UNIVERSIDAD DE CÓRDOBA
ÁREA DE MÁQUINAS Y MOTORES TÉRMICOS
Departamento de Química Física y Termodinámica Aplicada

SECOND GENERATION BIOFUELS FROM MICROBIAL OIL

Ph.D.Thesis

David E. Leiva-Candia

Supervisors: María del Pilar Dorado Pérez, Universidad de Córdoba
Colin Webb, University of Manchester

TITULO: *Second generation biofuels from microbial oil*

AUTOR: *David E. Leiva Candia*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

www.uco.es/publicaciones
publicaciones@uco.es

TÍTULO DE LA TESIS: Second generation biofuels from microbial oil

DOCTORANDO/A: David Eduardo Leiva Candia

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El doctorando David E. Leiva Candia ha demostrado, a lo largo de esta tesis, un notable interés en la investigación, que se ha plasmado en exhaustivas búsquedas bibliográficas a lo largo de todo el periodo, elaboración minuciosa de diseños de experimentos, toma de decisiones certeras y análisis concienzudo de los resultados obtenidos. Su trabajo en el laboratorio ha sido muy cuidadoso, mostrando grandes habilidades para el trabajo en equipo e incluso la dirección de investigación. Se ha responsabilizado del correcto funcionamiento de diversos equipos de experimentación y ha contribuido al buen funcionamiento del equipo investigador. Fruto de esta investigación ha sido la publicación de varios artículos en revistas internacionales de alto prestigio, así como capítulos de libro y comunicaciones en diversos congresos, nacionales e internacionales.

Su esfuerzo por realizar una investigación de calidad le ha llevado realizar una estancia de tres meses en la Universidad de Manchester, Reino Unido y otra de similar duración en la Universidad Agrícola de Atenas, Grecia, donde ha alcanzado las destrezas precisas para llevar a buen puerto el desarrollo de esta tesis.

Consideramos que el doctorando ha cubierto los objetivos de su formación, que presenta un trabajo novedoso y de alta calidad y que está capacitado para continuar avanzando en esta línea, dirigiendo su propia investigación y colaborando con otros investigadores en materias afines.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, _17_ de __enero__ de __2014__

Firma del/de los director/es

Fdo.:_Colin Webb__ Fdo.: _María del Pilar Dorado Pérez__

Table of contents

List of tables

List of figures

1. Agradecimientos

La realización de esta tesis implica un apartado enorme de agradecimientos ya que mucha gente aportó no sólo un grano de arena sino un castillo completo.

Comenzaré por agradecer a mi madre que fue y sigue siendo mi guía en la vida, siempre confiando y apoyando en buenos y malos momentos. Sigo escuchando la frase que me decías: ¡TU PUEDES HIJO!. Sé que desde el cielo lo estarás disfrutando casi tanto como yo. A mi padre que me enseñó que sin sacrificio no hay logros, que el dolor del trabajo es pasajero pero el dolor por no conseguir las metas se queda para toda la vida. ¡Gracias viejos queridos!.

A mis hermanos, sobrinos, cuñadas y toda mi familia chilena, por entender que mi camino estaba lejos de ellos físicamente, pero siempre cerca en el corazón, ¡gracias a todos!.

A mi esposa Ana por darme siempre el empujoncito que necesitaba, por tu compañía, por tu cariño, por tu comprensión, por tu paciencia y por ser como eres. Tú eres la gran gestora de esta tesis. Sobre todo gracias por darme los hijos que me has dado, ¡Te amo y gracias por estar conmigo!.

A mis hijos Nico y Leonor por darme un poco de su tiempo para irme de estancias y tiempo de más para conseguir esta tesis, por siempre

recibirme con un beso y una sonrisa en los momentos que no salían las cosas e igualmente cuando el cansancio me sobrepasaba. Además agradecer a mis dos pequeños científicos, porque gracias a ellos muchas veces logré ver cosas complejas como algo sencillo, como cuando les comenté que los bichitos no se querían comer el sustrato, Leonor me dijo: pues papá dale patatitas que están muy buenas y Nicolás me dijo: seguro es porque tiene cosas verdes. ¡¡¡¡No estaban tan lejos de la realidad!!!. Mis futuros doctores os espero.

