiments

The experiments conducted with the yeasts and fungi are summarized in **Figure 4.1** and explained in more detail in **Paper II**. The experiments involving microalgae are summarized in **Figure 4.2** and explained in more detail in **Papers III** and **IV**.

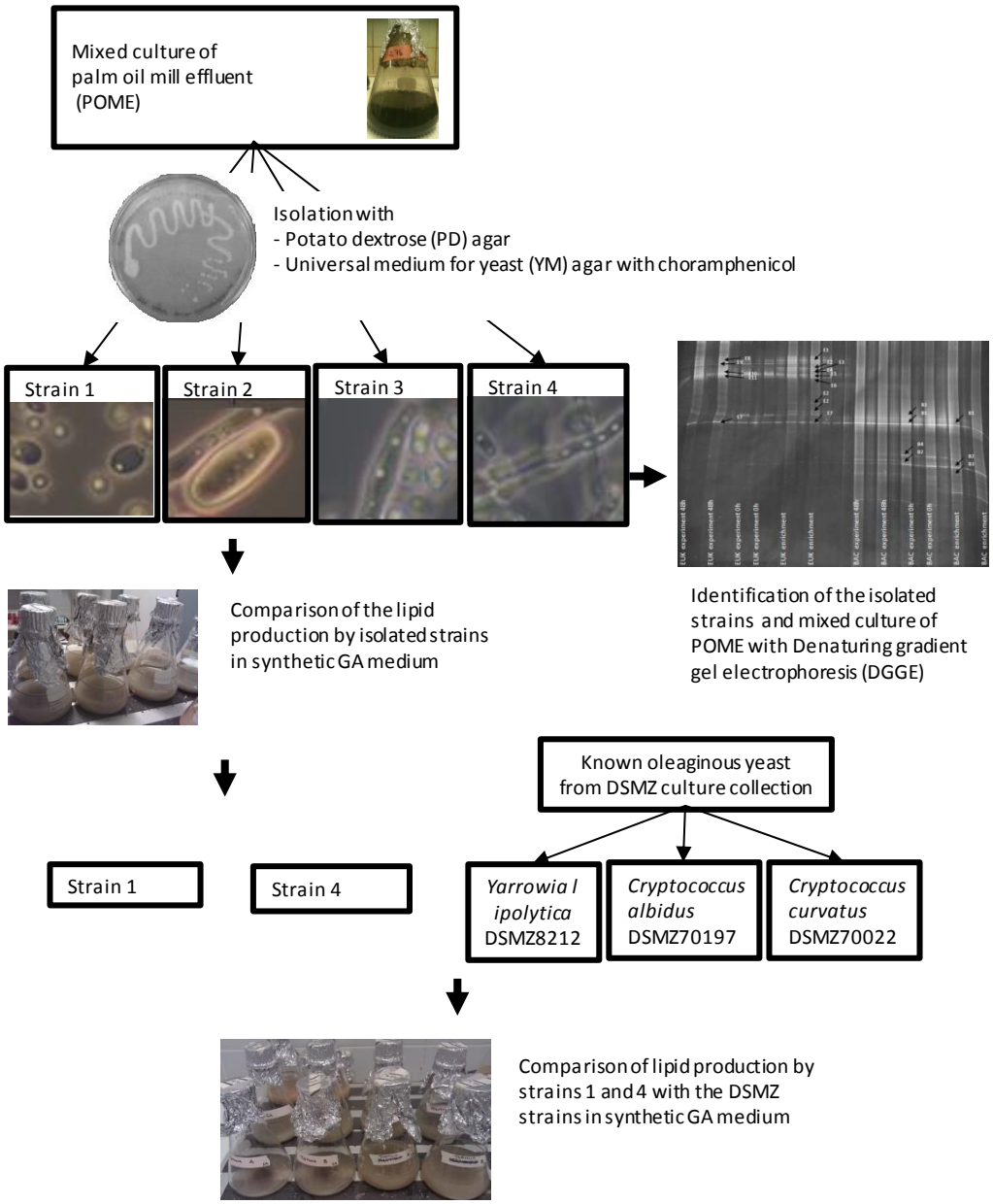

Figure 4.1. *Flowchart of the experiments conducted with the yeasts and fungi.*

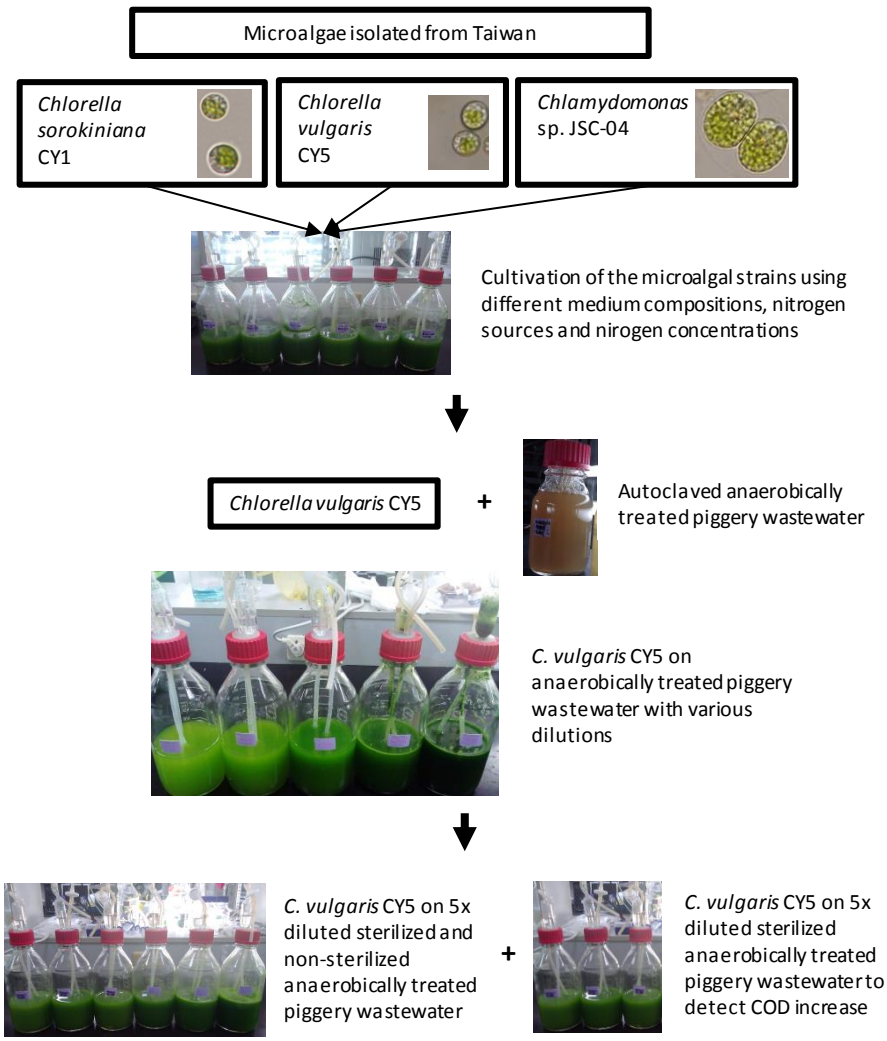

Figure 4.2. *Flowchart of the experiments conducted with the microalgae.*

4.4 Measurements

Due to the different laboratory facilities, the measurements for the experiments conducted with the yeasts and fungi in Finland were mainly different from the analyses used to characterize the microalgal cultivations in Taiwan. The methods and instruments used are summarized in **Table 4.3**.

Table 4.3. *Methods and instruments used in this work.*

Analysis	Method for the experiments with yeasts & fungi (Papers I & II)	Method for the experiments with microalgae (Papers III & IV)
	Physicochemical and instrumental analyses	
pH	WTW pH330i meter with WTW SenTix 41 electrode	PB-10 pH meter with pH/ATC electrode
Dissolved oxygen	WTW Oxi 330i meter with CellOX 325 electrode	
Light intensity/Irradiance		LI-250 light meter with LI-190SA pyranometer sensor
Biomass	Determination of total residue and total fixed residue in water, sludge and sediment & Freeze-drying (SFS3008, 1990)	Kett Infrared Moisture Determination Balance FD-720 & Optical density at waveleght 680 nm
Biological oxygen demand (BOD)	Determination of biochemical oxygen demand (BOD) of water, Dilution method (SFS 3019, 1979)	
Chemical oxygen demand (COD)	Determination of chemical oxygen demand (COD_{Cr}) in water with closed tube method, Oxidation with dichromate (SFS 5504, 1988)	Standard method 5220C: Closed Reflux, Titrimetric Method (APHA, 2012)
Total dissolved carbon (TDC)		LiquiTOC II, standards $KHC_8H_4O_4$ & Na_2CO_3 (**Paper IV**)
Soluble nitrogen (N_s)	Hack Lange LCK 238 kit	
Total Kjehldahl nitrogen (TKN)		Standard method 4500-N(org)C: Organic Nitrogen/Semi-Micro-Kjeldahl (APHA, 2012)
Total nitrogen (TN)		LiquiTOC II, $(NH_4)_2SO_4$ and KNO_3 as standards (**Paper IV**)
Total ammonium		TKN & LiquiTOC II, $(NH_4)_2SO_4$ as a standard
Nitrate	Dionex DX-120 with IonPac™ AG23	Optical density at wavelength 220 nm
Nitrite	Dionex DX-120 with IonPac™ AG23	ICS-3000 with IonPac™ AS14
Soluble phosphorus	Hack Lange LCK 349 kit	
Phosphate	Dionex DX-120 with IonPac™ AG23	ICS-3000 with IonPac™ AS14
Chloride	Dionex DX-120 with IonPac™ AG23	ICS-3000 with IonPac™ AS14
Lipids	Gravimetric method with modified Blight and Dyer extraction (Santala et al., 2011) & Chromatographic method with direct trans-esterification (Su et al., 2007)	Chromatographic method with direct trans-esterification (Su et al., 2007)
Lipid composition	Thin layer chromatography, silica plate with n-hexane:diethyl ether:acetic acid (80:20:2 v/v/v) (**Paper I**)	
Total sugars		Phenol–sulfuric acid method (Dubois et al., 1956)
Sugar content		Modified quantitative saccharification, ICS-5000 with CarboPac SA10G (**Paper III**)
Hydrolyzation of polysaccharides		Heated in trifluoroacetic acid and neutralized with $CaCO_3$ (**Paper III**)
Proteins		HCl hydrolysis (Černá, 2011) ICS-5000 with AminoPac™ PA10 (Ho et al., 2014)
Phase contrast microscope	Zeiss Axioskop 2	Nikon ECLIPSE 80i & Nile Red -staining
	Microbiological analyses	
DNA extraction	PowerSoil™ DNA isolation kit	
primers for polymerase chain reaction	prokaryotes: GC-BacV3f & 907r (Muyzer et al., 1993) & eukaryotes: Euk1A & Euk516r-GC (Díez et al., 2001) with temperature program described by Koskinen et al. (2007)	
Denaturing gradient gel electrophoresis	INGENYphorU2x2 (Koskinen et al., 2007)	
Sequence identification	Bioedit & BLAST softwaves with GenBank database	

5 Results and Discussion

5.1 Possible Carbon and Nutrient Sources for Microbial Lipid Production

The characteristics of the MWW, CTMP, and POME (**Table 5.1**) were rather similar to those reported in the literature (Karttunen et al. 2004; Lam & Lee, 2011; Bajpaj 2000). The PW was anaerobically pretreated, so it was less concentrated than the piggery effluents described in the reviewed literature (Ji et al., 2013; Whang et al., 2012).

Table 5.1. *Composition of the municipal wastewater (MWW), chemithermomechanical pulp mill wastewater (CTMP), palm oil mill effluent (POME), and anaerobically pretreated piggery wastewater (PW) used in this work (**Papers I** & **III**).*

	MWW	CTMP1	CTMP2	POME	PW	autoclaved PW
pH	7.3	7.3	7.8	5.1	7.7	9.8
BOD_7 [2] (mg/L)	120	3 600	3 500	40 000	n.a. [1]	n.a. [1]
COD_{tot} [3] (mg/L)	220	7 600	8 800	43 000	332	377
COD_s [3] (mg/L)	120	7 000	6 400	19 000	298	308
N_s (mg/L)	50	30	30	400	n.a. [1]	n.a. [1]
TKN [4] (mg/L)	n.a. [1]	n.a. [1]	n.a. [1]	n.a. [1]	348	287
NO_3^- (mg/L)	n.a. [1]	n.a. [1]	n.a. [1]	n.a. [1]	5.5	7.1
P_s (mg/L)	3	5	5	100	n.a. [1]	n.a. [1]
PO_4^- (mg/L)	n.a. [1]	n.a. [1]	n.a. [1]	n.a. [1]	101.4	28.4
Cl^- (mg/L)	n.a. [1]	n.a. [1]	n.a. [1]	n.a. [1]	105	106
Lipid content (wt%)	9.3±1.4	18.5	12.4	19.6±0.8	n.a. [1]	n.a. [1]
Lipid concentration (g/L)	0.021±0.002	0.23	0.17	8.4±1.2	n.a. [1]	n.a. [1]

[1] n.a. = not analyzed; [2] BOD_7 = Biological oxygen demand with 7 days incubation; [3] COD_s/COD_{tot} = soluble-/total chemical oxygen demand; [4] TKN = total Kjehldahl nitrogen

POME had the highest COD and BOD contents, whereas POME and PW had the highest nutrient concentrations of the studied wastewaters (**Table 5.1**). A high C/N ratio can been used to enhance the *de novo* lipid accumulation of yeasts, as mentioned in chapter 2.3.1. C/N ratios of 20-160 have been reported to be the most optimal for efficient lipid accumulation (Angerbauer et al., 2008; Hassan et al., 1996; Matsui et al., 2011; Papanikolaou & Aggelis, 2002; Papanikolaou et al., 2001, Zhu et al., 2008). The COD_s/N_s ratios of the wastewaters were 2 g/g for MWW, 200 g/g for CTMP,

and 50 g/g for POME. The COD_{tot}/TKN ratio for PW was 1.3 g/g. Due to the high C/N ratios, POME and CTMP are likely more suitable for microbial lipid production with yeasts than MWW or PW. No additional chemicals were used in the palm oil production (Hassan et al., 2014), indicating that POME does not contain any microbial inhibitors, so it should be a very promising organic substrate for microorganisms. CTMP wastewater, on the other hand, may contain compounds (such as pinosylvins and flavonoids) that inhibit biological functions (Rintala & Puhakka, 1994). Based on this, POME is more suitable for microbial lipid production than CTMP wastewater. In addition, there is a need to develop POME treatment because the commonly used open pond systems do not meet the current discharge limits, and they release methane into the atmosphere (Gobi & Vadivelu, 2013).

The lipid content and TAG concentration of POME were much higher compared to the lipid content of MWW and CTMP, as shown in **Figure 5.1**. The lipids of PW were not studied. The compounds present in wastewater may originate from the production process generating the wastewater, or they can be intermediate or final metabolic products of the microorganisms present in the wastewater. The identification and quantification of these compounds is essential in enabling the efficient separation and utilization of the compounds for the production of valuable commodities (Revellame et al., 2012).

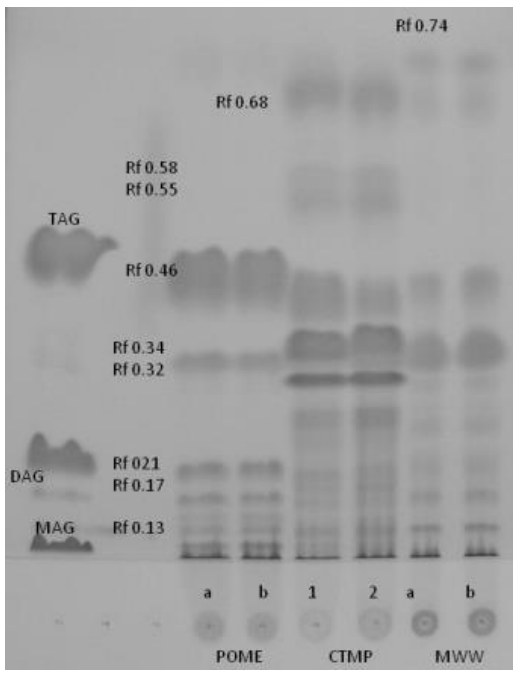

Figure 5.1. *Lipid composition of POME, CTMP, and MWW samples on thin layer chromatography (TLC, silica plate with n-hexane – diethyl ether – acetic acid (80:20:2 v/v/v) with iodine staining). (**Paper I**)*

POME contained mainly TAGs and some FFAs, DAGs, MAGs, and trace quantities of steryl esters (**Figure 5.1**). The lipid composition of POME was similar to palm oil (results not shown), but POME contained more MAGs and sterols. CTMP wastewater contained mainly FFAs and sterols and some TAGs, steryl esters, and possibly some FAME (Rf 0.58 & 0.55 in **Figure 5.1**). The lipids in CTMP wastewater are believed to originate mainly from the trees processed in the pulp mill. MWW contained a significantly lower concentration of TAGs than POME and CTMP. MWW also contained a larger fraction of FFAs and sterols, and possibly some steryl esters. In MWW, lipids are believed to originate from households, including kitchen waste, human excreta, cosmetics, and pharmaceutical products. Having the highest lipid content, POME would be more suitable for *ex novo* lipid accumulation than MWW and CTMP.

5.2 Isolation of Eukaryotes from Wastewater

Not only it is very difficult for oleaginous microorganisms to grow well in wastewater if the indigenous microorganisms of the wastewater are present, but it is also difficult to study mixed cultures of wastewater due to the complexity of the community (Daims et al., 2006). Indigenous microorganisms usually grow more efficiently in the competitive environments of unsterilized wastewaters compared to known oleaginous strains, which often originate from laboratory cultures and are not accustomed to competing with other organisms for substrate, nutrients, and space. For example, chemical and molecular analyses conducted by Hall et al. (2010) demonstrated that the microorganisms present in the wastewater outcompeted the bioaugmented oleaginous consortium used in their experiments. In this study, the experiments conducted using POME as a carbon and nutrient source and the POME enrichment culture as inoculum did not demonstrate an increase in lipid concentration; however, the micrographs (**Figure 5.2**) illustrated lipid inclusions within the relatively large cells, indicating that the eukaryotic organisms present in the mixed culture were able to accumulate lipids (**Paper II**). Therefore, the eukaryotes from POME were isolated using four types of agar plates: potato dextrose (PD), universal medium for yeast (YM), YM with chloramphenicol, and YM with xylan as a carbon source instead of glucose (**Paper II**). In further studies, it would be beneficial to also include a plate containing FAs as the carbon source to isolate microorganisms that have the potential for *ex novo* lipid accumulation.

The microbial community composition of the POME enrichment was complex, and the culture contained a large number of different bacteria and eukaryotes. Based on the micrographs, the eukaryotes appeared to have potential for lipid production. Therefore, only the eukaryotes identified from the POME culture enrichment are presented in **Table 5.2**. In total, four eukaryotic strains were successfully isolated from the POME enrichment with the agar plates. These four strains were identified as *Candida silvae* NRRL Y-6725 (with 100% similarity), *Galactomyces geotrichum* LMA-20 (with 99.8% similarity), *Lecythophora hoffmannii* CBS245.38T (with 96.7% similarity), and *Graphium penicillioides* JCM9300 (with 99.3% similarity) (**Paper II**). Of these strains,

only *C. silvae* was also detected in the initial POME enrichment shown in **Table 5.2**, indicating that the share of the other isolated strains was very low in the original culture.

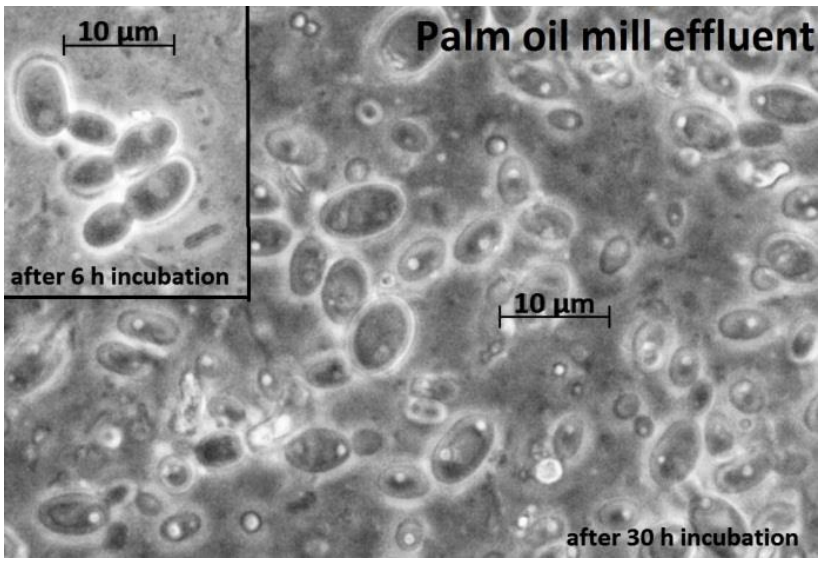

Figure 5.2. *A phase contrast microscope image of the mixed microbial community grown on POME. Lipid inclusions were formed inside the cells during the incubation, as no bright inclusions were seen inside the microorganisms at the beginning of the incubation (after 6 h of incubation), whereas many these types of inclusions could be seen inside the microorganisms after 30 hours of incubation. (**Paper II**)*

Table 5.2. *Selected eukaryotes identified from the POME enrichment with DGGE (**Paper II**).*

Sequence length	The closest match in GenBank database		
	Similarity	Affiliation	Class/family
465	100.0%	*Candida tropicalis*	Saccharomycetes/mitosporic Saccharomycetales
503	99.0%	*Hartmannella vermiformi*	Tubulinea/Hartmannellidae
461	90.0%	*Acanthamoeba hatchetti*	Tubulinea/Acanthamoebidae
495	88.9%	*Pichia occidentalis*	Saccharomycetes/Pichiaceae
407	88.7%	*Issatchenkia* sp.	Saccharomycetes/Saccharomycetaceae
473	97.0%	*Candida intermedia*	Saccharomycetes/mitosporic Saccharomycetales
522	100.0%	*Dimastigella trypaniformis*	Kinetoplastea/Bodonidae
472	88.3%	*Pichia jaroonii*	Saccharomycetes/Pichiaceae
501	90.4%	*Cyberlindnera jadinii*	Saccharomycetes/Phaffomycetaceae
461	99.8%	*Candida silvae*	Saccharomycetes/mitosporic Saccharomycetales
494	99.2%	*Pichia fermentans*	Saccharomycetes/Pichiaceae

5.3 Selecting a Yeast Strain for Lipid Accumulation

The isolated strains were cultivated in a GA medium to study their suitability for lipid production. The initial COD_s, N_s, and P_s concentration and the initial COD_s/N_s ratio in the GA medium were 60±4 g/L, 550±30 mg/L, 290±10 mg/L, and 100 g/g, respectively. In the first 7 day cultivation experiment including all four isolated strains, *C. silvae* and *G. penicillioides* showed higher lipid production, and their lipid contents per dry weight were above 15wt% (**Table 5.3**). Therefore, they were selected to be compared with the well-known oleaginous yeast strains (*Yarrowia lipolytica* DSMZ8212, *Cryptococcus curvatus* DSMZ70022, and *Cryptococcus albidus* DSMZ701097) in a longer 15-day cultivation experiment in a GA medium.