A mis suegros José Luis y Pepi por asumir el papel de padres y darme su apoyo incondicional cuando más lo necesitaba. A mis cuñadas María, Susana, Dani, Juan Antonio y mi compadre Cana por escuchar sobre mi tema de tesis y poner cara de interés cuando me pasaba de emoción, ¡Gracias por eso y por el apoyo!. A toda mi familia y amigos de España por acogerme sin conocerme y su apoyo incondicional en todo momento.

Al grupo de investigación Biosahe por ser como sois, buenos compañeros y grandes investigadores. A Sara ¡¡por enseñarme que no soy tan loco!! que siempre hay alguien todavía más, por entregarme sus conocimientos y su amistad sin nada a cambio. A mami Pilar por ver algo en mi que aún no veo, por siempre darme palabras de apoyo y confianza para que despegue, por darme desafíos y por dejarme ser como soy ¡Gracias!. A Isa y Javi por sus conversaciones de la vida que siempre ayudan. A Loli por dejarme aprender de ella, a Marta, Kika, Azahara, Laure y Pepe por su comprensión y cariño. A Jesús López por

entregarme parte de su sabiduría y darme la oportunidad de conocerlo junto a su familia, a la cual estimo, en especial a su esposa María. En general a todos aquellos que han formado o formarán parte de éste grupo tan compacto y especial que tenemos. Por sobre todo quiero agradecer que nos permitamos reír juntos sobre las cosas, por muy duras que sean.

A mis compañeros de departamento y área, Fernando, Inés, Manuel, Francisco y Paco, gracias por compartir vuestra amistad y paciencia. A Paco por contarme sus telenovelas amorosas todos los lunes y compartir su gusto por la misma película que yo, "princesa por sorpresa", que ya es difícil encontrar alguien con esos gustos, ¡gracias amigos! Al departamento de Química Física ¡a todos Gracias!. Apartado especial a Don José Agüera por dejarme aprender de él, estoy seguro que ya estás trabajando para disminuir la entropía del universo desde el cielo, suerte en ello, ya nos veremos allí y podré seguir aprendiendo de ti.

A mis directores Pilar y Colin por su tiempo, apoyo y paciencia, por dejarme aprender sus grandes conocimientos. En especial como directora a Pilar que tuve que llegar a llamarla Mami, porque así me trata. Recordar que cuando comencé me dijiste que un doctorando al final de su tesis debe saber más que su director pero ahora que llegue al final te respondo, ¡¡no lo conseguí!!, pero es porque tenía la meta muy alta, aunque igual me siento satisfecho y aún me queda tiempo para seguir aprendiendo de ti.

A los investigadores y amigos que conocí en ambas estancias en Manchester y Atenas. Ernesto, Sofia, Vaso, Nikos, Harris, Mary y todos los demás. En especial a Apostolis por darme el achuchón que necesitaba para acabar la tesis y por adentrarme en el tema de las fermentaciones que me encanta.

Finalmente agradecer a todos por entenderme y dejarme vivir mi locura que es una parte muy importante para mi desarrollo emocional.

¡¡¡Gracias!!!

Resumen

El biodiésel se puede producir a partir de diferentes fuentes oleaginosas, siendo las más utilizadas los aceites vegetales, debido a su disponibilidad y a aportar un biocombustible con propiedades similares al gasóleo. Sin embargo, el uso de aceite vegetal genera controversia debido a su baja sostenibilidad, el conflicto potencial con el sector alimenticio y la utilización de tierra cultivable para uso energético. Por ello, en esta tesis se hace una revisión sobre alternativas novedosas a las materias primas tradicionales para producir biodiésel. Por motivos de sostenibilidad, el uso de prácticas agrícolas en los cultivos oleaginosos, principalmente fertilizantes, debe reducirse al mínimo. En esta tesis se ha apreciado que no afecta a la calidad del biodiésel producido, lo cual redunda en un beneficio para el medio ambiente.