Table 5.3. *COD_s removal, biomass production, and lipid production of the strains isolated from POME compared to the known oleaginous strains (**Paper II**). The highest results are bolded.*

Species	Time (d)	COD_s (removal-%)	Biomass (g/L)	Lipids (wt%)	Lipids (g/L)	Lipids (mg/L/d)
Batch cultivation for 7 days incubation in GA medium						
C. silvae (Strain 1)	7	90.4	11.7±0.8	**15.8±1.9**	1.85	264
G. geotrichum (Strain 2)	7	86.0	10.92±0.5	7.4±1.4	0.81	116
L. hoffmannii (Strain 3)	7	95.0	13.0±0.5	9.4±1.3	1.22	174
G. penicillioides (Strain 4)	7	83.6	12.9±0.1	**18.4±1.0**	2.37	339
Batch cultivation for 15 days incubation in GA medium						
C. silvae (Strain 1)	15	89.8	7.7	17.9±0.1	1.38	92
G. penicillioides (Strain 4)	15	94.7	5.1	**29.1±3.0**	1.48	99
Y. lipolytica	15	93.8	13.5±0.8	15.7±3.4	2.12	141
C. curvatus	15	94.7	17.5±0.2	**20.2±2.9**	3.54	**236**
C. albidus	15	88.0	9.3±0.6	9.4±0	0.87	58

Increasing the incubation time increased the cellular lipid content of *C. silvae* and *G. penicillioides* (**Table 5.3**), but due to a lower biomass concentration, the lipid productivity was lower in the 15-day cultivation assay than in the 7-day cultivation assay. Both of the isolated strains contained more lipids than the oleaginous strains *Y. lipolytica* and *C. albidus*. *G. penicillioides* contained even more lipids per dry weight than *C. curvatus* (29.1±3.0% vs 20.2±2.9%), but the overall lipid concentration was lower due to a lower biomass concentration. With the oleaginous strains, the C/N ratio of 100 g/g may have already been too high for enhanced lipid accumulation. For example, Beopoulos et al. (2009b) reported *Y. lipolytica* to produce more citric acid and less lipids with C/N ratios of 70-100 g/g. In these experimental conditions (**Paper II**) of the known oleaginous strain, only *C. curvatus* contained more than 20 wt% lipids, which has generally been the limit for referring to a microorganism as oleaginous (Ratledge & Wynn, 2002).

5.4 Selecting a Microalgal Strain for Lipid Accumulation

Three microalgal strains isolated from a southern Taiwanese freshwater area were studied in a 1 L batch of photobioreactors (**Paper III**). The studied strains included *Chlorella sorokiniana* CY1, *Chlorella vulgaris* CY5, and *Chlamydomonas* sp. JSC-04. The biomass and lipid production of the strains were studied using different media (BBM or BG-11), nitrogen sources ($NaNO_3$, NH_4Cl, or $CO(NH_2)_2$/L at an initial concentration of 40 mg N per L), and nitrogen concentrations (63, 125, 250 or 500 mg N per L using $NaNO_3$). The results for these experiments are summarized in **Table 5.4**.

All of the strains produced more biomass in the nutrient-rich medium BG-11 than in the less nutrient-rich BBM. In the cultures with BBM, the nitrogen was exhausted after 3 days of incubation, while there was still some dissolved nitrogen left in the cultures with the BG-11 medium after 20 days of incubation. Normally, nitrogen limitation has been considered to enhance lipid accumulation in many organisms (Feng et al., 2012; Griffiths et al., 2012; Xin et al. 2010b), but in this study, the BG-11 medium with a higher nitrogen concentration enabled higher lipid productivities. On the other hand, in the experiment studying the effect of different nitrogen concentrations in BG-11, a higher lipid content per dry cell weight was generally obtained at lower nitrogen concentrations. It is well-known that salinity may also trigger lipid accumulation (Campenni et al., 2013; Chen et al., 2013; Mohan & Devi, 2014; Shah et al., 2014). In addition to nitrogen concentration, the only obvious difference between the two media was the higher sodium and chlorine concentrations in BG-11 than BBM. Thus, the higher salinity may have caused the higher lipid production in the medium with the higher nitrogen concentration. BG-11 contained 490 mg/L sodium and 260 mg/L chlorine, while Mohan & Devi (2014) showed that 1 g/L NaCl (equals 390 mg/L Na & 610 mg/L Cl) can be used to enhance microalgal lipid accumulation.

Changing the nitrogen source mainly affected the culture pH. The nitrate increased the culture pH, and the ammonium severely decreased the pH. The pH remained stable when urea was used (**Paper III**). Similar changes in pH with various nitrogen sources have been reported by Hulatt et al. (2012): an increase in pH was caused by OH^- production during nitrate uptake, a decrease in pH was caused by H^+ production during ammonium uptake, and no change in the pH was caused by the consumption of uncharged urea (Goldman & Brewer, 1980). In the current study, the microalgae did not grow on BBM with ammonium due to a severe decrease in the culture pH. Similarly, Hulatt et al. (2012) reported a collapse of *C. vulgaris* cultures due to a pH decrease after 3 to 4 days of cultivation when ammonium was used as the nitrogen source. Changing the nitrogen source from nitrate to urea did not affect the algal lipid content; however, it decreased the biomass production of the *Chlorella* species and increased the biomass production of the *Chlamydomonas* sp. Overall, the experiments with the three algal species studied in the synthetic medium demonstrated *C. vulgaris* CY5 to be the species with the most potential for lipid accumulation. Therefore, *C. vulgaris* was selected to be used in further wastewater studies.

Table 5.4. *Biomass and lipid production with C. sorokiniana CY1, C. vulgaris CY5, and Chlamydomonas sp. JSC-04 using different media, nitrogen sources, and nitrogen concentrations after 12 days of incubation. (**Paper III**). The highest results are shown in bold and highlighted by underlining.*

Inoculum	Medium	Biomass (g/L)	Lipid content (wt%)	Lipid productivity (g/L/d)	Main observations
Effect of medium					
CY1	BBM	2.5	32.3	69.8	More lipids with BG-11, with higher nitrogen concentration than BBM. BG-11 contains also more salts.
CY1	BG-11	**3.8**	**37.7**	**120.7**	
CY5	BBM	2.0	32.4	54.6	
CY5	BG-11	3.5	35.2	104.9	
JSC-04	BBM	2.4	22.9	45.9	
JSC-04	BG-11	3.4	22.9	66.7	
Effect of nitrogen source (initially 40 mg/L nitrogen)					
CY1	BBM & $NaNO_3$	2.5	32.3	**69.8**	With NH_4^+ pH decreased too low
CY1	BBM & urea	2.3	**33.7**	67.2	
CY5	BBM & $NaNO_3$	2.0	32.4	54.6	
CY5	BBM & urea	1.8	31.8	49.3	
JSC-04	BBM & $NaNO_3$	2.4	22.9	45.9	
JSC-04	BBM & urea	**2.8**	21.8	53.1	
Effect of nitrogen concentration (original BG-11 contained 250 mg N/L)					
CY1	BG-11 & 63 mg N/L	3.1	57.2	144.9	Mainly lower the nitrogen concentration, higher the lipid content.
CY1	BG-11 & 125 mg N/L	3.0	44.3	110.1	
CY1	BG-11 & 250 mg N/L	**3.8**	37.7	120.7	
CY1	BG-11 & 500 mg N/L	3.5	40.0	115.7	
CY5	BG-11 & 63 mg N/L	3.4	**57.5**	**161.8**	
CY5	BG-11 & 125 mg N/L	3.5	35.2	104.9	
CY5	BG-11 & 250 mg N/L	3.4	23.3	64.9	
CY5	BG-11 & 500 mg N/L	3.0	44.3	111.3	

5.5 Suitability of Wastewater for Lipid Production with Microalgae

An organic carbon source for microalgae is not as essential as it is for yeasts because microalgae can grow photosynthetically using inorganic carbon. Due to its high content of nutrients, anaerobically treated PW was studied as a nutrient source for microalgae. The wastewater contained mainly ammonium as a nitrogen source. Wang & Curtis (2015) stated that the pH imbalance in microalgal cultivations is not solely caused by nitrogen metabolism but also by a change in the heterotrophic carbon source. Due to the effect of the carbon source on pH, PW containing ammonium and organic carbon was used for microalgal cultivation, although *C. vulgaris* did not grow well in the previous experiment with ammonium as the sole nitrogen source (**Paper III**). First, an experiment with PW was conducted to study the suitability of sterilized anaerobically pretreated piggery wastewater for microalgal lipid production at various dilutions (1-20x). The aim was to determine the optimal initial nutrient concentration for microalgal lipid production and to eliminate the effect of possible inhibitory compounds. The culture pH was not adjusted initially; however, after a few hours, the cultures started to flocculate, and the microalgae appeared to be unhealthy. Therefore, the culture pH was adjusted to 6.6±0.4 after 4 hours of incubation. The

highest lipid production was obtained with 5x diluted wastewater (**Figure 5.3**). The toxicity of high ammonium concentration (Zheng et al., 2012b) might have caused the lag-phase at the beginning of the cultivation with the undiluted wastewater (**Figure 5.3c**). Due to a higher ammonium concentration and biomass production in less diluted wastewater (1x & 2x), the culture pH decreased more than in the cultures with more diluted wastewater (5x, 10x & 20x). The average lipid productivity from day zero to day 8 with 5x diluted wastewater was 101 mg/L/d, while in the other dilutions it was 59±19 mg/L/d. Unfortunately, the COD_s concentration increased with time, and therefore simultaneous wastewater treatment and lipid production in one step system appeared to be impossible. An increase in COD_s was caused by exocellular carbohydrates, as shown by the increase in the sugar concentration over time (**Figure 5.3b**).

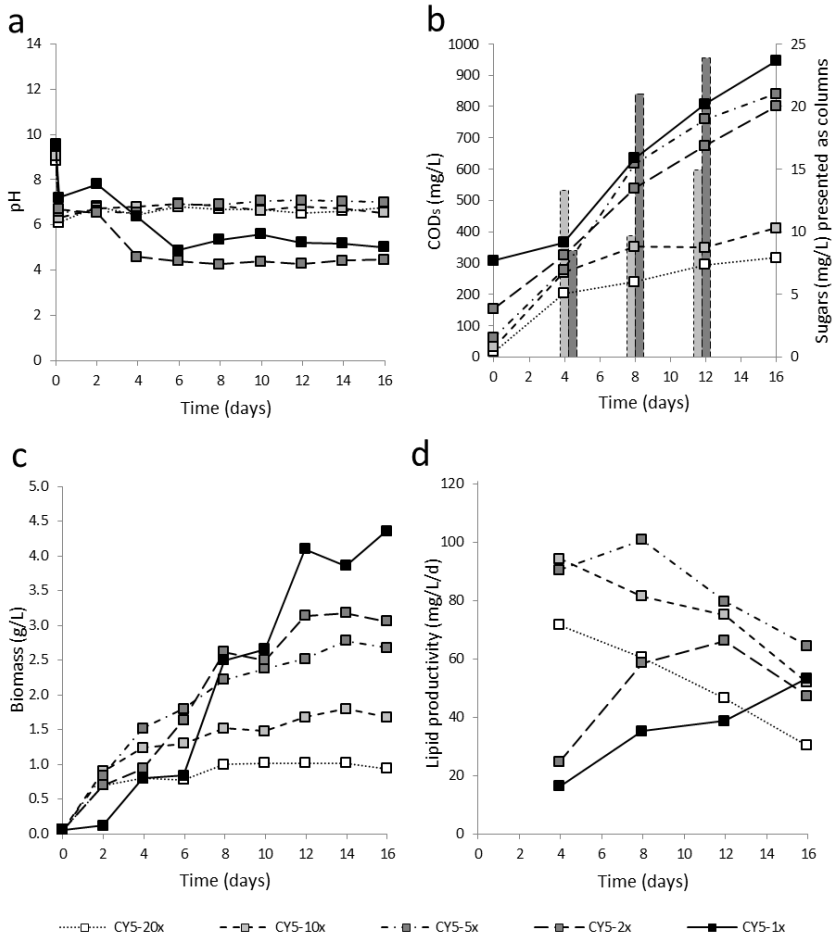

Figure 5.3. *Culture pH [a], COD_s and total sugars [b], biomass [c], and lipid productivity [d] of C. vulgaris CY5 cultivated in diluted (1-20x) and sterilized anaerobically treated piggery wastewater (**Paper III**).*

The sterilization of wastewater is not a cost-effective option in full-scale oleaginous microalgal biomass production, and therefore microalgae must be able to compete with the mixed population of microorganisms present in the unsterilized wastewater. In the second experiment with microalgae and wastewater, the effect of the wastewater microorganisms on the growth of *C. vulgaris* was studied by using sterile and non-sterile wastewater for cultivation. The number of microorganisms also affects competitiveness; therefore, increasing the number of microalgae by increasing the initial inoculum concentration was studied to determine whether or not it would benefit the growth of the microalgae in the competitive wastewater environment.

The wastewater pH increased when it was autoclaved, and the increase was likely caused by NH_3 gas formation from NH_4^+ ions (Karttunen et al., 2004), as the NH_4^+ concentration decreased after the wastewater was autoclaved. Due to the increase in wastewater pH after autoclaving, the pH of sterilized wastewater was adjusted to a neutral pH using H_2SO_4. The adjustment was conducted to minimize the effect of the initial pH on the cultivation results with sterilized and non-sterilized wastewater (**Paper IV**). With sterilized wastewater, the culture pH decreased below 4 after 1 day of incubation, while pH 4.5-8 has been reported to be optimal for the cultivation of *C. vulgaris* (Mayo & Noike, 1994). Therefore, the culture pH was adjusted above 4.5 with NaOH. The cultivations with non-sterilized wastewater did not require any pH adjustment, and these results indicate a possible decrease in chemical costs if the wastewater is not autoclaved; however, it would be useful to also study the growth of microalgae in the sterilized wastewater without any initial pH adjustment to verify whether or not that would eliminate the need to use chemicals for pH adjustment.

With sterilized and non-sterilized wastewater, the NH_4^+ was mostly consumed within the first 4 days (**Figure 5.4a & b**). The aim of wastewater treatment is to remove nutrients and carbon, but in this study with *C. vulgaris* cultivated in PW, the COD_s concentration increased with time. The experiment with non-sterilized wastewater was repeated with a 150 mg/L inoculum concentration in three parallel vessels to study the composition of the produced COD_s more carefully and to verify the results with non-sterilized wastewater. In three parallel vessels, a similar trend for the removal of nitrogen and an increase in the dissolved carbon concentration were detected (**Figure 5.5** vs. **Figure 5.4**). The DOC concentration increased with time, while the DIC concentration decreased (**Figure 5.5c**).

A healthy microalga naturally excretes extracellular DOC during photoautotrophic growth (Malinsky-Rushansky & Legrand, 1996), and the quantity and type of the released carbohydrates depends on the photosynthetic activity (Maksimova et al., 2004), general physiology and the taxa of the microalga. The effect of temperature and light intensity on the carbon excretion of phytoplankton has been known for some time (Zlornik & Dubinsky, 1989), and stressful conditions may enhance the release of extracellular carbohydrates. Stressful conditions can be caused by oxidative stress due to an inhibiting substance or a changing growth mode from mixotrophic to heterotrophic (Mohamed, 2008; Shipin et al., 1999). The initial concentrations of TDC, DOC, and DIC in anaerobically treated 5x diluted PW were 54.7±0.4 mg/L, 12.7±0.3 mg/L, and 42.0±0.1 mg/L,

respectively. Due to a low initial concentration of organic carbon in 5x diluted PW, the growth mode changed from mixotrophic to photoautotrophic rather quickly after beginning the experiment. The change in the growth mode is possibly one of the reasons that the algae began to excrete polysaccharides.

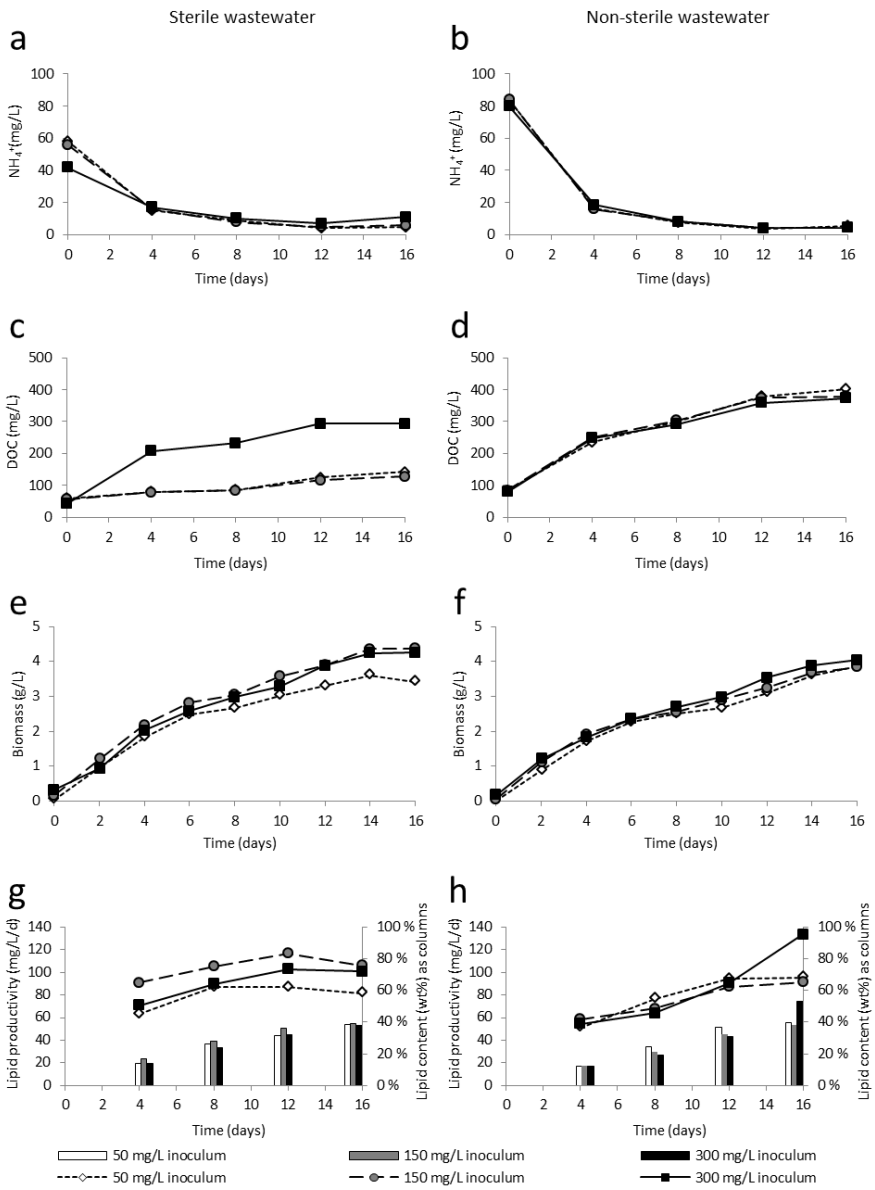

Figure 5.4. *NH_4^+ [a, b], DOC [c, d], biomass as dry weight [e, f] and lipid content and productivity [g, h] for Chlorella vulgaris CY5 cultivated in the sterilized and the non-sterilized 5x diluted anaerobically treated piggery wastewater (**Paper IV**).*

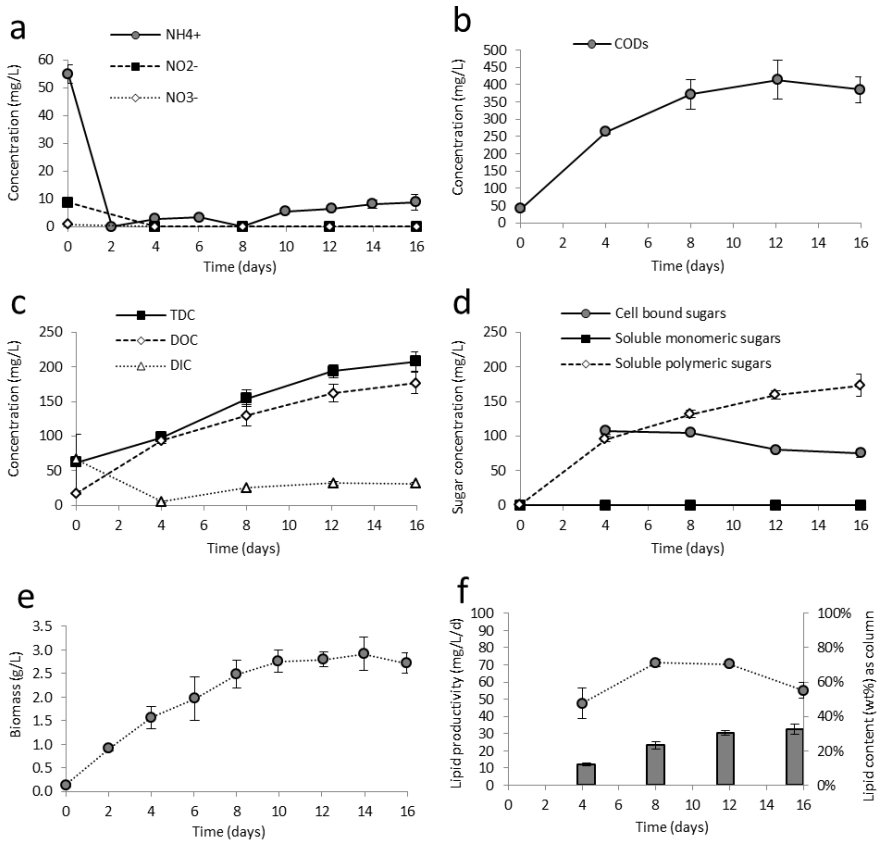

Figure 5.5. *Nitrogen [a], COD_s [b], dissolved carbon [c], total sugars (cell bound sugars, monomeric sugars, polymeric sugars [d], biomass as dry weight [e], and lipid content and productivity [f] for Chlorella vulgaris CY5 in non-sterilized 5x diluted anaerobically treated piggery wastewater. (**Paper IV**).*

As microalgae are assumed to produce approximately 2 g of biomass from 1 g of carbon (Chisti, 2007), less than 100 mg/L from the final biomass (2.7±0.2 g/L) was heterotrophically produced by *C. vulgaris,* and the rest of the algal biomass was produced autotrophically. In addition to the algal cells, the produced biomass also contained other microorganisms, which were naturally present in the unsterilized wastewater. Li et al. (2011b) studied nutrient removal and biodiesel production with *Chlorella* sp. in sterilized and non-sterilized highly concentrated MWW. In their control study with non-sterilized wastewater under illumination, the bacterial biomass production was approximately 10% of the biomass obtained with wastewater inoculated with algae. Therefore, it is assumed that most of the biomass in this study with unsterile wastewater was also a microalgal biomass.