Otro pilar en que se sustenta esta tesis trata sobre el biodiésel obtenido a partir de aceite microbiano producido por levaduras oleaginosas, junto a la reutilización de residuos agroindustriales. Estos microorganismos pueden acumular diferentes cantidades de lípidos con perfil de ácidos grasos variable, según el sustrato utilizado o las condiciones de crecimiento. Así, se aprecia que la glicerina, subproducto de la producción de biodiésel, combinado o no con hidrolizado de torta de prensado de semillas oleaginosas, es una fuente de carbono adecuada para muchas levaduras oleaginosas en la producción de aceite. Finalmente, se concluye que una optimización de las condiciones de cultivo (modo de cultivo, temperatura, etc.) para cada una de las levaduras puede mejorar la acumulación de lípidos intracelulares.

Summary

Biodiesel can be produced from different oleaginous sources. Currently, the most extended biodiesel originates from vegetable oils, due to their availability and similar properties to diesel fuel. However, the use of vegetable oil as feedstock to produce biodiesel is controversial due to biodiesel low sustainability, potential conflict with food and the use of arable land for energy purposes. In this context, this thesis reviews novel alternatives to the traditional raw materials used to produce biodiesel. Moreover, for sustainability reasons, the use of agricultural practices in oilseed crops, focused on fertilizers, should be reduced. In the present thesis, no influence over the quality of biodiesel has been found, thus environment benefits are achieved.

The study of microbial oil produced from oleaginous yeasts in tandem with reutilization of agro-industrial waste is another key point of this thesis. Oleaginous yeast accumulates intracellular lipids through the fermentation of various agro-industrial wastes. These microorganisms accumulate different amounts of lipid with variable fatty acid composition, according to the substrate used or the growth conditions. Thus, it appears that glycerol, a by-product from the biodiesel industry, in combination or not with hydrolysates from oilseed meal, is suitable as a carbon source for many oleaginous yeasts for the production of lipids. Finally, it may be concluded that the optimization of the culture conditions (culture mode, temperature, etc.) for each oleaginous yeasts can improve the intracellular lipid accumulation.

Preface

Worldwide, the global energy requirement for transport is increasing. Despite the high pollution derived from the use of compression ignition engines, vehicles with diesel engines are the most widely used in Europe. For this reason, the development of alternative energies to fossil fuels is of vital necessity. In this context, biodiesel appears as a realistic solution to environmental and energy dependence problems derived from the use of fossil diesel fuel. Biodiesel can be produced from different oleaginous sources. Currently, the most extended biodiesel originates from vegetable oils, due to similar properties to diesel fuel. However, the use of vegetable oil as feedstock to produce biodiesel is controversial due to biodiesel low sustainability and potential conflict with food and fiber production for the use of arable land. Another issue that needs to be fixed is the cost of production, mostly influenced by the price of the raw material. For these reasons, the study of new oleaginous sources to produce biodiesel is of special interest for the scientific community.

In this context, this PhD thesis is seeking to provide solutions to the problems created in the current biodiesel industry. This thesis comprises four chapters, composed of book chapters edited by internationally reputed publishers and articles published in peer-reviewed journals belonging to the first quartile in the category Energy & Fuels of the Journal Citation Reports, according to the Institute for Scientific Information (ISI Thompson Reuters) database.

Chapter 1 presents the state of the art concerning non-edible raw materials to produce biodiesel, ranging from non-edible vegetable oils, animal fats, waste oils, soapstocks, oleaginous insects and oleaginous microbial oils. This review was conducted with the objective of finding out the most suitable non-edible oleaginous sources to further use in the production of biodiesel. Technical and economical issues related to raw material processing and subsequent conversion in biodiesel were taken into consideration.

As biodiesel costs are key for its commercialization, the reduction of inputs, like fertilization, during the cultivation constitutes an interesting alternative to lower the final cost. Chapter 2 comprises the study of the effect of nitrogen fertilization in rapeseed oil and biodiesel properties. This oilseed was selected because it is the favorite oleaginous crop for biodiesel production in Europe. Moreover, European standard for biodiesel, EN 14214, was built up based on rapeseed oil-derived biodiesel. The objective of this study was to provide information about the influence of N fertilization on biodiesel production and properties, with the aim of reducing production costs while keeping biodiesel adequate quality.