Due to the increasing dissolved carbon content, measured as TDC and COD_s, the content of proteins and sugars in the liquid phase of the culture with *C. vulgaris* and PW was measured

(**Paper IV**). Only very low concentrations of proteins and monosaccharides were detected (results not shown). The samples were acid hydrolyzed to detect the polysaccharides, and the polysaccharide concentration was shown to increase with time. When the soluble sugars were converted to COD equivalents (Haandel & Lubbe, 2007), the increase of soluble sugars and measured COD_s were similar from the beginning of the incubation until day 12 (**Paper IV**). The ratio of different sugars did not vary during the incubation, and the polysaccharides consisted of 21±2% arabinose, 37±0.6% galactose, and 41±2% rhamnose (**Paper IV**). Other microalgae have also been reported to extract carbohydrates containing various sugars, including the aforementioned ones (Bafana, 2013; Maksimova et al., 2004; Villay et al., 2013).

The inoculum concentration did not have an effect on cultures with non-sterilized wastewater, and sterilization increased the biomass and lipid production (**Figure 5.4**). Similarly, Li et al. (2011b) obtained more biomass with *Chlorella* sp. cultivated on sterilized highly concentrated MWW than in the non-sterilized wastewater. In our three parallel vessels, similar trends in the increase of biomass and lipid production were detected, as in the experiment comparing sterilized and non-sterilized wastewater (**Figure 5.5** vs. **Figure 5.4**). When *C. vulgaris* was cultivated in non-sterilized wastewater, the cumulative average lipid productivity from day zero to day 4 was 47±9 mg/L/d and 71±2 mg/L/d from day zero to 8. The average daily lipid productivity between days 4 and 8 was as high as 95±12 mg/L/d. The lipid productivities were higher with sterilized wastewater, as shown in **Table 5.5**, while the lipid productivities with non-sterilized wastewater were also rather high. The difference in the lipid productivity between sterilized and non-sterilized wastewater was not significant, considering the high cost and energy demand of sterilization and the fact that the pH control was not required in the cultures grown in non-sterilized wastewater.

Table 5.5. *Cumulative and average daily lipid productivities (mg/L/d) of C. vulgaris cultivated in sterilized and non-sterilized piggery wastewater using various dilutions and inoculum concentrations. The highest results are bolded.*

Vessel		Cumulative daily lipid productivity (mg/L/d)				Average daily lipid productivity (mg/L/d)		
Wastewater	**Inoculum**	**4d**	**8d**	**12d**	**16d**	**4d-8d**	**8d-12d**	**12d-16d**
20x diluted sterile	**50 mg/L**	71.6	60.5	46.5	30.3	49.3	18.6	-18.3
10x diluted sterile	**50 mg/L**	94.2	81.3	75.1	51.9	68.5	62.7	-17.9
5x diluted sterile	**50 mg/L**	90.4	100.7	79.6	64.4	26.7	**121.66**	18.8
2x diluted sterile	**50 mg/L**	24.7	58.6	66.2	47.2	92.4	81.6	-9.9
1x diluted sterile	**50 mg/L**	16.3	35.1	38.7	53.4	53.9	45.9	97.3
5x diluted sterile	**50 mg/L**	62.9	86.7	87	81.7	110.4	87.6	65.7
5x diluted sterile	**150 mg/L**	90.8	105.4	116.8	106.3	83.1	**139.8**	74.8
5x diluted sterile	**300 mg/L**	70.9	89.6	102.8	100.6	108.3	129.1	94.2
5x diluted non-sterile	**50 mg/L**	51.3	76.7	94.4	95.3	102.1	130.0	97.8
5x diluted non-sterile	**150 mg/L**	58.8	68.2	87.5	91.3	77.6	126.0	102.7
5x diluted non-sterile	**300 mg/L**	54.3	63.8	90.4	113.6	73.3	143.4	263.5*
5x diluted non-sterile	**150 mg/L**	47.4±9.0	71.1±2.2	70.5±1.1	55.1±4.5	**94.9±11.6**	69.3±1.8	8.9±18.2

* Result unreliable due to unidentified peaks in lipid detection

The biomass concentrations and lipid contents obtained in this study with *C. vulgaris* in sterile and non-sterile wastewater were rather high compared to the literature, as shown in **Table 5.6**. Zhu et al. (2013a/b) have also obtained almost 3 g/L biomass concentrations and lipid contents above 30 wt% with *C. zofingiensis* in PW; however, their PW contained almost ten times more COD (3500 mg/L vs. 300 mg/L) and more than a two times lower nitrogen concentration (150 mg/L vs. 350 mg/L) compared to the PW used in this study. The PW used in this study was anaerobically pretreated, and therefore contained less COD. Zhu et al. (2013a) obtained the 3 g/L biomass concentration with 1.8 times dilution (1900 mg COD/L & 80 mg N/L). For the PW experiments presented in this work, the 5x dilution (70 mg TKN/L) was chosen, and the nitrogen concentration was similar. Zhu et al. (2013a) obtained a 110 mg/L/d lipid productivity with an initial 1900 mg COD/L concentration and 10 days of incubation, while 105 mg/L/d with 8 days of incubation and 117 mg/L/d with 12 days of incubation was obtained in this study. In the experiment by Whang et al. (2012), the initial COD concentration varied between 0.25-1 g/L, and no CO_2 was added. They obtained only a 0.1-0.3 g/L biomass after 10 days of incubation, so the lipid productivity with 12-22 wt% lipid content was only 6.3 mg/L/d; however, Whang et al. (2012) reported the removal of both nutrients and COD, indicating that the growth of the microalgae was heterotrophic.

Table 5.6. *Biomass concentrations and lipids contents obtained with Chlorella spp. cultivated in various wastewaters.*

Strain	Medium	Time (d)	Biomass (g/L)	Lipid content (wt%)	Reference
Chlorella sp.	Dairy manure (24000mgCOD/L, 3500mgN/L)	21	1.5-1.7	9-13.7	Whang et al., 2010
Chlorella sp.	Swine manure (7800mgCOD/L, 2100mgN/L)	7	0.5-1.6	27-30	Hu et al., 2012
C. vulgaris	Dairy wastewater (1500mgCOD/L, 97mgN/L)	4	0.9-1.9	10.3-14.4	Qin et a., 2014
C. vulgaris	Piggery wastewater (sterile)	12	3.9	36	**Paper IV**
C. vulgaris	Piggery wastewater (non-sterile)	12	3.2	32	**Paper IV**
C. vulgaris	Piggery wastewater (non-sterile)	12	2.8±0.2	30±1	**Paper IV**
C. vulgaris	Piggery wastewater (570mgTC/L, 60mgN/L)	20	0.5	29	Abou-Shanab et al., 2013
C. vulgaris	Piggery wastewater (840mgCOD/L, 510mgN/L)	30	1	28	Ji et al., 2013
C. zofingiensis	Piggery wastewater	10	1.0-3.0	33.9-45.8	Zhu et al., 2013a
C. zofingiensis	Piggery wastewater (3500mgCOD/L, 150mgTN/L)	10	2.9-3.0	33.0-33.3	Zhu et al., 2013b
C. pyrenoidosa	Piggery wastewater (11000mgCOD/L, 980mgN/L)	10	0.1-0.3	12-22	Whang et al., 2012

In the light dependent reactions of photosynthesis, algae use CO_2 to produce mainly carbohydrates, but fatty acids, amino acids, and organic acids can also be produced (Richmond,

2003). For optimizing the microalgal cultivation on wastewater for simultaneous wastewater treatment and lipid-rich biomass production, the growth conditions must be carefully considered. Nitrogen limited conditions can be obtained by diluting the wastewater, but it also decreases the carbon content, which affects the metabolism of microalgae (Wen & Chen, 2000). The solution to the problem could be to use a two-phase process, which consists of separate oleaginous biomass production steps and wastewater treatment steps or using other microorganisms with the microalgae to remove COD from the wastewater.

Although, the highest cumulative daily lipid productivity from day zero to day 8 with microalgae *C. vulgaris* in non-sterilized wastewater was 71.1±2.2 mg/L/d, with the yeast *G. penicillioides* in the GA medium, the cumulative lipid productivity from day zero to day 7 was 339 mg/L/d, which is almost 5 times higher. Using co-cultures of yeast and microalgae could be a possible solution. Co-cultures of yeasts and microalgae were also tested in this study, as explained in the next chapter (some of the results have not been previously published, and others have been published in conference proceedings and in a patent application).

5.6 Mixed Cultures in Wastewater for Lipid Accumulation

The suitability of MWW, CTMP, and POME for microbial *de novo* lipid production was studied using mixed microbial communities (Koskinen et al., 2013; Marjakangas et al., 2012; Marjakangas et al., 2013). The conditions for sufficient lipid production with activated sludge microorganisms in MWW and CTMP indicated that the lipid production process combining a mixed culture of microorganisms and real wastewater is difficult to optimize and requires further development (Laaksonen, 2011; Soini, 2012). Studies with POME demonstrated suitability for microbial lipid production, and therefore the patent application "*Process for producing lipids from palm oil production residues*" was prepared (Koskinen et al., 2013). However, lipid production process using POME also requires further development.

5.7 Co-Culture of Yeast and Microalgae in Wastewater for Lipid Accumulation

Although the presence of bacteria and other eukaryotes may distract the growth of the algae, in some cases, bacteria have been reported to enhance the growth of the algae (Cho et al., 2015; Natrah et al., 2014). In addition, using co-cultures of several microalgae have also increased the lipid production, due to the complementary use of substrates (Stockenreiter et al., 2010). *C. vulgaris* CY5 was cultivated separately with yeasts and fungi in 2x diluted wastewater in similar 1 L batch reactors with similar conditions as the other batch reactor studies presented in **Papers III** and **IV**. The yeast strain used in this assay was isolated from a culture containing *C. curvatus* and

fungi from a culture containing *G. penicillioides*. The strains were not identified after isolation, and therefore these isolated strains are referred to as yeastA and fungiA. The number of microalgal cells was calculated with a hemacytometer, and the algal biomass was calculated based on the assumption that one *C. vulgaris* cell weighs 25.7±0.28 pg (de-Bashan et al., 2005). The cultivation conditions used (light intensity 150 µmol photons/m^2/s, aeration 0.1 vvm with 2.5% CO_2, initial TDC 61±3 mg/L, and initial NH_4^+ 102±3 mg/L) favored the growth of phototrophic organisms, and therefore the produced biomass consisted mainly of microalgal cells, as shown in **Table 5.7**.

Table 5.7. *Cell count, biomass, and lipids for the experiment with a co-culture of C. vulgaris with the unidentified yeast and fungi in 2x diluted anaerobically pretreated piggery wastewater.*

Vessel	Cells count (10^6 cells/mL)	Calculated algal biomass (g/L)	Measured biomass (g/L)	Lipid content (wt%)	Cumulative average lipid productivity (g/L/d)
algae	n.c.*	n.c.*	3.1	25	47
algae + yeastA	131±23	3.4±0.6	3.5±1.4	33±2	73±35
algae + fungiA	87±28	3.1±0.4	3.6±0.3	26±3	59±11

*n.c. = not calculated

The biomass and lipid results shown in **Figure 5.6** demonstrated a variation between parallel bottles, and to make further conclusions from these results, *C. vulgaris* should be cultivated again with the yeastA and fungiA and more parallel bottles should be included. At the end of the incubation on day 16, the average values for biomass, lipid content, and lipid productivity were higher with the co-cultures compared to the culture inoculated with only *C. vulgaris* (**Table 5.7**). Chi et al. (2011) obtained 18.7 wt% lipid content with the yeast *C. curvatus* and the microalga *C. sorokiniana* and 28.6 wt% with the yeast *R. glutinis* and the microalga *C. sorokiniana* cultivated in a mixture of food waste and municipal wastewater. Their results are in the same range as obtained in this study. Also, many other studies have demonstrated an increase in the biomass concentration and lipid productivity when growing a co-culture of microalgae and yeasts instead of one of the organism types alone (Ling et al., 2014; Zhang et al., 2014; Yen et al., 2015; Xue et al., 2010).

For further studies, a two-step process with microalgae and other eukaryotes may also be an interesting option. In a two-step process, carbohydrates and lipids could be produced by microalgae using the nutrients from wastewater and the carbon from flue gases, while in the second step, for example, yeasts could be used to produce lipids from the nutrients and carbohydrates from the produced microalgal biomass (and wastewater). For instance, McCurdy et al. (2014) stated that the two-step process with yeasts and algae is a possible solution for the low biodiesel yields and rigorous processing issues in commercial production. Due to the high potential of the co-cultures, they should be further studied.

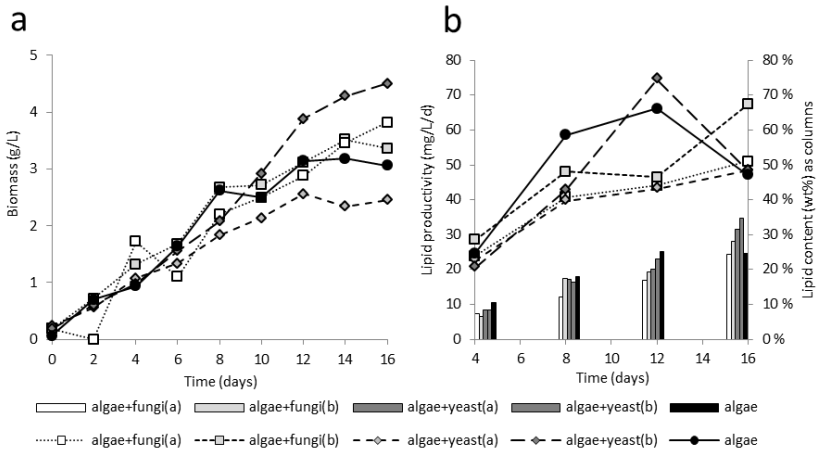

Figure 5.6. *Biomass [a], and lipid content and productivity [b] for the co-culture of algae separately with fungi and yeast in sterilized 2x diluted anaerobically treated piggery wastewater. Two parallel cultivations were conducted for algae separately with yeast and fungi (a & b), but only one cultivation for algae alone (**Paper IV**).*

5.8 Up-Scaled Reactor Experiments with Yeasts and Microalgae

The cultivation of yeasts and microalgae in somewhat larger bioreactors was also studied. The selected yeast ordered from the culture collection was cultivated in a 5 L (working volume 2 L) column reactor with aeration and the microalga *C. vulgaris* CY5 in a 50 L (working volume 35 L) column reactor with illumination and aeration with 5% CO_2 (***Figure 5.7***). An issue in these studies was the foaming of the wastewater cultivations. Microorganisms with very high lipid content float more easily because of the high buoyancy of the biomass caused by the low density of triglycerides (Eroglu & Melis, 2009). In the experiments with *C. vulgaris* and PW, aeration caused foaming in the wastewater, and most of the microalgal cells rose above the wastewater surface, which limited the nutrient availability for the algae and resulted in poor algal growth (**Figure 5.8**). *C. vulgaris* and the mixed microbial community present in the PW wastewater removed NH_4 from the PW, but the COD_s concentration increased with time in similar manner as in the smaller scale cultivations. Lipid content was less than 10% and it decreased with time. Cultures were taken over by some other microorganisms than algae after 4-6 days of incubation as the color of the cultivations changed from green to brown, and lipid content decreased remarkakby. Further studies should be conducted, and use of antifoaming agents, flocculants, or a biofilm-based cultivation system should be considered.

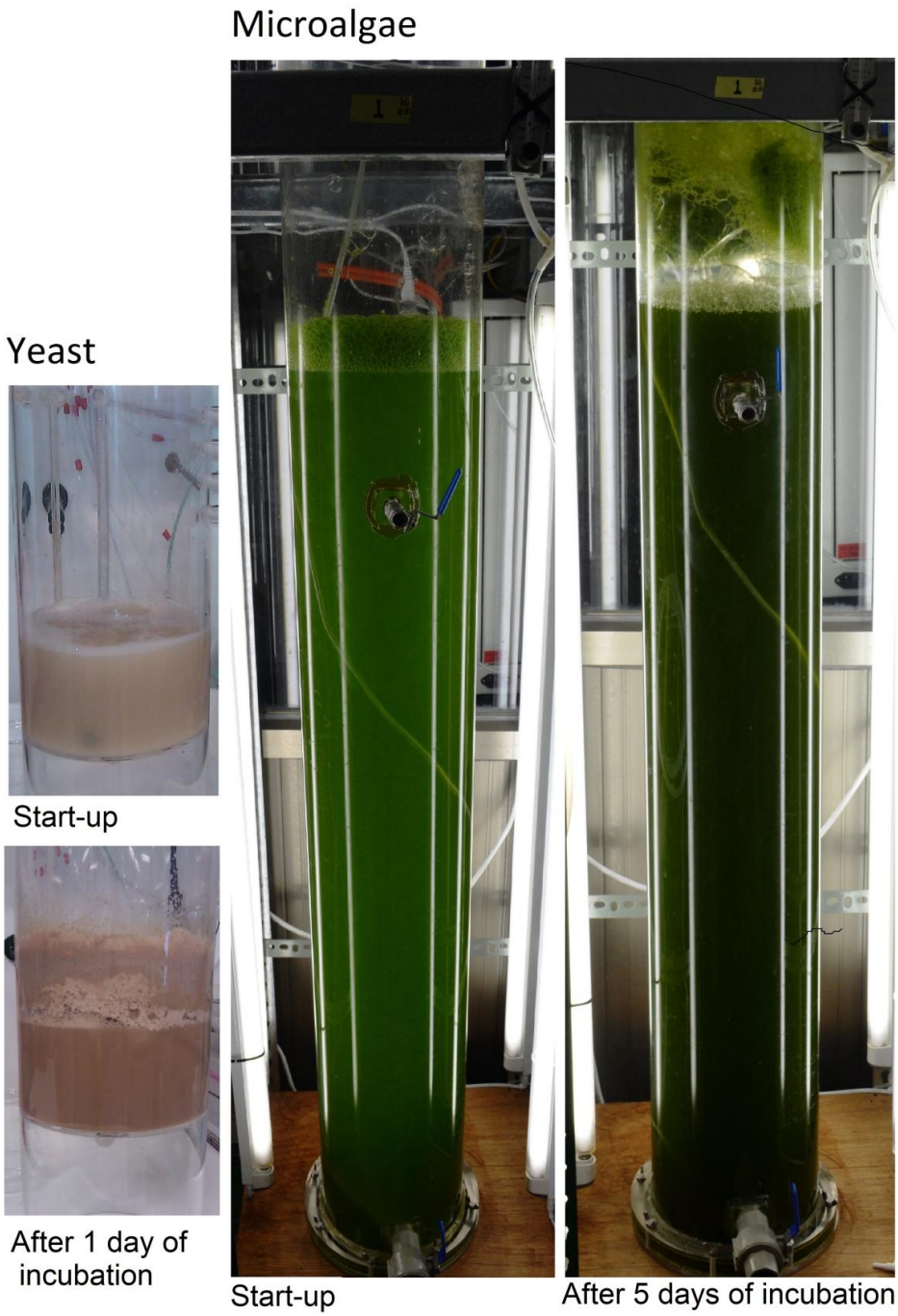

Figure 5.7. *Reactors used in the cultivation of the yeast and microalga on wastewater. Photographs demonstrate the initial situation and the situation after few days of incubation, when a part of the cells have risen above the wastewater surface due to the foaming.*

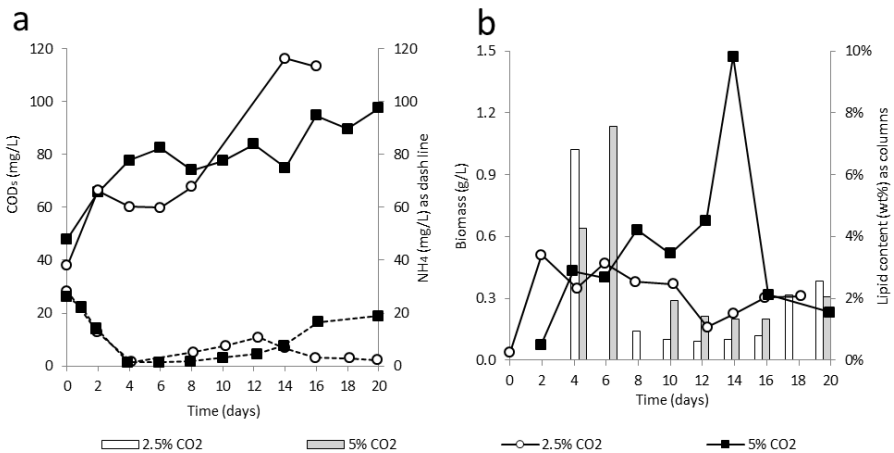

Figure 5.8. *COD_s and NH_4 concentrations (mg/L) [a], and biomass (g/L) and lipid content (wt%) [b] for the C. vulgaris and 5x diluted anaerobically treated piggery wastewater in the column reactor. Cultivations were conducted with 2% and 5% (v/v) CO_2.*

6 Summary and Conclusions

This work demonstrates that indigenous eukaryotic microorganisms can be used to produce oleaginous biomass for biofuel production by reusing the carbon and nutrients from wastewater. Due to a high C/N ratio and a lack of processing chemicals, POME was considered to be the carbon and nutrient source with more potential for oleaginous biomass production than MWW and CTMP (**Paper I**). In addition, POME had the highest TAG content, and TAGs are very good raw materials for biodiesel and renewable diesel production (**Paper I**).

It is difficult to cultivate oleaginous microorganisms efficiently in real wastewaters due to their complex chemical and microbiological nature; however, the diverse microbial community enriched from POME indicated the ability for lipid accumulation, as the presence of lipid-containing microorganisms was verified by microscopy observations (**Paper II**). Due to the high residual lipid content of POME, an increase in the lipid content and the concentration of the microbial biomass could not be demonstrated when the microbial culture enriched by POME itself was grown on POME (**Paper II**). Therefore, microorganisms isolated from POME were cultivated in a synthetic growth medium to study their biomass and lipid production abilities. In the studied conditions, the fungus *G. penicillioides* isolated from POME was able to accumulate more lipids than the well-known oleaginous yeast *C. curvatus* (**Paper II**). Oleaginous microorganisms accustomed to laboratory environments may have difficulties to grow in the competitive environment of real non-sterilized wastewaters, and therefore indigenous POME microorganisms with a lipid accumulation capability have an enormous potential in sustainable oleaginous biomass production. To the author's knowledge, the lipid accumulation potential of *G. penicillioides* has not been previously reported.