Chapters 3 and 4 are focused on the study of promising feedstocks for biodiesel production, mainly based on microbial oil from oleaginous yeast, besides the recycling of wastes. Depending on the selected microorganism, microbial oil may possess similar chemical composition to that of the most commonly used vegetable oils in the biodiesel industry. Moreover, oleaginous yeasts could accumulate intracellular lipids through cultivation on various agro-industrial wastes. Oleaginous yeasts may accumulate different amounts of microbial oil with varying fatty acid profiles, depending on the substrate or growing

conditions. For this reason, chapter 3 presents the most relevant issues concerning yeast oil production using agro-industrial waste as culture medium and evaluates the potential of this microbial oil as feedstock for biodiesel production. Chapter 4 focuses on the design of a new concept of biodiesel industry, where the main target is the production of microbial oil using a combination of by-products derived from the biodiesel industry, namely glycerol and the sunflower oil industry, namely sunflower meal. This study demonstrates that the valorization of agro-industrial wastes and by-product streams from the biodiesel industry could lead to the development of technologically viable new concept of biodiesel industry.

Finally, a chapter comprising a compendium of the conclusions derived from this thesis, followed by a bibliographic reference chapter are included.

Prefacio

A nivel mundial, el requisito global de energía para el transporte es cada vez mayor . A pesar de la elevada contaminación derivada del uso de los motores de encendido por compresión (MEC), este tipo de vehiculos son los más utilizados en Europa. Por esta razón , el desarrollo de energías alternativas a los combustibles fósiles es de vital necesidad. En este contexto, el biodiésel se presenta como una solución a los problemas ambientales y la dependencia energética derivados de la utilización de combustible de origén fósil . El biodiésel puede ser producido a partir de diferentes fuentes oleaginosas, siendo actualmente la más utilizada la que se origina a partir de aceites vegetales , debido a las propiedades similares al gasóleo. Sin embargo, el uso de aceite vegetal como materia prima es controversial debido a la baja sostenibilidad, el potencial conflicto con el sector alimenticio y la utilización de tierra cultivable para uso energético. Otro factor a estudiar es el costo de la producción , sobre todo por el alto precio de la materia prima. Por estas razones , el estudio de nuevas fuentes de oleaginosas para producir biodiésel es de especial interés para la comunidad científica .

En este contexto, la presente tesis doctoral pretende aportar soluciones a los problemas creados en la industria de biodiésel actual. Esta tesis consta de cuatro capítulos , compuestos de capítulos de libros editados por editoriales y artículos de prestigio internacional publicados en revistas que pertenecen al primer cuartil en la categoría de Energy &

Fuels del Journal Citation Reports , según el Institute for Scientific Information (ISI Thompson Reuters).

El Capítulo 1 presenta el estado del arte en relación con las materias primas no comestibles para producir biodiésel , que van desde aceites vegetales no comestibles, grasas animales, aceites usados, insectos oleaginosos y aceites microbianos. Esta revisión se realizó con el objetivo de descubrir las materias primas más adecuados para su uso posterior en la producción de biodiésel, tomando en consideración problemas técnicos y económicos relacionados con el procesamiento de la materia prima y su posterior conversión en biodiésel.

Como los costos de biodiésel son la clave para su comercialización , la reducción de los insumos, como la fertilización, durante el cultivo constituye una alternativa interesante para reducir el costo final. El Capítulo 2 comprende el estudio del efecto de la fertilización nitrogenada en propiedades del aceite y biodiésel de colza. Esta oleaginosa fue seleccionado porque es el cultivo preferido para la producción de biodiésel en Europa. Por otra parte , es el que más se ajusta al estándar europeo para biodiésel en cuanto a propiedades (EN 14214). El objetivo de este estudio es proporcionar información sobre la influencia de la fertilización nitrogenada sobre la producción y las propiedades del biodiésel, con el objetivo de reducir los costes de producción , manteniendo biodiésel de calidad adecuada.