An advantage of microalgae compared to yeasts and fungi is their ability to use inorganic carbon sources. This enables the combination of wastewater and flue gas treatment. From the preselected oleaginous microalgal strains, *C. vulgaris* accumulated more lipids than *C. sorokiniana* or *Chlamydomonas* sp. (**Paper III**). For further studies, *C. vulgaris* demonstrated its suitability for sustainable oleaginous biomass production by using aerobically pretreated piggery wastewater as

a nutrient and carbon source in cultivations in which CO_2 is also added as an inorganic carbon source (**Papers III** & **IV**). The lipid productivity of *C. vulgaris* was also rather high when non-sterilized wastewater was used (**Paper IV**). Sterilization affected the chemical composition of the wastewater, and therefore a chemical pH adjustment was required in the cultivation of *C. vulgaris* in sterilized wastewater; however, no chemical pH adjustment was required for cultivating *C. vulgaris* and the indigenous wastewater organisms in non-sterilized wastewater (**Paper IV**). To the author's knowledge, the lipid accumulation potential of *C. vulgaris* in sterilized and non-sterilized wastewater has not been previously compared. During the photoautotrophic growth, the microalgae excreted polysaccharides, as *C. vulgaris* did in this study. This demonstrates that the cultivation conditions for simultaneous wastewater treatment and oleaginous microalgal biomass production must be optimized carefully to enable the efficient removal of dissolved carbon compounds, nitrogen, and phosphorus. This issue has not been sufficiently acknowledged in the field of microalgal biotechnology. Therefore, based on results of this study, it is recommended that the possible release of carbon should be measured in all of the experiments studying simultaneous microalgal lipid production and wastewater treatment.

In the preliminary unpublished co-culture studies, heterotrophic yeast or fungi with autotrophic microalgae had higher lipid content than microalgae alone. Based on the results obtained by growing either microalgae or yeasts alone, higher lipid contents but lower biomass concentrations were obtained with microalgae (**Papers III & IV**) than with yeasts (**Paper II**). Microalgae also extracted organic carbon compounds to their surroundings, and therefore more studies on using co-cultures of yeast/fungi and microalgae should be conducted to simultaneously produce oleaginous biomass and to meet wastewater treatment targets.

Further studies are still required to enable commercial cultivation of oleaginous microbial biomass in wastewaters for biodiesel and renewable diesel production. Oleaginous microorganisms can be found from wastewaters, but their lipid accumulation and growth ability in non-sterilized wastewater should be studied more. Co-culturing the yeasts, fungi, and microalgae could be a more promising option than the cultivation of any of these microorganisms alone. One option is to co-culture yeasts and microalgae in a one step process for simultaneous lipid production and waste treatment. Other option is to use a two step process. In the first step, microalgae could simultaneously remove CO_2 from flue gas and nutrients from the wastewater, and produce biomass containing hydrocarbons and lipids. Lipids could be extracted from the microalgal biomass and the biomass residue could be used as a carbon source for the second step of the process, in which yeasts simultaneously utilize the carbon from the wastewater and microalgal residue, and accumulate lipids. In this kind of system, the wastewater and flue gas would be valuable resources for cultivation of microorganisms instead of being waste, which requires costly treatment. Several issues should still be studied in the laboratory scale, but to be able to commercially produce microbial biodiesel or renewable diesel, lipid production studies on larger scale should be conducted. There might be some problems in larger scale, which have not been detected in laboratory, such as the foaming of the wastewater and microbial cultures. If we want to take a step towards more sustainable world, one

option is to conduct more research at different scales on the use of eukaryotes in wastewater for production of microbial biodiesel or renewable diesel.

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ORIGINAL PAPERS

I

LIPID PROFILE CHARACTERIZATION OF WASTEWATERS FROM DIFFERENT ORIGINS

by

Efimova, E., Marjakangas, J.M., Lakaniemi, A.-M., Koskinen, P.E.P. & Puhakka J.A., 2013

Water Science & Technology, Vol. 68, No. 11, pp. 2505–2514,
doi:10.2166/wst.2013.538

II

LIPID PRODUCTION BY EUKARYOTIC MICROORGANISMS ISOLATED FROM PALM OIL MILL EFFLUENT

by

Marjakangas, J.M., Efimova, E., Lakaniemi, A.-M., Koskinen, P.E.P., Chang, J.-S. & Puhakka J.A., July 2015

Biochemical Engineering Journal, Vol. 99, pp. 48–54,
doi:10.1016/j.bej.2015.03.006

Biochemical Engineering Journal 99 (2015) 48–54

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Lipid production by eukaryotic microorganisms isolated from palm oil mill effluent

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ARTICLE INFO

Article history:
Received 28 November 2014
Received in revised form 9 February 2015
Accepted 8 March 2015
Available online 11 March 2015

Keywords:
Filamentous fungi
Lipid accumulation
Microbial growth
Palm oil mill effluent
Physiology
Yeast

ABSTRACT

Microbial oil production combined with wastewater management is one option for a more sustainable future. Micrographs of microbial cultures enriched from palm oil mill effluent (POME) showed lipid inclusion in the eukaryotic cells, indicating the cells can accumulate lipids. However, enriching the culture did not increase the total lipids. Therefore, eukaryotic microorganisms were isolated from POME to investigate whether these microorganisms are potential lipid producers. Four strains were isolated, and their lipid synthesis capabilities were compared with known oleaginous yeasts in a synthetic oil-free medium. Two strains (identified as *Galactomyces geotrichum* and *Graphium penicillioides*) had the potential to accumulate lipid accumulation based on the increase in triacylglycerol content. *G. penicillioides* was the most promising strain for lipid production as this strain accumulated more lipids than the well-known oleaginous yeast *Cryptococcus curvatus* (29.1 ± 3.0 wt% vs. 20.2 ± 2.9 wt%). To our knowledge, oil synthesis and accumulation by *G. penicillioides* have not previously been reported.

1. Introduction

Due to the increasing global demand for energy, fossil fuels must be substituted, or their use must be combined with alternative energy sources [1]. In the future, the ideal wastewater treatment plant would conserve the energy content of wastewater into a useful form instead of consuming energy. For example, wastewater sludge is used as a source of biogas, electricity, or liquid biofuels [2]. One possibility is to use wastewater to produce oleaginous biomass, from which lipids can then be extracted and used to produce biodiesel or renewable diesel [3].

Several oleaginous microorganisms have been studied for lipid accumulation. Known oleaginous yeasts include genera such as *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodosporidium*, *Cryptococcus*, *Trichosporon*, and *Lipomyces* [4]. Oleaginous microorganisms typically accumulate more than 20% of their dry weight as lipids [5]. The occurrence and activity of oleaginous yeasts in wastewater environments have not been systematically studied.

The palm oil industry produces a large volume of effluents with high organic carbon content [6]. Palm oil mill effluent (POME) is a colloidal suspension containing water (95–96%), oil (0.6–0.7%), and solids (4–5%) [7]. The estimated palm oil production of the two largest palm oil producers, Indonesia and Malaysia, was 33.5 and 21.25 million tons in 2014, respectively [8]. Each ton of crude palm oil produces at least 2.5 t of POME [6]. Thus, the estimated POME production in Indonesia and Malaysia was at least 80 and 50 million tons, respectively. POME is biodegradable. Gobi and Vaidivelu [9] reported that POME is a potential substrate for microbial biomass production.

The objective of this study was to produce lipids to generate biofuel by utilizing the carbon and nutrients of POME. Our hypothesis was that the microorganisms that most efficiently use POME are found in it, due to the long-term selective enrichment in this environment. Lipid production was studied with a mixed culture of indigenous microorganisms enriched from POME and pure cultures of microorganisms isolated from this mixed culture. The biomass production and lipid accumulation ability of isolated strains were compared with known oleaginous yeasts

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http://dx.doi.org/10.1016/j.bej.2015.03.006
1369-703X/

(*Yarrowia lipolytica* DSMZ8212, *Cryptococcus curvatus* DSMZ70022, and *Cryptococcus albidus* DSMZ70197) in a synthetic medium. Polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) was used to characterize the microbial community composition of the POME enrichment culture and to identify the isolated strains.

2. Materials and methods

2.1. Palm oil mill effluent (POME) and culture enrichment

The POME used in this study originated in a Malaysian palm oil mill, and then was transported and stored frozen. The POME had a high organic matter load (38–39 g BOD/L, 43–50 g COD_{tot}/L) and contained a significant amount of lipids (19–20 wt% of total dry weight, 7.2–9.6 g/L) [10]. The soluble COD:N:P mass ratio of POME was 200:4:1 [10].

To increase the number of POME-degrading microorganisms, the microorganisms present in the POME were enriched with a series of shake flask incubations. The enrichment was conducted by incubating 100 mL of non-sterilized POME (which acted as the source of the microorganisms and the substrate) in 250 mL Erlenmeyer flasks on an orbital shaker at 250 rpm and 27 °C. Thereafter, 10 mL of enrichment culture was transferred once a week into 90 mL of fresh non-sterilized POME for several months. The microbial community composition of the culture enrichment was characterized with PCR-DGGE to detect possible oleaginous microorganisms.

2.2. Growth kinetics of indigenous organisms on POME

A batch experiment studying the ability of indigenous organisms to use POME and accumulate lipids was conducted in 250 mL Erlenmeyer flasks with 150 mL initial culture volume. The POME enrichment culture (10% v/v) was used as inoculum to grow a microbial biomass on the POME (90% v/v) at 27 °C and with 250 rpm mixing. The culture pH was not adjusted.

2.3. Isolation of eukaryotic microorganisms from POME

Eukaryotic microorganisms were isolated from the POME using four types of agar plates (potato dextrose [PD: 4 g/L potato extract = 200 g/L potato infusion, 2 g/L dextrose, 15 g/L agar], universal medium for yeast [YM: 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone from soybeans, 10 g/L glucose, 15 g/L agar], YM with chloramphenicol [50 mg/L] and modified YM [with xylan used as carbon source instead of glucose]) at 27 °C. An array of different agar plates was used to isolate as many different eukaryotes as possible. The agar plates were inoculated by taking 10 μL POME enrichment culture after 7 days of incubation with a sterile disposable plastic cultivation loop and spreading the culture on one quarter of the agar plate. To obtain single colonies, the sample was diluted with the streak plate method using a flame-sterilized cultivation loop on the three other quarters of the plate. Three replicate plates for each plate type were inoculated. The colonies on the plates were checked daily for 3 weeks. All dissimilar colonies were isolated and transferred to new similar agar plates (two separate colonies of each type on two separate agar plates). To confirm whether the colonies were bacteria or eukaryotes, the colonies were monitored with phase contrast microscopy. Eukaryotic colonies were transferred to new corresponding agar plates at least four consecutive times to ensure the purity of the strain.

2.4. Known lipid accumulating microorganisms used as reference

The following yeast strains were obtained from the culture collection of Deutsche Sammlung von Mikroorganismen und Zellkulturen GMBH (DSMZ): *Y. lipolytica* DSMZ8212, *C. curvatus* DSMZ70022, and *C. albidus* DSMZ70197. These strains, which accumulate high concentrations of lipids [4], were used as reference organisms for the isolated strains to study lipid production capability.

2.5. Biomass production and lipid composition of isolated strains

Four strains isolated from POME (Strains 1–4) were cultivated in batch mode to delineate their biomass and lipid production abilities. The strains were pre-incubated in YM at 27 °C on an orbital shaker at 250 rpm for 3 days. These cultures were used as inoculum for cultivation in GA medium (40 g/L glucose; 2.5 g/L yeast extract; 1 g/L $(NH_4)SO_4$; 0.83 g/L $MgCl_2{\cdot}6H_2O$; 1 g/L K_2HPO_4; 0.5 g/L KH_2PO_4; 0.2 g/L $CaCl_2{\cdot}2H_2O$). In each flask (250 mL), the initial culture volume was 100 mL with 98% (v/v) GA medium and 2% (v/v) inoculum. The cultivations were conducted at 27 °C with 250 rpm mixing. Duplicate flasks with each strain were incubated for 7 days.

2.6. Lipid production and fatty acid profiles of isolated strains and oleaginous strains

The biomass production and lipid accumulation capability of the two strains isolated from POME (Strains 1 and 4) were compared with the three strains ordered from the culture collection (*Y. lipolytica* DSMZ8212, *C. curvatus* DSMZ70022, and *C. albidus* DSMZ70197). All strains were pre-incubated in YM at 27 °C on an orbital shaker at 250 rpm for 3 days. Using these cultures as inoculum, each strain was incubated separately in 250 mL of GA medium at 27 °C with 250 rpm mixing. In each flask, the initial culture volume was 100 mL with 98% (v/v) GA medium and 2% (v/v) inoculum. Duplicate flasks of each strain were incubated for 15 days.

2.7. Analyses

Culture pH was measured with a WTW pH330i meter and a WTW SenTix 41 electrode. The dissolved oxygen (DO) concentration was measured with the WTW Oxi 330i meter and the CellOX 325 electrode.

The biological oxygen demand ($BOD_{7,ATU}$) with 7 days of incubation was determined according to Finnish standard SFS 3019 [11]. Allyl-thiourea (ATU) was used in the BOD determination to inhibit nitrification. The total chemical oxygen demand (COD_{tot}) and the COD of soluble compounds (COD_s) were determined with the closed tube dichromate method according to Finnish standard SFS 5504 [12]. The COD_s samples were filtrated through an Acrodisc PSF syringe filter with a 0.45 μm Supor (PES) membrane before the analysis.

Soluble nitrogen (N_s) and phosphorus (P_s) were determined with commercial Hach Lange LCK 238 and LCK 349 kits (Dusseldorf, Germany), respectively. Before the N_s and P_s analyses, the samples were filtrated with the 0.45 μm Acrodisc PSF syringe filter. The Hach Lange DR 200 Dry Thermostat Reactor (Dusseldorf, Germany) was used to heat the samples, and the results were analyzed with the Hach Lange DR 2800 Portable Spectrophotometer.

Samples for lipid extraction (5–10 mL) were centrifuged (Sigma 4K15, Osterode, Germany) at 5000 × *g* for 10 min. For gravimetrical lipid analysis, lipids were extracted with the modified Bligh and Dyer method using chloroform, methanol, and phosphate buffered saline (PBS, pH 7.4) as described by Santala et al. [13]. Extracted lipids were analyzed with thin-layer chromatography (TLC) using silica gel glass plates described by Efimova et al. [10].

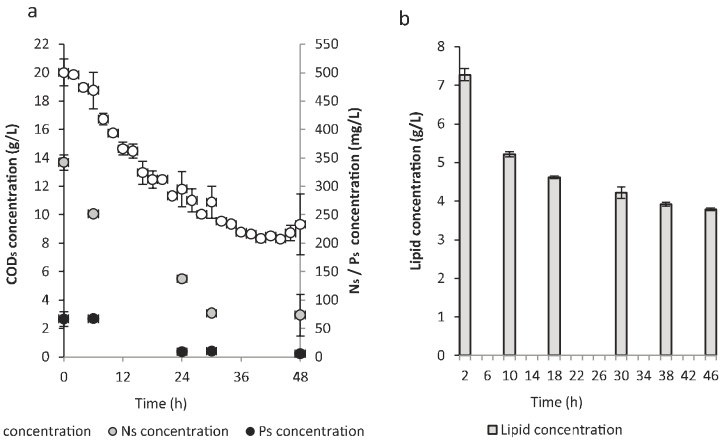

Fig. 1. (a) COD_s, N_s, and P_s removal from POME by the microorganisms enriched in POME and (b) lipid concentration of POME during the incubation.

Lipid content and composition were also determined as fatty acid methyl esters (FAMEs) with the direct trans-esterification method described by Su et al. [14]. However, sonication of the cells was omitted. Extracted FAMEs in *n*-hexane were analyzed with gas chromatography (GC-2014, Shimadzu, Kyoto, Japan) with a flame ionization detector (FID). Nitrogen was used as the carrier gas with a flow rate of 1.5 mL/min. Temperature was programmed to increase from 150 °C to 180 °C with rate 10 °C/min, from 180 °C to 220 °C with rate 1.5 °C/min and from 220 °C to 260 °C with rate 30 °C/min. The injection temperature was maintained at 250 °C and the detector temperature at 280 °C. Lipid content was determined from the FAME peak areas by using methyl pentadeconate as an internal standard.

Phase contrast microscopy (Zeiss Axioskop 2, Carl Zeiss, Göttingen, Germany) was used to study cell size and morphology. The microorganisms from the POME enrichment cultures were profiled with PCR-DGGE followed by sequencing. Samples taken to identify the microorganisms were stored in a freezer (−20 °C) before DNA was extracted with the PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA). Partial rRNA genes from isolated DNA were amplified using the PCR program described by Koskinen et al. [15]. For the partial eukaryotic 18S rRNA genes, Euk1A and Euk516r-GC were used as primers [16], while for the partial bacterial 16S rRNA genes, GC-BacV3f and 907r were used [17]. DGGE was conducted with the INGENYphorU2×2 system (Ingeny International BV, Goes, the Netherlands) as described by Koskinen et al. [15], except a denaturing gradient from 30% to 70% was used. The dominant (clearly visible) bands were excised from the DGGE gel, and their DNA was reamplified for sequencing as described by Koskinen et al. [15]. Pure culture samples were sequenced straight from the PCR-amplified extracted DNA without the PCR-DGGE step. Sequencing was done by Macrogen Inc. (Seoul, South Korea). The sequences were analyzed with BioEdit software and compared to the data in GenBank with BLAST software.

3. Results and discussion

3.1. Microbial growth on POME

Microorganisms enriched from POME oxidized 60% of the COD_s and removed 70% of N_s and 90% of P_s from POME (Fig. 1a). Damayanti et al. [18] reported that their POME sample contained 45 g/L of COD_{tot} of which 26 g/L was slowly biodegradable, 17 g/L inert particulate organic matter, and 2.8 g/L inert suspended organic matter. In our experiment, the initial COD_{tot} concentration was 41.5 ± 0.1 g/L, from which 27.3 ± 1.3 g/L was not degraded after 48 h.

The lipid concentration, which was measured with the modified Bligh and Dyer method, was 7.3 ± 0.1 g/L after 2 h and decreased to 3.8 g/L after 46 h (Fig. 1b). In a similar experiment with a 7-day incubation period (data not shown), the COD_s was mostly removed within the first few days, and the lipid concentration decreased to 3.8 g/L as in the experiment with 48 h incubation. The lipid samples contained residual plant biomass and microbial cells. Due to separation difficulties, the actual lipid content of the microbial biomass was not determined. The POME contained oil and carbohydrates. Glucose likely enhanced the lipid accumulation into yeasts [19]. Although the lipid concentration decreased, phase contrast micrographs showed that the lipids accumulated in eukaryotic cells as separate lipid inclusions (Fig. 2). According to Iassonova et al. [20], oleaginous yeasts may synthesize or adsorb fatty acids. Thus, the POME microorganisms might have adsorbed the residual lipids and/or synthetized new fatty acids using the organic constituents of POME.

POME microorganisms grew on POME (Fig. 1). Further, the micrographs (Fig. 2) showed that some eukaryotes in this culture accumulated lipids; however, the total lipids did not increase.

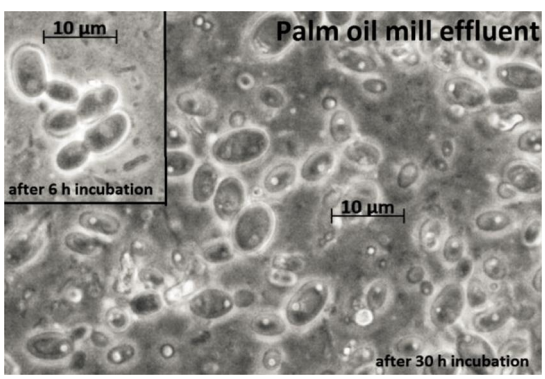

Fig. 2. Micrographs of the microorganisms in the POME taken with a phase contrast microscope.

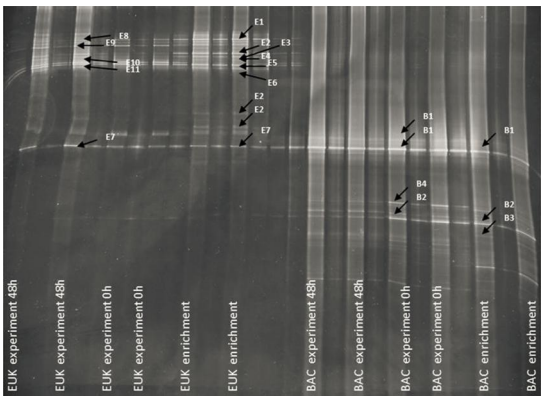

Fig. 3. Community profiles of POME enrichments used as inoculum and the samples from the beginning and the end of the batch experiment for COD_s and lipid removal. Bands labeled with black arrows were identified and are described in Table 1. BAC: amplification conducted with GC-BacV3f and 907r primers, EUK: amplification conducted with Euk1A and Euk516r-GC primers.

Microorganisms naturally present in POME likely have a growth advantage in POME compared to pure oleaginous microorganisms, although pure cultures of oleaginous yeasts have previously been grown on sterilized POME [21,22]. In a large-scale process, sterilization of wastewater is not economically feasible. To enable a sustainable POME-based lipid production process, microorganisms that grow competitively in non-sterilized POME and accumulate lipids are needed. Therefore, we studied the lipid-producing microorganisms naturally present in POME.

3.2. Identification of microorganisms in POME enrichment cultures

The microbial community composition of the POME enrichment culture was as shown in Fig. 3 and Table 1. To the authors' knowledge, microbial communities of POME have not been reported before. The prokaryotes belonged to Alpha- and Deltaproteobacteria and Flavobacteria. The eukaryotes belonged mainly to the genera *Saccharomycetes*. *Candida* and *Pichia* spp. have been reported to produce lipases and accumulate lipids. For example, *Candida tropicalis* can accumulate lipids up to 58% of its dry weight [23]. *Pichia occidentalis*, also known as *Issatchenkia occidentalis*, can be used for biological detoxification of lignocellulosic hydrolysate, because it can degrade volatile fatty acids as well as furfural, acetic acid, and guaiacol [24]. POME enrichment also included possible amebae *Hartmannella vermiformis* and kinetoplastid flagellate *Dimastigella trypaniformis*. These eukaryotes are possible predators in the cultures. The presence of possible predators indicates that during the process optimization steps the incubation time must be short to avoid predators feeding on the oleaginous microorganisms.

3.3. Identification of eukaryotic isolates

To isolate the eukaryotes from POME, PD agar, YM agar, YM agar with chloramphenicol, and modified YM agar with xylan instead of glucose were used for the plate cultivations. Several bacterial and eukaryotic organisms were detected from the plate cultivations at 27 °C. All eukaryotic colonies were chosen for purification. In total, four eukaryotic microorganisms (Strains 1–4) were successfully isolated from the POME enrichment cultures (Fig. 4). Strains 1, 3, and 4 were isolated with YM agar with chloramphenicol. Strain 2 was isolated with PD agar. Based on the plate cultivations with xylan as a carbon source, it is likely that POME enrichment did not

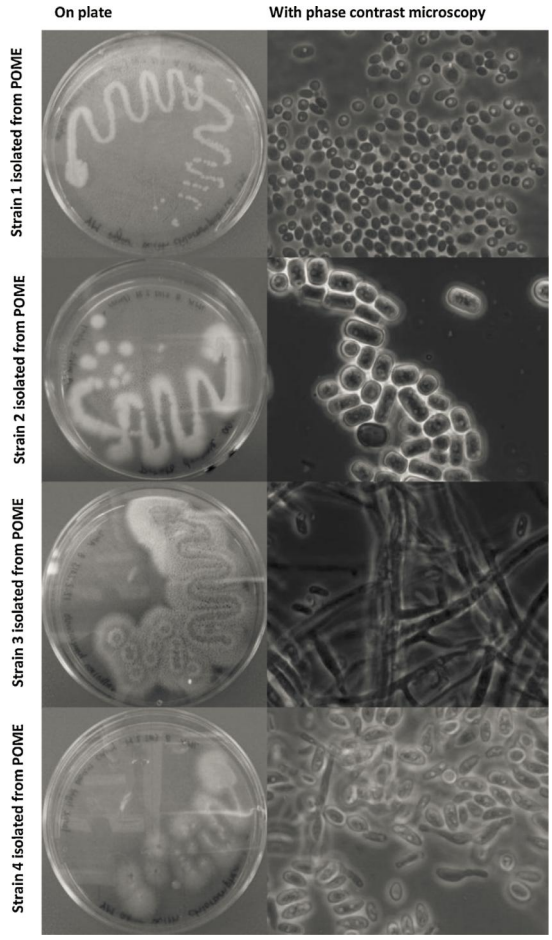

Fig. 4. Pictures of the strains isolated from POME on plate and with phase contrast microscopy.

contain strains that use xylan as a sole carbon source. In the identification of the microorganisms from POME enrichment culture, 11 eukaryotic strains were detected. However, many strains present in wastewater do not grow on solid agars with artificial medium.

Based on partial 18S rDNA sequencing, Strain 1 had 100% similarity to *Candida silvae* NRRL Y-6725, Strain 2 had 99.8% similarity to *Galactomyces geotrichum* LMA-20, Strain 3 had 96.7% similarity to *Lecythophora hoffmannii* CBS245.38T, and Strain 4 had 99.3% similarity to *Graphium penicillioides* JCM9300. Only *C. silvae* was detected from the initial sample of the enrichment culture. The other strains have likely been present in cell concentrations below the detection limit of PCR-DGGE.

Strain 1, *C. silvae*, was morphologically spherical, similarly as *C. silvae* in the micrographs in the database of CBS-KNAW Fungal Biodiversity Centre [25]. Strain 2, *G. geotrichum*, was slightly angular as in the micrographs in the database [25]. During the cultivation of Strain 3, *L. hoffmannii*, spherical sections in the mycelium were noted; this phenomenon was also observed by de Errasti et al. [26] with *L. hoffmannii* isolated from stems of exotic tree from Argentina. Strain 4, *G. penicillioides*, was similar to a *Graphium* species isolated from baobab trees in South Africa and Madagascar [27]. In addition to the similarity of single cells and mycelium in micrographs,

Table 1
Selected microorganisms identified from POME incubations by comparing the sequences from DGGE bands with the GeneBank data.

Band label[a]	Sequence length	The closest match in GenBank database		
		Similarity (%)	Affiliation (GenBank accession number)	Class/family
B1	490	96.1	*Chryseobacterium* sp. R9-11A (HQ154575)	Flavobacteria/Flavobacteriaceae
B2	484	96.9	*Vampirovibrio chlorellavorus* (HM038000)	Deltaproteobacteria/Bdellovibrionaceae
B3	355	93.0	*Sphingomonadaceae bacterium* HINF002 (AB426560)	Alphaproteobacteria/Sphingomonadaceae
B4	476	97.9	*Bdellovibrio bacteriovorus* (AF148939)	Deltaproteobacteria/Bdellovibrionaceae
E1	465	100.0	*C. tropicalis* (DQ515959)	Saccharomycetes/mitosporic Saccharomycetales
E2	503	99.0	*H. vermiformis* (FR832469)	Tubulinea/Hartmannellidae
E3	461	90.0	*Acanthamoeba hatchetti* (AF019068)	Tubulinea/Acanthamoebidae
E4	495	88.9	*P. occidentalis* (AB053240)	Saccharomycetes/Pichiaceae
E5	407	88.7	*Issatchenkia* sp. NRRL Y-12827 (EF550383)	Saccharomycetes/Saccharomycetaceae
E6	473	97.0	*Candida intermedia* (X89518)	Saccharomycetes/mitosporic Saccharomycetales
E7	522	100.0	*D. trypaniformis* (AY028447)	Kinetoplastea/Bodonidae
E8	472	88.3	*Pichia jaroonii* (AB436770)	Saccharomycetes/Pichiaceae
E9	501	90.4	*Cyberlindnera jadinii* (AB054569)	Saccharomycetes/Phaffomycetaceae
E10	461	99.8	*C. silvae* (AB053248)	Saccharomycetes/mitosporic Saccharomycetales
E11	494	99.2	*Pichia fermentans* (AB053241)	Saccharomycetes/Pichiaceae

[a] Band label in the DGGE gel presented as Fig. 3 (B for bacteria and E for eukaryote).

the Strain 4 colonies darkened after several days of incubation as reported for the *Graphium* species isolated from the tropical tree [27]. Thus, the morphologically strains isolated in this study were similar to the strains identified from the gene database and previous scientific publications.

3.4. Biomass production and lipid composition of isolated eukaryotes in synthetic medium

After 7 days of incubation in GA medium, the biomass concentrations of the strains isolated from POME were higher than 10 g/L (Table 2). However, the biomass concentrations were determined only by weighing the amount of freeze–dried biomass in 50 mL Falcon tubes. According to Samuelsson et al. [28], determining biomass with freeze–drying may overestimate the biomass concentration. Lipid compositions were analyzed with TLC from the lipids extracted with the modified Bligh and Dyer method. A proportion of the triacylglycerols increased during the cultivation with Strains 2 and 4, but not with Strains 1 and 3 (Fig. 5). With Strains 2 and 4, the proportion of diacylglycerols decreased when the triacylglycerol content increased. With Strain 1, the proportion of diacylglycerols decreased, but the proportion of monoacylglycerols and free fatty acids increased. Triacylglycerols are the major component of microbial lipid storage, and their increase indicates lipids have accumulated [4].

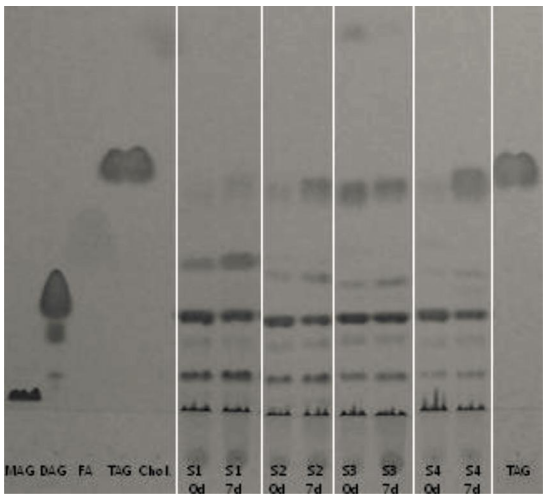

Fig. 5. TLC composition of the lipid samples taken at the beginning (0 day) and the end (7 days) of the first experiment with the strains isolated from POME. Standards: Gly: glycerol standard, MAG: 1-oleoy-rac-glycerol, DAG: 1,3-diolein, FA: palmitic acid, TAG: trioleoylglycerol, Ac-Chol: cholesteryl stearate.

Table 2
COD_S removal, biomass production, and lipid production of the strains isolated from POME.

Species	Time (Days)	COD_S Removal (%)	Biomass (g/L)	Lipids (wt%)	Lipids (g/L)
Strain 1	7	90.4	11.7 ± 0.8	15.8 ± 1.9	1.85
Strain 2	7	86.0	10.92 ± 0.5	7.4 ± 1.4	0.81
Strain 3	7	95.0	13.0 ± 0.5	9.4 ± 1.3	1.22
Strain 4	7	83.6	12.9 ± 0.1	18.4 ± 1.0	2.37

Lipids were also analyzed with direct transesterification as total fatty acids. The total fatty acid content of Strains 2 and 3 was low, while Strains 1 and 4 contained more than 15 wt% of fatty acids (Table 2). However, in Strains 1 and 4, the fatty acid content was still less than 20 wt%, the limit for calling microorganisms oleaginous [5].

3.5. Lipid production and fatty acid profiles of the isolated eukaryotes and the culture collection strains in synthetic medium

The lipid accumulation capabilities of the isolated strains with higher lipid content (Strains 1 and 4) were compared to the known oleaginous strains (*Y. lipolytica*, *C. curvatus*, and *C. albidus*) using 15 days of incubation. Lipid accumulation ability is strain dependent and is often enhanced by a high C/N ratio [3]. For example, a molar C/N ratio of 150:1 was reported to be optimal for lipid accumulation by *C. tropicalis* [23]. In the GA medium, the initial concentrations of COD_S, N_S, and P_S were 60 ± 4 g/L, 550 ± 30 mg/L, and 290 ± 10 mg/L,

Table 3
COD_S removal, biomass production, and lipid production of the strains isolated from POME compared to the known oleaginous strains.

Species	Time (Days)	CODs Removal (%)	Biomass (g/L)	Lipids (wt%)	Lipids (g/L)
Strain 1	15	89.8	7.7	17.9 ± 0.1	1.38
Strain 4	15	94.7	5.1	29.1 ± 3.0	1.48
Y. lipolytica	15	93.8	13.5 ± 0.8	15.7 ± 3.4	2.12
C. curvatus	15	94.7	17.5 ± 0.2	20.2 ± 2.9	3.54
C. albidus	15	88.0	9.3 ± 0.6	9.4 ± 0%	0.87

Table 4
Fatty acid profiles of the strains isolated from POME compared to the known oleaginous strains.

Species	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Strain 1	2.2 ± 0.1	–	–	9.8 ± 0.4	3.0 ± 0.2	1.3 ± 0.1
Strain 4	9.8 ± 0.1	–	2.6 ± 0.1	7.7 ± 0.1	9.0 ± 0.1	–
Y. lipolytica	2.6 ± 0.5	2.3 ± 0.5	1.1 ± 0.3	6.2 ± 1.7	3.5 ± 0.5	–
C. curvatus	4.8 ± 0.7	–	1.4 ± 0.1	10.1 ± 1.6	4.0 ± 0.5	–
C. albidus	1.3 ± 0.1	–	–	6.1 ± 0.1	2.0 ± 0.1	–

respectively. The initial COD_s:N_s:P_s ratio was 207:1.9:1, while the COD_s:N_s:P_s ratio of POME was 190:4:1 [10].

Based on the TLC plates, the triacylglycerol content increased with all known oleaginous strains and with the isolated Strain 4, but not with Strain 1 (data not shown). Increasing the incubation time increased the total fatty acid content of Strains 1 and 4 (Table 3). The lipid content was the highest with the Strain 4 isolated from POME (29.1 ± 3.0 wt%) followed by known oleaginous strain *C. curvatus* (20.2 ± 2.9%). The lipids accumulated by the strains were mainly palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), and linoleic acid (C18:2) (Table 4). These fatty acids and linolenic acid (18:3) are the most common fatty acids in biodiesel [29]. The most abundant fatty acid in the strains was oleic acid; however, for Strain 4, the most abundant fatty acid was palmitic acid followed by linoleic acid. The highest oleic acid content was obtained with *C. curvatus* (10.1 ± 0.1 wt%). The fatty acid composition is dependent not only on cultivation conditions, such as temperature [30,31], but also on the substrate, as shown with *Y. lipolytica* [32].

Increasing the incubation period from 7 days to 15 days increased the cellular lipid content but decreased the lipid concentration per culture volume. The lipid content in this study was low compared to other studies with various yeast strains reviewed by Papanikolaou and Aggelis [32]. Accumulation of microbial lipids depends on cultivation conditions [33,34] as well as the physiology and production of secondary metabolites [35]. Lipid accumulation of yeasts and molds is affected by the nitrogen source, carbon source, C/N ratio, temperature, agitation, and culture pH [3]. The agitation used in this study was not enough to keep the cultivations aerobic, and most likely, the culture pH was not optimal for lipid accumulation. Of the known oleaginous strains, only *C. curvatus* had lipid content higher than 20 wt% (Table 2). Of the isolated strains, only Strain 4, *G. penicillioides*, is oleaginous (lipid content >20 wt%). The cultivation conditions should be further optimized to obtain higher lipid content. In this study, pH was adjusted manually, and therefore, reactor studies with automatic continuous pH adjustment should be conducted. Further, engineering design research is required for selective enrichment of *G. penicillioides* to disclose the potential for lipid accumulation in an open POME environment in wastewater treatment systems.

4. Conclusions

A mixed culture of indigenous POME microorganisms grew on POME, but the total lipids did not increase. However, the micrographs showed that some eukaryotes present in the culture accumulated lipids during growth on POME. Therefore, eukaryotes were isolated from the POME culture. From these organisms, Strain 4 (99.3% similarity to *G. penicillioides*) was the most potent for lipid accumulation in synthetic medium. Strain 4 accumulated more lipids than the known oleaginous strain *C. curvatus* (29.1 ± 3.0 wt% vs. 20.2 ± 2.9 wt%) under similar conditions. Due to the lower biomass yield, the lipid concentration with *G. penicillioides* was lower than with *C. curvatus* (1.48 g/L vs. 3.54 g/L). In POME cultivations, *G. penicillioides* is likely to have a growth advantage compared to *C. curvatus* and may have the potential to produce oleaginous biomass from POME.

Acknowledgements

The authors gratefully acknowledge financial support from Neste Oil Corporation, the Finnish Funding Agency for Technology and Innovation, and the Industrial Research Fund of Tampere University of Technology.

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III

SELECTING AN INDIGENOUS MICROALGAL STRAIN FOR LIPID PRODUCTION IN ANAEROBICALLY TREATED PIGGERY WASTEWATER

by

Marjakangas, J.M., Chen, C.-Y., Lakaniemi, A.-M., Puhakka, J.A., Whang, L.-M. & Chang, J.-S., September 2015

Bioresource Technology, Vol. 191, pp. 369–376,
doi:10.1016/j.biortech.2015.02.075

Bioresource Technology 191 (2015) 369–376

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Selecting an indigenous microalgal strain for lipid production in anaerobically treated piggery wastewater

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HIGHLIGHTS

- Biomass and lipid production ability of three microalgal strains was studied.
- High lipid content was achieved with *C. vulgaris* in pre-treated piggery wastewater.
- *C. vulgaris* in piggery wastewater excreted xylose, mannose, and arabinose.

ARTICLE INFO

Article history:
Received 17 December 2014
Received in revised form 17 February 2015
Accepted 19 February 2015
Available online 26 February 2015

Keywords:
Lipid production
Chlorella vulgaris
Piggery wastewater

ABSTRACT

The aim of this study was to select a potential microalgal strain for lipid production and to examine the suitability of anaerobically treated piggery wastewater as a nutrient source for production of lipid-rich biomass with the selected microalga. Biomass and lipid productivity of three microalgal strains (*Chlorella sorokiniana* CY1, *Chlorella vulgaris* CY5 and *Chlamydomonas* sp. JSC-04) were compared by using different media, nitrogen sources, and nitrogen concentrations. The highest lipid content and productivity (62.5 wt%, 162 mg/L/d) were obtained with *C. vulgaris* with BG-11 with 62 mg N/L. Secondly, *C. vulgaris* was cultivated in sterilized, diluted (1–20×), anaerobically treated piggery wastewater. Biomass production decreased and lipid content increased, when wastewater was more diluted. The highest lipid content of 54.7 wt% was obtained with 20× dilution, while the highest lipid productivity of 100.7 mg/L/d with 5× dilution. Piggery wastewater is a promising resource for mass production of oleaginous microalgal biomass.

1. Introduction

In the modern world, energy expenditure continues to increase. Fossil fuel reserves are depleting and demand for alternative fuels is enormous. Biodiesel and renewable diesel are very good options for diesel-grade fuels for transportation (Demirbas, 2009). The term biodiesel generally refers to the fuel produced by esterification of vegetable oils and/or animal fats, while the term renewable diesel usually refers to a high quality fuel produced by hydrogenation of the lipids (Prince, 2009). Renewable diesel is fully compatible with existing fuel logistics, distribution and vehicle engines and results in lower air emissions than fossil diesel (Demirbas, 2009). The use of vegetable oils or sugars from food crops for production of transportation fuels has been questioned. Therefore, more and more attention has been focused on single cell oil production, i.e., lipid production in microbial cells. An advantage of microalgae compared to yeasts and other heterotrophic microorganisms for single cell oil production is algae's ability to fix CO_2, thus excluding the need for organic carbon source (Mata et al., 2010).

Microalgae's potential for third-generation biofuel production has been studied widely using different microalgae, cultivation strategies, photobioreactor designs and harvesting technologies (Chen et al., 2011). With various cultivation strategies, the disadvantage of heterotrophic growth, compared to autotrophic growth,

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http://dx.doi.org/10.1016/j.biortech.2015.02.075
0960-8524/

is to find low cost carbon source which do not compete with food production (Safi et al., 2014). Therefore, utilization of waste and residual materials as a carbon source has gained much attention. Several wastewaters have already been studied as a nutrient and/or carbon source for microalgae. For example, alcohol distillery wastewater, pig and poultry manure, palm oil mill effluent and textile wastewater have been studied (Fenton and hUallacháin, 2012; Ji et al., 2013; Kamarudin et al., 2013; Lim et al., 2010; Solovchenko et al., 2014; Whang et al., 2009). With several wastewater and microalga combinations, different strategies have been studied to enhance lipid accumulation. These include e.g., pH, temperature, nitrogen concentration, iron concentration, salinity stress, light intensity, photoperiod, CO_2 supplementation, nutritional mode (Converti et al., 2009; Mohan and Devi, 2014; Liu et al., 2008; Shah et al., 2014). Nevertheless, nitrogen limitation seems to be the most effective method to enhance lipid accumulation.

Use of microalgae for biofuel production has its pros and cons as reviewed by Safi et al. (2014). As an advantage, microalgae cultivation does not compete with agricultural land usage, conflict with food production or cause deforestation. However, the production costs are still not competitive with fossil fuels (Sander and Murthy, 2010). Therefore, waste and residue materials are attractive raw material options for the production of biofuels. Wastewaters can be used as a source of nutrients and carbon, while flue gases can be a sustainable source of CO_2. Nitrogen concentration and form of nitrogen (urea, ammonium, nitrate, nitrite) varies significantly among various wastewaters. Pig and poultry manure have already been found to be viable nutrient sources for algal cultivation (Fenton and hUallacháin, 2012). Thus, the aim of this study was to screen a suitable microalgal strain for oleaginous microalgal biomass production and to study the potential of using piggery wastewater as nutrient source for the selected oleaginous microalga. Simultaneous lipid accumulation in the microalgal cells and nitrogen removal from anaerobically treated piggery wastewater were also investigated.

2. Methods

2.1. Microalgal strains

Three different microalgal strains isolated from freshwater area in Southern Taiwan were studied. Strains had been identified as *Chlorella sorokiniana* CY1, *Chlorella vulgaris* CY5 and *Chlamydomonas* sp. JSC-04.

2.2. Medium

The media used to cultivate the pure cultures were Bold Basal Medium (BBM) and Blue Green Medium (BG-11). Compositions of the media are shown in Table 1.

2.3. Piggery wastewater

Wastewater used in this study is originated from a Taiwanese wastewater treatment plant treating piggery effluent. Treatment process consists of pH control basin, followed by four anaerobic basins, three aerobic basins and a settler. Grab sample of anaerobically treated piggery wastewater was taken after the fourth anaerobic basin prior to the aerobic treatment.

Wastewater was sterilized with autoclave (121 °C, 20 min) to be able to study the chemical suitability of anaerobically treated piggery wastewater for cultivating *C. vulgaris* CY5 without the inference of the microbial community of the wastewater. Unfortunately, the sterilization caused changes to the chemical composition of the wastewater. Sterilization increased the pH of the wastewater from 7.7 to 9.8. Change in pH and temperature induced stripping of ammonia–nitrogen, because form of ammonia between NH_4^+ ions and NH_3 gas depends on pH and temperature (Olguin et al., 2001). Sterilization also increased the soluble and total COD, and decreased the phosphate concentration (Table 2). To be able to concentrate in the growth of microalgae, sterilization was chosen to be used despite the changes in the chemical composition.

Table 1
Medium compositions.

Component	BBM (g/L)	BG-11 (g/L)
K_2HPO_4	0.075	0.04
KH_2PO_4	0.175	
$NaNO_3$	0.25	1.5
NaCl	0.025	
$C_6H_8O_7$		0.006
$NaCO_3$		0.02
$MgSO_4{\cdot}7H_2O$	0.075	0.075
$CaCl_2{\cdot}2H_2O$	0.025	0.036
EDTA	0.05	0.001
KOH	0.031	
$C_6H_8FeNO_7$		0.006
$FeSO_4{\cdot}7H_2O$	0.00498	
H_2SO_4	10 mL	
H_3BO_3	0.01142	2.86
$ZnSO_4{\cdot}7H_2O$	0.001412	0.222
$MnCl_2{\cdot}4H_2O$	0.000232	1.81
$CuSO_4{\cdot}5H_2O$	0.000252	0.079
$Ca(NO_3)_2{\cdot}6H_2O$	0.00008	
$Co(NO_3)_2{\cdot}6H_2O$		0.049
$Na_2MoO_4{\cdot}2H_2O$	0.000192	0.39

2.4. Experimental conditions

All of the batch experiments were conducted in 1 L glass vessels (15.5 cm in length and 9.5 cm in diameter) equipped with an external light source (14 W fluorescent light/TL5). Light intensity was 150 μmol/m^2/s and aeration rate 0.1 vvm with 2.5% CO_2. Initial volume of medium was 800 mL and volume of inoculum was selected to attain initial optical density of 0.1 at wavelength of 680 nm (OD_{680}). Cultures were incubated for 16–20 days at room temperature (20–25 °C) and the aim was to optimize the conditions for biomass and lipid production.