Los capítulos 3 y 4 se centran en el estudio de las materias primas prometedoras para la producción de biodiésel , con base principalmente en aceite microbiano producido a partir de levaduras oleaginosas en conjunto con la reautilización de residuos agroindustriales. Las levaduras oleaginosas acumulan lípidos intracelulares a través de la fermentación de diversos desechos

agroindustriales. Las levaduras oleaginosas pueden acumular diferentes cantidades de aceite microbiano con perfiles de ácidos grasos diferentes, dependiendo del sustrato o las condiciones de crecimiento . Por esta razón , el capítulo 3 se presentan los aspectos más relevantes en relación con la producción de aceite de levaduras utilizando residuos agroindustriales como medio de cultivo y evalúa el potencial de este aceite microbiano como materia prima para la producción de biodiésel. El Capítulo 4 se centra en el diseño de un nuevo concepto de la industria de biodiésel, donde el objetivo principal es la producción de aceite microbiano utilizando una combinación de los subproductos derivados de la industria del biodiésel , como es la glicerina y la industria de aceite de girasol, como es la torta del prensado de la semilla de girasol. Este estudio demuestra que la valorización de los residuos agroindustriales y subproductos de la industria del biodiésel podría conducir al desarrollo de nuevo concepto tecnológicamente viable de la industria.

Por último, se incluye un capítulo que agrupa un compendio de conclusiones derivadas de esta tesis doctoral, seguido por un capítulo de referencias bibliográficas.

Chapter 1

"Feedstock to produce next generation biodiesel"

Chapter 1. Feedstocks to produce next generation biodiesel

Compiled from:

"Latest trends in feedstocks for biodiesel production" published in Biofuels, Bioproducts and Biorefining. DOI: 10.1002/bbb.1435

"New frontiers in the production of biodiesel: biodiesel derived from macro and microorganism" accepted in liquid biofuels: emergence, development and prospects

Abstract The biodiesel industry is gaining interest in the past years due to the depletion of the easily extracted-petroleum, the increasing demand to the automotive market and the environmental damage. It is acknowledged that the main obstacle to biodiesel marketing is the cost of production, which is mostly due to the price of the raw material (usually vegetable oils). Edible seed oil biodiesel has been criticized due to its low sustainability and potential conflict with food and fiber production for the use of arable land, besides high water and fertilizer requirements. In this context, biodiesel from non-edible sources, like animal fat, waste oil, insect oil or single cell oil constitutes an alternative biofuel that omits the previous drawbacks. In this review and taking into account the previous consideration, the most interesting feedstocks for biodiesel production are shown. While frying oils and animal fats constitute the most extensively studied non-edible raw materials for biodiesel production, soapstocks are gaining interest among the scientific community. Finally, promising feedstocks for biodiesel

production, such as microbial oil (also named single cell oil), insect oil or microdiesel are reviewed.

1.1 Introduction

British Petroleum (BP) Statistical Review of World Energy estimated the accessible crude oil resources about 171.1 thousand million tones at the end of 2010. Considering the current world consumption about 11.6 million tons of crude oil per day, fossil resources will only be available for a short period of time [1, 2]. Furthermore, the combustion of fossil resources generates massive emissions of greenhouse gases that are contributing to the irreparable change to the global climate. Consequently, a sustainable and competitive alternative energy based on renewable and abundant feedstocks, like biomass [3] or other regenerative sources [2], is highly demanded.

Nowadays, biodiesel is the only direct substitute for diesel fuel in compression ignition engines and the interest on this biofuel has been growing up in the last decades because it may effectively reduce the dependence on imported fossil oil in the transport sector, in which the security of energy supply problem is most acute. Moreover, the use of biodiesel reduces greenhouse gases and the main harmful emissions to comply the new EU limits for exhaust emissions [4]. Finally, biodiesel development could provide opportunities for local and regional development, especially in rural and isolated areas.