First *C. sorokiniana* CY1, *C. vulgaris* CY5 and *Chlamydomonas* sp. JSC-04 were cultivated in BBM and BG-11 medium to compare the growth of the three microalgae and study the effect of medium composition. In the second experiment all three microalgal strains (*C. sorokiniana* CY1, *C. vulgaris* CY5 and *Chlamydomonas* sp. JSC-04) were cultivated in BBM using various nitrogen sources to study the effect of nitrogen source. In the original BBM 0.25 g $NaNO_3$/L is used as nitrogen source, while in this experiment 0.16 g NH_4Cl/L and 0.088 g $CO(NH_2)_2$/L were used as nitrogen sources separately.

Table 2
Wastewater composition of the anaerobically treated piggery wastewater before and after sterilization with autoclave.

Component	Anaerobically treated piggery wastewater	Sterilized anaerobically treated piggery wastewater
pH	7.7	9.8
COD_{tot} (mg/L)	332	377
COD_s (mg/L)	298	308
TKN (mg/L)	348	287
NH_4-N (mg/L)	233	210
NO_3^- (mg/L)	5.5	7.1
PO_4 (mg/L)	101.4	28.4
Cl^- (mg/L)	105	106

The concentrations of the tested nitrogen sources were selected to have the same initial nitrogen concentration (40 mg N/L). In the third experiment, strains *C. sorokiniana* CY1 and *C. vulgaris* CY5 were incubated in BG-11 with various $NaNO_3$ concentrations to study the effect of nitrogen concentration. In the original BG-11 medium nitrogen concentration was 250 mg/L (1.5 g $NaNO_3$/L), while in this experiment concentrations of 63, 125, and 500 mg N/L were used.

Based on the experiments in synthetic medium, the most potential microalgal strain for lipid production was selected and further studied in sterile anaerobically treated piggery wastewater. Therefore, *C. vulgaris* CY5 was incubated in sterilized anaerobically treated piggery wastewater with various wastewater dilutions to study the effect of wastewater strength. Studied wastewater dilutions were 20×, 10×, 5×, 2× and undiluted (1×). Cultivation conditions were as described above, except the inoculum was centrifuged (5000 rpm, 10 min) prior to the experiment to minimize transfer of nutrients from preculture into the experimental phase.

2.5. Analyses

Light intensity at the surface of the cultivation flasks was measured with LI-250 light meter with a LI-190SA pyranometer sensor (LI-COR, Inc., Lincoln, USA). Phase contrast microscopy (ECLIPSE 80i, Nikon, Japan) was used to study cell size and morphology. Culture pH was measured with Sartorius PB-10 pH meter with pH/ATC electrode (Germany).

Biomass concentration was determined by measuring optical density at wavelength 680 nm (i.e., OD_{680}) with Hitachi U-1900 Spectrophotometer (Japan). Dry biomass concentration was determined from 5 mL culture aliquot by centrifuging (12,000 rpm, 3 min) the biomass with Thermo Scientific Heraus Pico 17 microcentrifuge (Germany), washing the pellet twice with water, and drying the biomass with Kett Infrared Moisture Determination Balance FD-720 (Japan) until the sample mass was invariant.

Total and soluble chemical oxygen demands (COD_{tot} and COD_s) were determined according to the standard method 5220C: Closed Reflux, Titrimetric Method (APHA, 2012). Before analysis COD_s samples were centrifuged (12,000 rpm, 3 min) and filtrated with Millipore Millex-HV PVDF 0.45 μm syringe filter.

Nitrate concentration was determined by measuring optical density at wavelength 220 nm. Conversion equation $c(NO_3^-) = 17.587 \cdot OD_{220} - 0.1689$, where OD_{220} is the absorbance at wavelength 220 nm, was used to determine the nitrate concentration ($c(NO_3^-)$). Total nitrogen (TN) was determined with TOC/TN_b analyzer (LiquiTOC II, Elementar, Germany), using $(NH_4)_2SO_4$ and KNO_3 as standards. Total Kjeldahl Nitrogen (TKN) and ammonium (NH_4-N) were determined according to the standard method 4500-N(org)C: organic nitrogen/Semi-Micro-Kjeldahl (APHA, 2012).

Lipid composition was determined as fatty acid methyl esters (FAMEs), which were extracted from the microalgae cells with direct transesterification method. The microalgal cells were harvested with centrifugation (5000 rpm, 10 min), washed thrice with deionized water (5000 rpm, 5 min), and dried for 24 h with EYELA FDU-1100 Freeze Dryer (Japan). For transesterification 40 mg of microalgal cells were homogenized with MP Biometrical FastPrep®-24 (USA) with 2 g glass beams and 4 mL of 0.5 M KOH in methanol. For saponification, cell mixture (40 mg cells, 2 g glass beads, 8 mL 0.5 M KOH in methanol) was heated at 100 °C for 15 min. For esterification, 8 mL of 0.7 M HCl in methanol and 10 mL of 14% $C_2H_8BF_3O_2$ were added and solution was heated at 100 °C for 15 min. After cooling down to room temperature, 4 mL of saturated NaCl solution was added for emulsification and 4 mL of N-hexane was added for extracting the formed FAMEs. Solution was mixed for 5 min in Scientific industries Vortex-Genie 2 with Vertical Multi-Tube Holder (USA) and centrifuged (6000 rpm, 5 min) with Hettich Rotofix 32A (Germany) before the upper N-hexane layer was separated. Extracted FAMEs in N-hexane were analyzed with gas chromatography (GC-2014, Shimadzu, Japan) with flame ionization detector (FID). Nitrogen was used as carrier gas with flow rate of 1.5 mL/min. Temperature was programmed to increase from 150 to 180 °C with rate 10 °C/min, from 180 to 220 °C with rate 1.5 °C/min and from 220 to 260 °C with rate 30 °C/min. Injection temperature was maintained at 250 °C and detector temperature at 280 °C. Lipid content was determined from the FAMEs peak areas by using methyl pentadecanoate as an internal standard.

The carbohydrate content of the liquid samples filtrated with Millipore Millex-HV PVDF 0.45 μm syringe filter was determined by phenol–sulfuric acid method (Dubois et al., 1956). Carbohydrate composition was determined with ion chromatography (ICS-5000, Dimex, USA) with sugar column (CarboPac SA10G, Dimex, USA). Samples were detected with and without hydrolyzation to detect both monosaccharides and polysaccharides. Polysaccharides were hydrolyzed by adding 0.5 mL sample and 0.5 mL 6 M trifluoroacetic acid, and sample tubes were heated in glycerol at 100 °C for 24 h. Samples were neutralized with $CaCO_3$ prior to the analysis.

3. Results and discussion

3.1. Effect of media composition on microalgae growth and lipid production

During the cultivation of strains *C. sorokiniana* CY1, *C. vulgaris* CY5 and *Chlamydomonas* sp. JSC-04 in BBM and BG-11 medium, culture pH with BBM medium was 7.4 ± 0.3 and with BG-11 medium 7.7 ± 0.3. BG-11 contained more nutrients than BBM and the measured initial nitrate concentration, as total nitrogen, in BG-11 was 180 mg N/L whereas in BBM it was 30 mg N/L. Nitrogen was exhausted from BBM by day 3, whereas in BG-11 soluble nitrogen was still available after 20 days of cultivation. With each of the microalgal strains more biomass was produced with nutrient-rich BG-11 medium (Fig. 1a). Lipid productivity (g/L/d) was also remarkably higher with BG-11 medium than with BBM (Fig. 1b) with all the strains. Generally, nitrogen limitation has been thought to trigger lipid accumulation. In this experiment, however, higher lipid content was obtained using medium with higher nitrogen content (BG-11). For example, lipid content of *C. vulgaris* after 16 days incubation in BG-11 was 39.8 wt% and correspondingly in BBM 34.9 wt%. In addition to low nitrogen concentration, increased salinity can also trigger lipid accumulation. In this study, sodium and chloride concentrations were much higher in BG-11 than in BBM, being 490 vs. 77 and 260 vs. 27 mg/L, respectively. According to Mohan and Devi (2014), 1 g/L NaCl concentration, which equals 390 mg/L Na and 610 mg/L Cl, increased total lipid content of microalgae from natural water body from 15.2% to 23.4% and neutral lipid content from 6% to 9.2%. In their study 1 g/L NaCl concentration caused higher increase in lipid content than 0.5 or 2 g/L NaCl concentrations.

3.2. Effect of nitrogen source

During the cultivation of the three algal strains (*C. sorokiniana* CY1, *C. vulgaris* CY5 and *Chlamydomonas* sp. JSC-04) with different nitrogen sources, pH increased with nitrate (7.4 ± 0.2), remained at the original level with urea (6.7 ± 0.2) and severely decreased with ammonium (pH below 4 within two days). Hulatt et al. (2012) detected similar changes in pH when using nitrate, urea and ammonium as a nitrogen source for *C. vulgaris*. Similarly to the

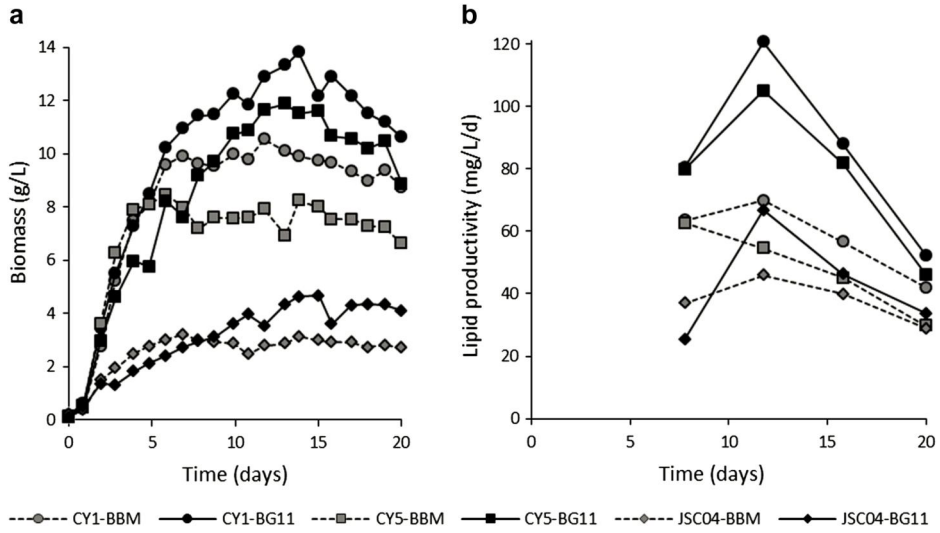

Fig. 1. Biomass (a) and lipid productivity (b) of *Chlorella sorokiniana* CY1, *Chlorella vulgaris* CY5 and *Chlamydomonas* sp. JSC-04 cultivated in BBM and BG-11.

results obtained by Hulatt et al. (2012), growth of microalgae in cultures with ammonium ceased due to drop in culture pH after few days of cultivation. On one hand, the growth should not have been limited by the ammonium, because the initial ammonium concentration was below 50 mg/L, which has been found to limit algal growth (Ip et al., 1982). On the other hand, growth must have been affected by the pH, because the pH between 3.5 and 4.5 have been reported to limit the growth of *C. vulgaris* (Huss et al., 1999).

After 12 days incubation, lipid content and lipid productivity obtained with all the strains were quite similar with urea and nitrate (Table 3). With *Chlorella* strains lipid productivity was slightly higher with nitrate than with urea, while with *Chlamydomonas* sp. lipid productivity was slightly higher with urea. Hsieh and Wu (2009) studied *Chlorella* sp. in artificial seawater with various urea concentrations (25–200 g/L). They obtained 0.46–2.03 g/L biomass productions and 66.1–32.6 wt% lipid contents. Their maximum lipid productivity was 0.124 g/L/d with 0.1 g/L urea. In our study, more biomass (2.40 g/L) but less lipids (0.833 g/L/d) was produced with *C. sorokiniana* in BBM with urea.

3.3. Effect of nitrogen concentration

Due to the higher lipid productivities obtained with BG-11 cultivations, BG-11 was used to study various nitrogen concentrations instead of BBM. Results for lipid contents and productivities in the earlier studies were higher with *Chlorella* species, and therefore, *Chlamydomonas* sp. was not included into this experiment.

Biomass concentrations higher than 3 g/L were obtained with all tested nitrogen concentrations (63, 125, 250 and 500 mg N/L). Doubling the initial nitrogen concentration of BG-11 to 500 mg N/L slowed down the biomass growth at the beginning of the experiment (Fig. 2c and d), while final biomass concentration was mainly higher at the higher nitrogen concentrations.

Lipid contents of both *Chlorella* strains were higher with the BG-11 medium with the lowest nitrogen concentration compared to the other nitrogen concentrations (Fig. 2c and d). Because changes in the nitrogen concentration of the medium did not have significant effect on biomass concentration, lipid productivity was also the highest with the lowest nitrogen concentration. The lipid productivity with *C. vulgaris* was higher than with *C. sorokiniana* (162 mg/L/d vs. 145 mg/L/d). Therefore, only *C. vulgaris* CY5 was used in the experiment with piggery wastewater. Strain *C. vulgaris* have been widely studied and it is also stated to be one of the most interesting microalgae (Safi et al., 2014).

Table 3
Maximum biomass concentration, lipid content per dry weight and lipid productivity obtained with *Chlorella sorokiniana* CY1, *Chlorella vulgaris* CY5 and *Chlamydomonas* sp. JSC-04 incubated in BG-11 with nitrate and urea as the nitrogen sources.

Strain	Nitrogen source	Biomass (g/L)	Lipid content (wt%)	Lipid productivity (g/L/d)
Chlorella sorokiniana CY1	Nitrate	2.5	32.3	69.8
	Urea	2.3	33.7	67.2
Chlorella vulgaris CY5	Nitrate	2.0	32.4	54.6
	Urea	1.8	31.8	49.3
Chlamydomonas sp. JSC-04	Nitrate	2.4	22.9	45.9
	Urea	2.8	21.8	53.1

3.4. Cultivation of microalgae in anaerobically treated piggery wastewater

Five different dilutions (20×, 10×, 5×, 2×, 1×) of the sterilized anaerobically treated piggery wastewater were studied as a cultivation medium for *C. vulgaris* CY5. Main nitrogen source in anaerobically treated piggery wastewater was ammonium, which was not very good nitrogen source for *C. vulgaris*, based on the previous experiment with various nitrogen sources. Anyhow, *C. vulgaris* was able to grow in sterile anaerobically treated piggery wastewater in preliminary studies (data not shown), and therefore, it was used in this experiment. Culture pH was adjusted to 6.5 ± 0.4 after the incubation was started (Fig. 3a), because the high pH of the wastewater (9.2 ± 0.4) caused microalgae to flocculate. On day 2 culture pH of the cultivations was already near neutral (6.9 ± 0.5).

3.4.1. Changes in ammonium concentration and organic carbon

TN was measured from the cultures after 4 and 8 days of cultivation. On day 4, TN concentration was below 5 mg/L in all

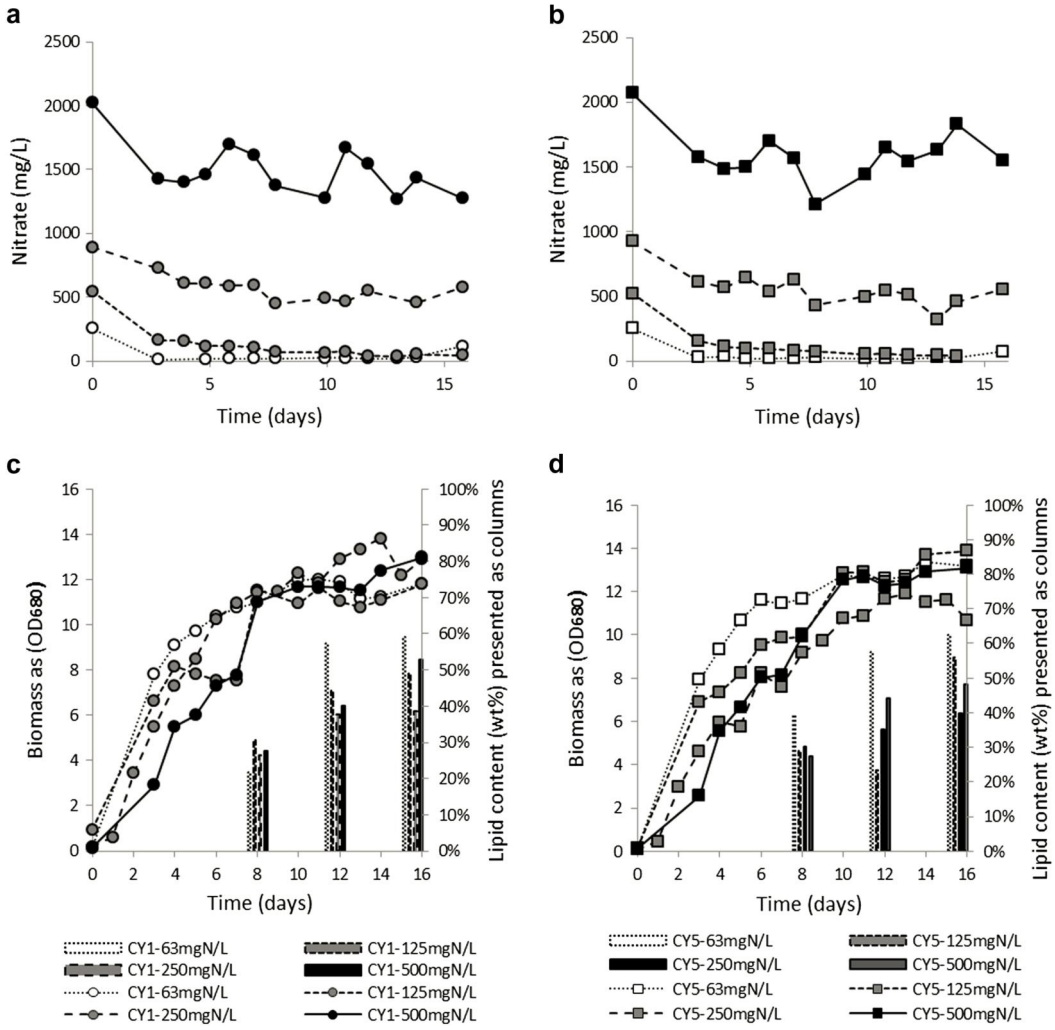

Fig. 2. Nitrate concentration (a and b), and biomass and lipid content (c and d) of *Chlorella sorokiniana* CY1 (a and c) and *Chlorella vulgaris* CY5 (b and d) cultivated in BG-11 with various nitrogen concentrations (63, 125, 250 and 500 mg N/L).

other cultures except in the culture with undiluted wastewater, where the TN concentration was still 27 mg/L. On day 8, nitrogen was also limiting the growth of *C. vulgaris* in diluted wastewaters as the TN concentration of culture had decreased to 6 mg/L. Based on the TN results, *C. vulgaris* CY5 can be used to remove nitrogen from the anaerobically treated piggery wastewater.

COD_s concentration increased during the incubation (Fig. 3b), which demonstrated release of carbon from microalgae to their surroundings. Dissolved organic carbon concentration increased with time also based on the determination with TOC/TN_b analyzer (data not shown). *C. vulgaris* YSW-04 has been shown to remove COD from pretreated piggery wastewater (Ji et al., 2013) and *Chlorella* sp. to remove 27.4–38.4% COD from diluted digested dairy manure (Whang et al., 2009). Also other studies have shown *Chlorella* sp. to be able to remove COD from palm oil mill effluent, textile wastewater and alcohol distillery wastewater (Kamarudin et al., 2013; Lim et al., 2010; Solovchenko et al., 2014). In this study, COD_s release was generally higher, when wastewater was less diluted. However, released COD_s was not proportional to biomass growth, as during the 16 days incubation with the dilutions of 20×, 10×, 5×, 2× and 1×, COD_s release was 344, 236, 298, 216 and 148 mg COD_s/g biomass, respectively. As an average 250 ± 140 mg COD_s was released per 1 g of biomass. Lancelot (1983) has found high negative correlation between phytoplankton extracellular release and mineral nitrogen concentration during the spring bloom. In this study, the higher COD_s release with higher wastewater dilution may also be related to nitrogen deficiency.

Microalgae usually release carbohydrate-rich dissolved organic matter during photoautotrophic growth. For example, *Rhodella violacea* have been found to produce exopolysaccharides containing uronic acid, xylose, arabinose, rhamnose, galactose, glucose, glucuronic acid (Villay et al., 2013). Also *Chlamydomonas reinhardtii* is known to secrete large amount of exopolysaccharides including

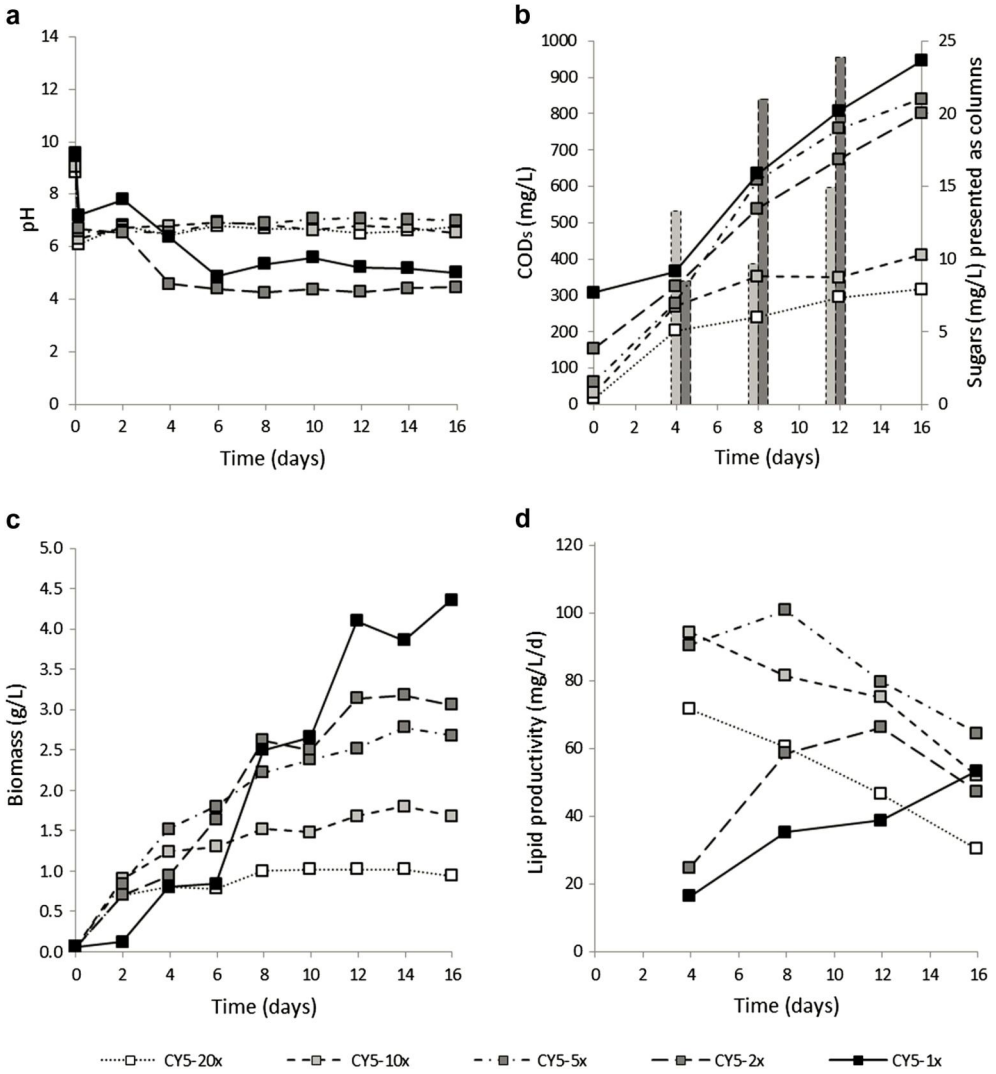

Fig. 3. Culture pH (a), COD_s and total sugars in the liquid phase (b), biomass concentration (c) and lipid productivity (g/L/d) (d) of *C. vulgaris* CY5 cultivated in sterilized anaerobically treated piggery wastewater with various dilutions (20×, 10×, 5×, 2×, 1×).

galacturonic acid, ribose, arabinose, xylose, glucose, galactose, and rhamnose sugars (Bafana, 2013). Yalcin et al. (1994) studied gum formation of *Chlorella* sp., and they found gum to contain glucuronic acid, arabinose, glucose, fucose and minerals. In our experiments, organic matter excreted by *C. vulgaris* contained very small concentrations of monosaccharides – fucose, sucrose, arabinose, xylose and mannose – and some polysaccharides which mainly consisted of arabinose. Total sugar concentrations for the cultures with 10× and 2× diluted wastewater were as presented in Fig. 3b.

According to Malinsky-Rushansky and Legrand (1996) excretion of organic compounds during the photosynthesis is normal for healthy population and they found extracellular release for *Chlorella*-like species to be 12.4% of total assimilated C^{14}. However, medium has significant effect on exopolysaccharide production (Bafana, 2013). For *R. violacea* optimal pH for exopolysaccharide production was 8.3, when pH from 6 to 10 was studied (Villay et al., 2013). Therefore, initial pH of the cultures in this study might have affected the exopolysaccharide production. In further experiments with *C. vulgaris* and sterile 5× diluted anaerobically treated piggery wastewater (data not shown), culture pH was initially adjusted to 8. In these experiments COD_s was measured at the beginning and after 4, 8, 12 and 16 days incubation and most of the increase in COD_s concentration occurred before day 4. Adjusting culture pH has a great effect on exopolysaccharide production. Unfortunately, release of dissolved organic carbon is a potential loss in conversion of light energy to utilizable biomass (Hulatt and Thomas, 2010). Due to the COD_s increase during cell growth, *C. vulgaris* CY5 seems to be not suitable to remove carbon from the anaerobically treated piggery wastewater, at least without more careful pH adjustment.

3.4.2. Biomass and lipid production

More biomass was produced when wastewater was less diluted, but with the undiluted wastewater 2 days lag phase in biomass production was observed at the beginning of the incubation (Fig. 3c). Doubling time in the exponential growth phase for *C. vulgaris* in anaerobically treated piggery wastewater with the tested dilutions was 2.51 ± 0.16 d, which was similar to the doubling time of *C. vulgaris* in BG-11 medium (2.47 ± 0.05 d). Biomass production of *C. vulgaris* in the wastewater was efficient and Kumar et al. (2010) have also found predigested piggery effluent without additional nutrients to be a very promising substrate for *C. vulgaris*.

Lipid content of the biomass (wt%) was higher with more diluted wastewater, which contained a less amount of nitrogen source. Results with the wastewater were consistent with the results from the experiments with various nitrogen concentrations in artificial medium, where higher lipid content was also achieved with lower nitrogen concentration. Lipid content of the cultures with diluted wastewater increased already between days 4–8, which did not occur with undiluted wastewater, because there was still 27 mg/L TN in the culture with undiluted wastewater on day 4. The effect of nitrogen concentration on lipid accumulation is very well-known, while other parameters also affect lipid accumulation. Diluting the wastewater decreased the concentration of all compounds, which decreased the biomass production. However, the other components may also affect the lipid accumulation. For example, the chlorine concentration decreased from 110 to 5.3 mg/L when wastewater was more diluted. Taking into account both the biomass production and cellular lipid content, the most suitable wastewater dilution was the 5× dilution, which gave the maximum lipid productivity of 101 mg/L/d on day 8. The 5× diluted wastewater and BBM had similar chlorine concentration (21 mg/L vs. 27 mg/L) and nitrogen concentration (42 NH_4-N mg/L vs. 41 NO_3-N mg/L).

In this study, the lipid content of *C. vulgaris* obtained with the diluted piggery wastewater was rather high compared to other studies. However, Kobayashi et al. (2013) compared three *C. sorokiniana* species with 10% anaerobic digester effluent from cattle manure digestion with BBM in 3 L hanging bag photobioreactors (21 d). They obtained 25–30% lipids with both wastewater and BBM. Similarly, our *C. vulgaris* strain with 16 days incubation obtained similar lipid contents with 5× diluted wastewater and BBM (38.5 vs. 35 wt%). On the other hand, lipid contents up to 58% have been achieved with *C. vulgaris* in optimized conditions (Mata et al., 2010).

Ji et al. (2013) also reported increased biomass growth when *C. vulgaris* have been cultivated in less diluted piggery wastewater. In their flask experiment the total nitrogen concentration of the filtrated (0.45 μm) wastewater was higher than in our study (512 vs. 287 mg/L). With 5× diluted wastewater they obtained less biomass (1 g/L) and lipids (0.26 g/L) than we did. Similarly to our study, Whang et al. (2009) found total fatty acid content to increase with increasing wastewater dilution, when they studied *Chlorella* sp. in diluted digested dairy manure. However, they also obtained less biomass (1.47–1.71 vs. 0.94–4.36 g/L) and lower lipid content (9.0–13.7% vs. 20–55 wt%) with 16 days incubation than we did. On the other hand, rather similar biomass concentrations were obtained with 10× diluted wastewater (1.57 vs. 1.68 g/L).

Release of COD may also explain the rather high lipid contents. Release of DOM is normal for healthy algae population (Malinsky-Rushansky and Legrand, 1996), but it may also demonstrated stress in the culture (Hulatt and Thomas, 2010) and lipid accumulation is enhanced by stressful conditions. Initial stress caused by high pH may have improved the accumulation of lipids and may be one of the reasons for the rather high lipid contents of this study. However, further experiments should be conducted to study the effect of pH for lipid accumulation.

4. Conclusions

This study with freshwater algae from Taiwan revealed that *C. vulgaris* has potential for lipid-rich biomass production. For *C. vulgaris* in artificial medium, nitrate was a better nitrogen source than urea, while ammonium was not suitable as a sole nitrogen source. However, ammonium-rich wastewater was suitable for lipid production. *C. vulgaris* removed nitrogen but not carbon from the wastewater, due to release of exopolysaccharides. The highest lipid content (54.7 wt%) was obtained with 20× diluted wastewater, whereas the highest lipid productivity (100.7 mg/L/d) with 5× dilution. *C. vulgaris* has potential for simultaneous oleaginous biomass production and nitrogen removal from anaerobically treated piggery effluent.

Acknowledgements

TUT's Graduate School is acknowledged for funding this research. Thanks also for Maj and Tor Nessling foundation, TUT foundation and A.R. Winter's memorial foundation for travel Grants. The authors also acknowledge the financial support received for this work from Taiwan's Ministry of Science and Technology (104-3113-E-006-003 and 103-2221-E-006-190-MY3). This work is also supported in part by the Taiwan's Ministry of Education under the ATU plan.

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IV

SIMULTANEOUS NUTRIENT REMOVAL AND LIPID PRODUCTION WITH *CHLORELLA VULGARIS* ON STERILIZED AND NON-STERILIZED ANAEROBICALLY PRETREATED PIGGERY WASTEWATER

by

Marjakangas, J.M., Chen, C.-Y., Lakaniemi, A.-M., Puhakka J.A., Whang, L.M. & Chang, J.-S., November 2015

Biochemical Engineering Journal, Vol. 103, pp. 177–184,
doi:10.1016/j.bej.2015.07.011

Biochemical Engineering Journal 103 (2015) 177–184

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular article

Simultaneous nutrient removal and lipid production with *Chlorella vulgaris* on sterilized and non-sterilized anaerobically pretreated piggery wastewater

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ARTICLE INFO

Article history:
Received 21 April 2015
Received in revised form 10 July 2015
Accepted 14 July 2015
Available online 23 July 2015

Keywords:
Aerobic process
Lipid production
Microalgae
Piggery wastewater
Sterilization
Wastewater treatment

ABSTRACT

Piggery wastewater is a potent nutrient source for microalgal lipid production. Wastewater has been usually sterilized when used for microalgal cultivation. This is uneconomical in large-scale applications. Therefore, lipid productivity of *Chlorella vulgaris* CY5 using sterilized and non-sterilized diluted anaerobically pretreated piggery wastewater was studied in batch reactors. The maximum average lipid productivity was obtained after 12 days of incubation and it was higher with the sterilized wastewater than with the non-sterilized one (117 g/L/d vs. 91.3 g/L/d), due to the higher biomass concentration. Because of the unexpected increase of dissolved organic carbon (DOC) in the cultures, second experiment was conducted to characterize the composition of produced DOC in non-sterilized wastewater. Carbohydrate content increased in the liquid phase but decreased in the biomass after nitrogen had been exhausted. After 12 days of incubation, soluble chemical oxygen demand (COD_s) was 414 ± 56 mg/L, biomass production was 2.8 ± 0.15 g/L, and lipid content was 30.3 ± 1.2 wt%. Average lipid productivity from day zero to day 12 was 70.5 ± 1.1 g/L/d. *C. vulgaris* removed nutrients from the non-sterilized wastewater and produced oleaginous biomass, although the lipid productivity was higher with sterilized wastewater.

1. Introduction

The demand for more eco-efficient society is enormous. Industrial, agricultural and municipal systems require modifications, such as reusing wastewaters and flue gases in energy generation to become more sustainable. Production of lipid-rich biomass to generate biofuels is one example [1–4]. Lipid-rich biomass can be produced with various microorganisms, such as bacteria, yeasts, fungi, and microalgae, but only photosynthetic organisms can be used to simultaneously fix CO_2 from the flue gas and remove nutrients from the wastewater [4].

Several factors, such as various microalgal species, cultivation strategies, reactor designs and harvesting technologies, have been used to produce third-generation biofuels with microalgae [5]. *Chlorella vulgaris* is one the most studied microalga due to its ability to grow in relatively harsh conditions and in the presence of culture invaders [6]. Biomass composition of *C. vulgaris* is highly dependent on cultivation conditions and medium composition [7]. In stressful growth conditions, such as nitrogen starvation, *C. vulgaris* accumulates lipids mainly in the cytoplasm and chloroplasts [8], but it may also produce starch granules inside the chloroplasts [6]. *C. vulgaris* accumulated high concentrations of lipids, especially in mixotrophic conditions, where both light, CO_2, and organic carbon were provided simultaneously [6]. In mixotrophic conditions algae can grow both using light as energy source and CO_2 as carbon source (autotrophic growth), and using organic carbon as energy and carbon source (heterotrophic growth) [4].

Integration of nutrient removal and biodiesel production with *Chlorella* spp. and piggery wastewater has been reported [3,9–11]. Microalgal lipid studies on piggery wastewater have been mainly conducted with sterilized wastewater. In addition to autoclaving,

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http://dx.doi.org/10.1016/j.bej.2015.07.011
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Table 1
Composition of the undiluted anaerobically treated piggery wastewater before (non-sterilized) and after sterilization (sterilized) with autoclave (121 °C, 20 min).

Component	Anaerobically treated piggery wastewater	
	Non-sterilized	Sterilized
pH	7.7	9.8
COD_{tot} (mg/L)	332	377
COD_s (mg/L)	298	308
TKN (mg/L)	348	287
NH_4-N (mg/L)	233	210
NO_3- (mg/L)	5.5	7.1
PO_4- (mg/L)	101.4	28.4

sterile-filtration, UV irradiation and addition of sodium hypochlorite have also been used to eliminate bacterial and protozoal invaders in dairy wastewater [12].

The objective of this work was to compare cultivation of *C. vulgaris* CY5 in sterilized and non-sterilized anaerobically pretreated piggery wastewaters, as sterilization of large volumes of wastewater is very costly. These experiments aim to delineate microalgal ability to grow in non-sterilized wastewater environment, and simultaneously accumulate lipids and remove nitrogen from the anaerobically pretreated piggery wastewater.

2. Materials and methods

2.1. Microalgae

Chlorella vulgaris CY5 isolated from a freshwater area in Southern Taiwan was used in this work. Prior to the growth experiments in wastewater the strain was pre-cultured in BG-11 medium, the composition of which has been described in our previous study [13]. Between the experiments, the strain was maintained in BG-11 agar plates (BG-11 with 15 g/L agar).

2.2. Piggery wastewater

Wastewater of this study originated from a Taiwanese wastewater treatment plant treating piggery effluent. Treatment process consisted of a pH control basin, followed by four anaerobic basins, three aerobic basins and a settler. A grab sample of anaerobically pretreated piggery wastewater was taken after the fourth anaerobic basin prior to the aerobic treatment. The growth of *C. vulgaris* was examined in both sterilized (autoclaved 121 °C, 20 min) and non-sterilized anaerobically pretreated piggery wastewater. Composition of the wastewater before and after autoclaving was as given in Table 1.

2.3. Experimental conditions

Experiments were conducted in 1 L glass vessels (15.5 cm in height and 9.5 cm in diameter) equipped with an external light source (14W fluorescent light/TL5). Light intensity was 150 μmol/m^2/s and aeration rate 0.1 vvm with 2.5% CO_2. Initial culture volume was 800 mL and cultures were incubated for 16 days at room temperature (20-25 °C). Microalgal biomass from the pre-cultures was centrifuged (5000 rpm, 10 min) and the settled biomass was used as inoculum to minimize transfer of nutrients from pre-culture medium into the wastewater used in the microalgal growth experiments.

First *C. vulgaris* CY5 was cultivated in sterilized and non-sterilized 5× diluted wastewater using various inoculum concentrations (50 mg/L, 150 mg/L & 300 mg/L). The pH of the cultures with sterilized wastewater was adjusted to below 8, with 4 M H_2SO_4 (6 mL of 4 M H_2SO_4 into 800 mL of wastewater) at the beginning of the cultivation experiments, to minimize the effect of initial culture pH on microalgal growth in the experiment studying the effect of sterilization. NaOH was used to adjust the culture pH during the experiments if pH decreased below 4, because low pH has been shown to inhibit growth of *C. vulgaris* [14]. Aim of this experiment was to study the effects of sterilization and inoculum size on biomass and lipid production. As the COD_s concentration increased during the cultivation, the cultivation of *C. vulgaris* CY5 with non-sterilized 5x diluted wastewater was repeated in triplicate batch reactors to further delineate lipid production process in non-sterilized wastewater and to characterize the composition of released organic matter. Therefore, carbohydrates from the liquid phase and composition of the biomass were determined in the second experiment. Results for the triplicate batch reactors were calculated as average values ± standard deviation of the results from these three reactors.

2.4. Analyses

Light intensity was measured with LI-250 light meter and LI-190SA pyranometer sensor (LI-COR, Inc., USA). Culture pH was measured with PB-10 pH meter equipped with pH/ATC electrode (Sartorius, Germany). Cell size and morphology were studied using phase contrast microscopy (ECLIPSE 80i, Nikon, Japan). Lipid inclusions were identified with the same microscope using fluorescence light and Nile Red staining. The staining was conducted by adding 1 μL of Nile Red per 1 mL of sample and then incubated at room temperature (20-25 °C) for 5 min before microscopy examination with fluorescence light.

Biomass concentration was measured as optical density at a wavelength of 680 nm (OD_{680}) and as dried biomass (g/L). For biomass composition analyses, biomass was harvested with centrifugation (5000 rpm, 5 min) and washed thrice with reverse osmosis treated water. From the harvested biomass, the content of lipids, carbohydrates and proteins was analyzed. Lipids were determined as fatty acid methyl esters (FAMEs) after extraction from the biomass with direct transesterification method [13]. Extracted FAMEs were analyzed by gas chromatography using a flame ionization detector (GC-2014, Shimadzu, Japan). Carbohydrate content of the biomass was determined with the modified quantitative saccharification method [15] as described by Ho et al. [16]. Total protein content of the biomass was measured from acid hydrolyzed sample diluted with NaN_3 solution [17] as described by Ho et al. [18].

Total and soluble chemical oxygen demand (COD_{tot} and COD_s) were analyzed with the standard dichromate method 5520C: Closed Reflux, Titrimetric method [19]. COD_s sample was filtrated with 0.45 μm syringe filter prior to the analysis. Total Kjeldahl nitrogen and ammonium (TKN and NH_4-N) from the wastewater were analyzed with the standard method 4500-N(org)C: organic nitrogen/Semi-Micro-Kjeldahl [19]. NO_3^- and PO_4^- ions from the filtrated (0.20 μm) wastewater were analyzed with an Ion Chromatograph (ICS-5000, Dionex, Italy). During the experiments, ammonium and carbon concentrations were determined with TOC/TN_b analyzer (LiquiTOC II, Elementar, Germany) from filtrated samples (0.45 μm). $(NH_4)_2SO_4$ was used as a nitrogen standard. Total dissolved carbon (TDC) was determined as a sum of dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC). $KHC_8H_4O_4$ was used as the standard for DOC and Na_2CO_3 as the standard for DIC. Carbohydrate content of non-hydrolyzed and hydrolyzed liquid samples (centrifugation at 5000 rpm for 10 min) was determined by phenol-sulfuric acid method [20] and sugar column as described by Marjakangas et al. [13]. The non-hydrolyzed samples were analyzed to detect the monosaccharides present in the liquid phase, while the hydrolyzed samples were analyzed to detect the polysaccharides in the liquid phase by hydrolyzing the polysaccharides into detectable monosaccharides.

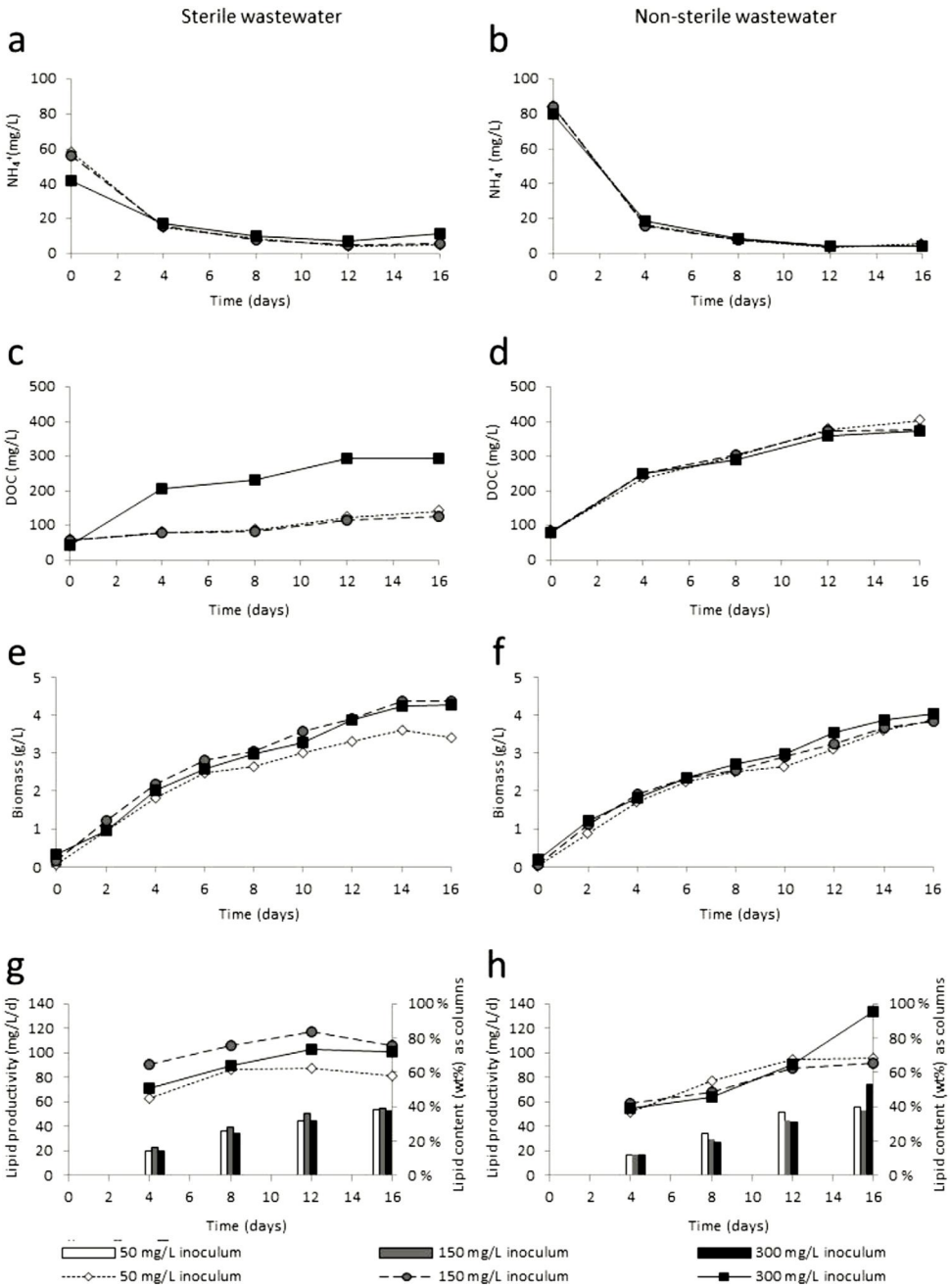

Fig. 1. NH_4^+ [a, b], DOC [c and d], biomass concentration (g/L) [e and f] and lipid content (wt%) and average lipid productivity from day zero onwards (g/L/d) [g and h] for *Chlorella vulgaris* CY5 cultivated in the sterilized and the non-sterilized 5× diluted anaerobically treated piggery wastewater.

3. Results and discussion

3.1. Effect of autoclaving on wastewater composition

Autoclaving increased the pH of the wastewater from 7.7 to 9.8 (Table 1). The NH_4^+ concentration decreased in sterilization. This was likely due to NH_3 gas formation from NH_4^+ ions caused by the increase in pH [21]. The initial NH_4^+ concentration in the cultures with the sterilized wastewater and with the non-sterilized wastewater were 52 ± 9 mg/L and 83 ± 2 mg/L, respectively. The DOC increased in the sterilization, and the initial DOC of the

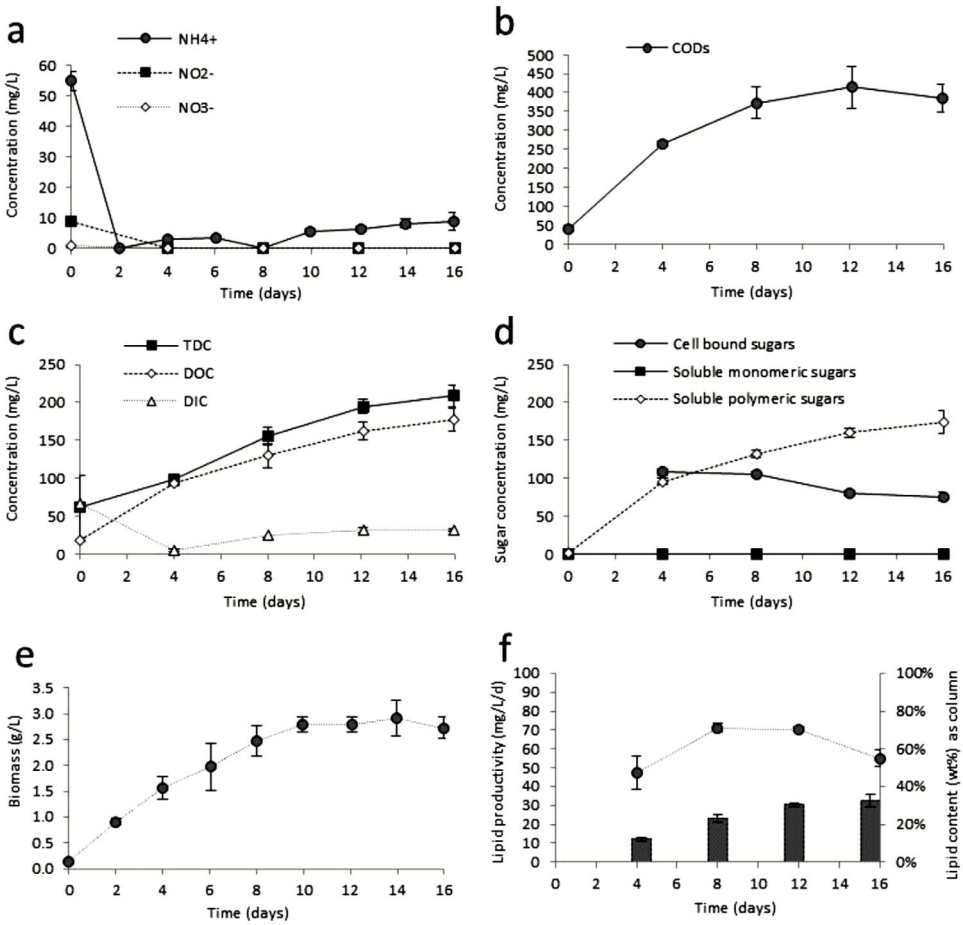

Fig. 2. Nitrogen concentration (NH_4^+, NO_2^-, NO_3^-) [a], COD_s concentration [b], dissolved carbon concentration (TDC, DOC, DIC) [c], total sugar concentration (cell bound sugars, monomeric sugars, polymeric sugars) [d], biomass concentration (g/L) [e] and lipid content (wt%) and average lipid productivity from day zero onwards (g/L/d) [f] for *Chlorella vulgaris* CY5 in non-sterilized 5× diluted anaerobically treated piggery wastewater.

sterilized and non-sterilized wastewater were 157 ± 14 mg/L and 130 ± 3 mg/L, respectively.

3.2. Changes in culture pH during the microalgal growth

After 1 day of incubation the culture pH with sterilized wastewater dropped below 4, and therefore, the culture pH with sterilized wastewater was neutralized using NaOH. The pH decreased to 2.8 on day 1 in the culture with sterilized wastewater and 300 mg/L inoculum. This very likely inhibited algal growth. The pH decrease was likely caused by lower buffer capacity of the sterilized wastewater and higher rate of ammonium oxidation with higher inoculum concentration, as consumption of ammonium as a nitrogen source decreases pH [14]. After the pH adjustment, pH with the sterile wastewater remained near neutral (7.3 ± 0.4). With the non-sterilized wastewater no pH adjustments was required. At the beginning, culture pH was 8.4 and it decreased slightly to 7.2 ± 0.1 during the first 4 days. For the rest of the incubation, the culture pH remained at 7.0±0.1. These results show that sterilization is not cost effective, not only due to the sterilization expenses but also due to the chemical costs of the required pH adjustment.

3.3. Removal of nitrogen during the microalgal growth

Ammonium was mostly oxidized during the first 4 days from both the sterilized and the non-sterilized wastewater (Fig. 1a and b). In the experiment repeated with the non-sterilized wastewater the initial ammonium concentration of the non-sterilized wastewater was lower than in the experiment comparing sterilized and non-sterilized wastewater (55.3 mg/L vs. 83 ± 2 mg/L) likely due to storing of the wastewater in a fridge for some time. Seasonal and even hourly variation in wastewater quality is a reality in municipal wastewater treatment plants [22]. Boursier et al. [23] also showed that piggery wastewater composition in respect to e.g. COD and total nitrogen from the same swine production unit can vary at different times. Thus, it is also important to understand how the differences in wastewater quality affect nutrient removal efficiency and microalgal biomass production. In the second experiment with the non-sterilized wastewater the ammonium was removed already by day 2 (Fig. 2a), which indicated faster nitrogen removal and microbial growth than that obtained in the first experiment with both the sterilized and the non-sterilized wastewater. Nitrate and nitrite were also removed below the detection limit

within the first few days in the second experiment with the non-sterilized wastewater (Fig. 2a). These results demonstrate that *C. vulgaris* effectively removed nitrogen from the piggery wastewater despite the small variations in the wastewater composition.

3.4. Removal and release of carbon during the microalgal growth

The concentration of dissolved organic matter was measured as COD_S and TDC, which is the sum of DOC and DIC. The concentration of dissolved carbon increased during incubation with both the sterilized and the non-sterilized wastewater (Fig. 1c and d), which is not desirable in wastewater treatment. With non-sterilized wastewater, the increase of DOC was similar with all inoculum concentrations, whilst in cultures with sterilized wastewater DOC increased much more when initial inoculum concentration was 300 mg/L than 50 or 150 mg/L. *C. vulgaris* species have been reported to produce more intracellular and extracellular polysaccharides as a protective response against oxidative stress [24]. In our study, the higher DOC with 300 mg/L inoculation was likely caused by low pH stress (2.8) on day 1. In further experiment with the non-sterilized wastewater, DOC also increased during incubation (Fig. 2b and c). The DIC decreased within the first four days and remained at low level for the rest of the incubation. COD_S also increased with time, while most of the increase occurred during the first eight days of cultivation (Fig. 2b).

Contrary to our results, in other studies, *Chlorella* spp. removed COD (and thus DOC) from piggery wastewater [3,10,25,26]. However, *C. vulgaris* has been demonstrated to release DOC in synthetic medium during photoautotrophic growth [27]. In fact, *Chlorella* like species has been reported to release up to 12.5% from the total assimilated carbon as extracellular organic compounds [28]. Many flask studies without additional CO_2 feeding to boost autotrophic growth demonstrated algae-mediated COD removal from piggery wastewater [9,11,25,26]. Some studies with additional CO_2 feeding also demonstrated removal of COD [3,10,12]. For example, Zhu et al. [10] studied *Chlorella zofingiensis* in piggery wastewater in tubular bubble column photobioreactor using compressed air aeration with 5–6% CO_2, sunlight intensity of 842 ± 778 μmol/m^2/s and initial COD of 3.5 g/L. Despite the CO_2 feeding, both nitrogen and COD by the microalgal culture were removed [10]. Wu et al. [29] studied *Nannocholropsis oculata* in combination of anaerobically/aerobically pretreated swine wastewater by providing CO_2, but they only reported removal of nitrogen and phosphorus.

The increase in the soluble carbohydrate concentration in this study was proportional with the biomass concentration; 134 ± 6 mg total sugars per 1 g of biomass was released (Fig. 2d). Sugars were not detected from the non-hydrolyzed supernatant samples whereas in the hydrolyzed supernatant samples, the carbohydrate concentration increased with time (Fig. 2d). This demonstrated that the released carbohydrates were polysaccharides. The hydrolyzed polysaccharides contained 21 ± 2% arabinose, 37 ± 0.6% galactose and 41 ± 2% rhamnose. According to Maksimova et al. [30], *Chlorella pyrenoidosa* released extracellular carbohydrates consisting of galactose, mannose, arabinose, xylose, ribose, fucose, and rhamnose. Yalcin et al. [31] found *Chlorella* sp. to produce gum, which contained sugars and minerals.

The release of carbohydrates from algae depends on the photosynthetic activity [30]. In our study, the culture conditions changed from mixotrophic to photoautotrophic due to the low initial concentration of organic carbon. Based on the assumption that microalgae can produce approximately 2 g of biomass from 1 g of carbon [32], less than 100 mg/L from the microalgal biomass in our study was produced heterotrophically from the wastewater, as the initial carbon content in the cultivations was below 50 mg DOC/L. Therefore, it can be concluded that most of the microalgal biomass was produced autotrophically. The increase of different soluble sugars between day 4 and 12 – converted to COD equivalents according to Haandel and Lubbe [33] – was 155 mg/L, while the measured increase in COD_S was 150 mg/L. This demonstrated that the increase in COD_S was mainly caused by the release of carbohydrates. Before day 4, increase in the different sugars converted to COD equivalents was also rather similar to the measured COD_S (250 mg/L vs. 220 mg/L), but not after 12 days of incubation (32 mg/L vs. −29 mg/L). In the experiment with sterilized wastewater the increase of DOC concentration and biomass concentration (Fig. 1c and f) did not correlate. In the dark reactions of photosynthesis, mainly carbohydrates, but also fatty acids, amino acids and organic acids, are produced from CO_2 [34]. Therefore, the growth conditions must be carefully considered when designing a simultaneous lipid-rich biomass production and wastewater treatment. Dilution decreases the nitrogen concentration, but also the carbon content, which causes changes in the metabolism of microalgae [35].

3.5. Production of biomass

With sterilized wastewater, final biomass was higher with inoculum concentration of 150 and 300 mg/L than with 50 mg/L (Fig. 1e). However, with non-sterilized wastewater the inoculum had no coherent effect on biomass production (Fig. 1f). Less biomass was produced when the wastewater was not sterilized. The difference between the biomass production with sterilized and non-sterilized wastewater became clearer after nitrogen was consumed. Final biomass concentrations after 16 days of incubation with 150 mg/L inoculum concentration were 4.4 g/L and 3.8 g/L, respectively. In the experiment repeated with the non-sterilized wastewater, the biomass measured as dry weight increased until day 10 (Fig. 2e), indicating that the exponential growth by day 10. The final biomass was 2.7 ± 0.2 g/L and it was lower than the biomass obtained in the first experiment with both of the sterilized and the non-sterilized wastewater.

In the non-sterilized wastewater microalgae competed for the nutrients and space with other microorganisms, which were not viable in sterilized wastewater. The microscopy examination illustrated the proportion of microalgal cells to decrease and the number of bacteria and eukaryotes to increase after nitrogen was removed (micrographs not shown). However, the cultures were not taken over by protozoa, as no major decrease in the biomass concentration was detected.

In a previous study using mixed algal cultures and biphasic cultivation, mixotrophic growth conditions were more suitable for biomass production, while autotrophic conditions with nitrogen deprivation were more suitable for lipid accumulation [36]. In this study, the sterilized wastewater contained slightly more COD than the non-sterilized wastewater (66.4 vs. 75.4 mg COD_{tot} /L), which has likely increased biomass production. However, the ammonium content was slightly lower in the sterilized wastewater than in the non-sterilized one (287 vs. 348 mg TKN/L), which likely reduced the biomass production. Based on our calculations of the differences in the biomass production on non-sterilized wastewater in the two separate experiments can partly be explained by the variation in the chemical composition of the wastewater. No clear correlation between the initial ammonium concentration and the biomass production could be seen. However, the ratios between biomass concentrations (g/L) on day 12 and initial carbon concentrations (g CODs/L) were quite similar in the two experiments (73 vs. 69), despite the fact that most of the microalgal biomass was produced autotrophically using inorganic carbon.

Although the availability of carbon and nutrients affects the growth, effect of sterilization on the microbial community of the wastewater had also clear effect on the biomass production. After nitrogen deprivation, bacteria likely outcompeted the microalgae

in non-sterilized wastewater, and therefore higher biomass concentration was obtained with sterilized wastewater. The results show that *C. vulgaris* was able to grow with the mixed culture of microorganisms originating from the anaerobically pretreated wastewater.

3.6. Composition of biomass and production of lipids

Biomass composition of *C. vulgaris* in the non-sterilized wastewater changed with time. Approximately 1 g/L (45±6%) of the biomass was not carbohydrates, proteins or lipids (Fig. 3). Lipid and protein content of the biomass increased with time, while carbohydrate content decreased with time. Based on the average lipid productivity from day zero onwards (Fig. 2f), day 8 or 12 would be the best day to harvest the biomass. On day 12 the biomass concentration had reached the maximum (Fig. 2e). On day 12 lipids included mainly C16:0, C18:2, C18:1, C18:3, C17:0, C18:0 and C16:1 (Table 2); proteins mainly glutamine, arginine, phenylalanine, glutamate and alanine; and carbohydrates mainly glucose and low level of galactose.

Cellular lipid content with the sterilized and the non-sterilized wastewater and all the studied initial inoculum concentrations was above 30 wt% after 12 days of incubation (Fig. 1g and h, Fig. 2f). Lipid productivity was higher with the sterilized wastewater than with the non-sterilized wastewater due to the higher biomass production. The highest lipid productivity measured from day zero onwards was obtained between day zero and 12. In cultures, where 150 mg/L inoculum was used, biomass concentration, lipid content and average lipid production in the sterilized wastew-

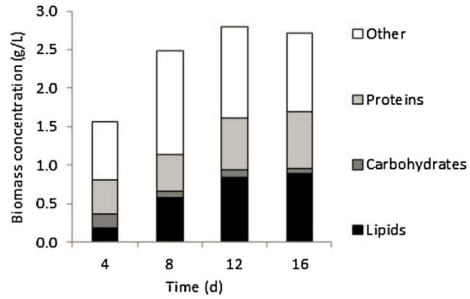

Fig. 3. Biomass composition of the experiment with *Chlorella vulgaris* CY5 in non-sterilized 5× diluted anaerobically treated piggery wastewater.

Table 2
Lipid content (average ± standard deviation) of *Chlorella vulgaris* CY5 incubated in 5 times diluted non-sterilized anaerobically treated piggery wastewater.

Time (d)	Lipid content(wt%)	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3
4	12.1 ± 0.9	3.4 ± 0.4	0.3 ± 0	1.1 ± 0	0.8 ± 0	2.0 ± 0.3	3.4 ± 0.2	1.4 ± 0.1
8	23.1 ± 2.3	6.4 ± 0.6	0.5 ± 0.1	1.6 ± 0	1.2 ± 0.1	5.2 ± 0.8	5.7 ± 0.2	1.9 ± 0.1
12	30.3 ± 1.2	8.0 ± 0.3	0.6 ± 0	2.0 ± 0.1	1.5 ± 0.1	7.0 ± 0.7	7.6 ± 0.2	2.3 ± 0.2
16	32.5 ± 3.2	8.2 ± 0.9	0.7 ± 0.1	2.2 ± 0.2	1.7 ± 0.1	7.7 ± 1.1	8.4 ± 0.7	2.5 ± 0.3

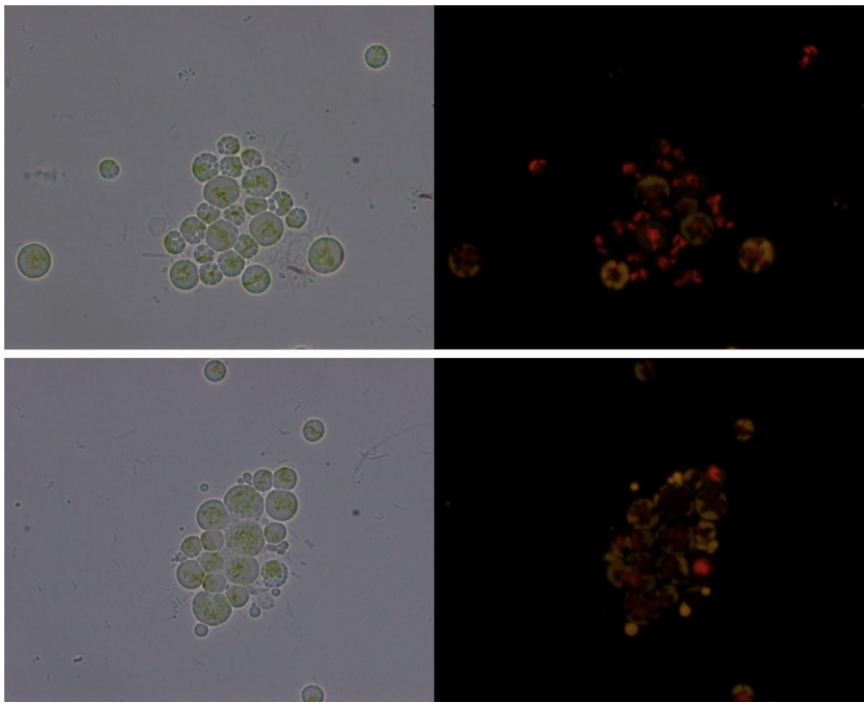

Fig. 4. Phase contrast micrographs (left-hand) and fluorescence micrographs (right-hand) of Nile Red stained *Chlorella vulgaris* CY5 cultivated in the 5× diluted non-sterilized anaerobically treated piggery wastewater (Cultivation condition: 1 L Batch reactor; CO_2 supply, 2.5%, 0.1 vvm; light source, TL5; light intensity 150 μmol/m^2/s).

Table 3
Biomass concentration (g/L) and lipid content (wt%) of *Chlorella* spp. cultivated in various wastewaters.

Medium	Strain	Time (d)	Biomass (g/L)	Lipid content (wt%)	Reference
Dairy manure	*Chlorella* sp.	21	1.5–1.7	9–13.7	[38]
Dairy wastewater (UV treated)	*C. vulgaris*	4	0.9–1.9	10.3–14.4	[12]
Piggery wastewater	*C. vulgaris*	20	0.5	29	[9]
Piggery wastewater	*C. vulgaris*	30	1	28	[25]
Piggery wastewater	*C. zofingiensis*	10	1.0–3.0	33.91–45.81	[3]
Piggery wastewater	*C. zofingiensis*	10	2.9–3.0	33.0–33.3	[10]
Piggery wastewater (sterile)	*C. vulgaris*	12	3.90	36	This study
Piggery wastewater (non-sterile)	*C. vulgaris*	12	3.24	32	This study
Piggery wastewater (non-sterile)	*C. vulgaris*	12	2.8 ± 0.2	30 ± 1	This study
Primary piggery wastewater	*C. pyrenoidosa*	10	0.1–0.3	12–22	[11]
Swine manure	*Chlorella* sp.	7	0.5–1.6	27–30	[26]

ater were 3.90 g/L, 36 wt% and 116 mg/L, and in the non-sterilized wastewater 3.24 g/L, 32 wt% and 87.5 mg/L, respectively. With three parallel non-sterilized wastewater cultivations, the corresponding results were 2.8 ± 0.2 g/L, 30.3 ± 1.2 wt% and 70.5 ± 1.1 g/L/d, respectively. The highest lipid content with non-sterilized wastewater (32.5 ± 3.2 wt%) was obtained on day 16 (Fig. 2f) and the highest average lipid productivity (71.2 ± 2.2 g/L/d) on day 8 (Fig. 2f). Micrographs (Fig. 4) with Nile Red stained biomass demonstrated the accumulation of lipids in *C. vulgaris* cells grown in non-sterilized piggery wastewater. However, the competition with the other microorganisms decreased the lipid productivity slightly in the non-sterilized wastewater compared to the sterilized wastewater.

Microalgal lipid production from piggery wastewater has previously been mainly studied using sterilized wastewater [3,9–11,25], although treatment of non-sterilized piggery wastewater in algal ponds has been studied without measuring the lipids [37]. For example, Whang et al. [11] studied lipid production of *C. pyrenoides* with piggery wastewater in 1 L conical flasks. Initial COD varied between 0.25–1 g/L and no CO_2 was added. The biomass after 10 days of incubation was 0.1-0.3 g/L, and therefore the lipid productivity was only 6.3 mg/L/d [11]. In their experimental conditions concentration of both nutrients and COD decreased, likely due to heterotrophic utilization of the organic carbon and nutrients.

Compared to the previous studies conducted with sterilized wastewaters, the biomass and lipid productivities obtained in this study both with the sterilized and the non-sterilized wastewater were comparable or higher (Table 3). Although the obtained lipid productivity was lower with the non-sterilized wastewater than the sterilized wastewater, the difference was not significant, when considering the high cost and energy demand of autoclave sterilization and the fact that pH control was not required in the cultures grown on the non-sterilized wastewater.

4. Conclusions

C. vulgaris CY5 is able to grow and produce lipids in non-sterilized anaerobically pretreated piggery wastewater. However, biomass production is slightly higher in the sterilized wastewater due to the absence of competition from nutrients and space with other wastewater microorganisms. The average lipid productivity from day zero to day 12 was higher with the sterilized wastewater compared to the non-sterilized one (117 g/L/d vs. 91.3 g/L/d). Whilst in further study with the non-sterilized wastewater the lipid content on day 12 was 30.3 ± 1.2 wt% and lipid productivity between day zero and 12 was 70.5 ± 1.1 g/L/d. Due to the low carbon content of the diluted piggery wastewater, microalgal growth was mainly photoautotrophic and resulted in excretion of DOC to the medium. Excretion of DOC should be taken into account when evaluating the suitability of microalgae for simultaneous wastewater treatment and lipid production, especially in the case of wastewaters with low concentration of organic carbon.

Acknowledgements

Thanks for TUT's Graduate School for funding the research exchange. Thanks also for Maj and Tor Nessling foundation, TUT foundation and A.R.Winter's memorial foundation for travel grants. The authors also acknowledge the financial support received for this work from Taiwan's Ministry of Science and Technology (104-3113-E-006-003, 103-3113-E-006-006 & 103-2221-E-006-190-MY3). This work is also supported in part by the Taiwan's Ministry of Education under the ATU plan.

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FI-33101 Tampere, Finland

ISBN 978-952-15-3631-1 (printed)
ISBN 978-952-15-3632-8 (PDF)
ISSN 1459-2045