The most common feedstocks used in biodiesel production are vegetable oils derived from edible plants, such as rapeseed, palm, soybean, sunflower and other oleaginous crops. However, biodiesel from edible oils

is controversial. During the last few years, some social movements accused biofuels from edible raw materials of being the main cause of increased global food market prices. The possible depletion of ecological resources due to intensive agricultural practices used in crop cultivation is another issue related with edible oil biofuel. Traditionally, land has not only been used for feeding purposes, i.e. coffee, tobacco, drinking alcohol, flowers or cosmetic cultivars, among many others. However, only energetic uses are under discussion. In any case, the production of food to feed the world population must be guaranteed.

Several studies have identified the price of feedstock as the most significant factor affecting the economic viability of biodiesel market [5, 6], reaching up to 70–95% of the total biodiesel production cost [7]. Thus, to provide competitive biodiesel, the price of raw material must be considered a key parameter [8]. Alternative non edible, low cost feedstocks have been analyzed to overcome the most important drawbacks of biodiesel from edible oils [9]. Non edible feedstocks may provide biofuel in a sustainable and reasonable priced way [10].

Even though research has increased in the last years, some biodiesel processing technologies are not yet available on a fully commercial scale, but it is estimated to enter the market in the coming years. Table 1.1 summarizes the most important differences between the feedstocks mentioned in this chapter for biodiesel production.

Table 1.1 Non-edible feedstocks used for biodiesel production

Feedstock	Advantages	Drawbacks	Research challenges	Ref
Waste frying oils	No difference in engine performance and exhaust emissions compared with other biodiesel	High content on FFA	To optimize and improve mass transfer using acid heterogeneous and enzymatic catalysis	[6, 11-15]
Animal fats	High concentration of saturated fatty acid: high oxidation stability and calorific value	High content on FFA for low cost fats Biodiesel with high viscosity and poor low temperature properties		[16-19]
Soapstocks	Soapstocks are a residue that the disposal is expensive	Hard transesterification process due to water and FFA content	To develop more efficient biodiesel conversion process	[20-22]

Feedstock	Advantages	Drawbacks	Research challenges	Ref
Non-edible oils	Can grow up in marginal lands Several research works carried out with new crops	Can present high FFA Competition for water and soil with edible crops	To find out the vegetable crops that produce triglycerides with optimal fatty acid composition	[23]
Microalgae oil	Several research works carried out in this field Some secondary products, such as algae biomass, can be used as fertilizer or for anaerobic digestion	Final cost of biodiesel	To optimize harvesting, dewatering and lipid extraction To find out the adequate amount of CO2, nutrients and light To develop microalgal species with high lipid production Possibility of chemically induced or auto-flocculation of microalgal cells	[24, 25]
Yeast oil	Fast growth rate	Low yield of lipids High cost of technology	To improve the growth rate and lipid accumulation of yeast in lignocellulosic substrates To study the metabolic pathway of lipid accumulation	[26]

Feedstock	Advantages	Drawbacks	Research challenges	Ref
Insect oil	Can be fed with agricultural, industrial or urban residues The resulting protein can also be used as a protein source in animal feed		To improve lipid content and fatty acid composition for biodiesel production	[27]
Molds oil		The oil accumulated is composed by more unsaturated fatty acid than that of yeast	To obtain high oil yield growing up in wastes as feedstocks or lignocellulosic substrate	[28]
Bacterial oil	Easy DNA modifications High assortment of species that can grow in different feedstocks		Genetic modifications to improve oil content yields and grow rate To improve the growth rate and lipid accumulation of yeast in wastes or lignocellulosic substrates	[29, 30]
Microdiesel	Avoid the need of reagents of transesterification	Low yields	To transfer interesting metabolic pathways of low efficient microorganisms to more efficient ones To improve microdiesel yield	[31]

1.2 Non-edible vegetable oils

Some non-edible plant oils may be potentially used to produce biodiesel. The demand of edible oils as food, and their subsequent high price, prevents their use as fuel particularly in developing countries [32]. Non-edible oil plants are naturally accessible worldwide [33]. Although there is no direct food *versus* fuel competition, indirect competition for the land may be controversial.

In the last few years, several studies have been carried out to establish the suitability of non-edible oilseed crops to produce biodiesel [10, 23, 34, 35]. Non edible oleaginous crops used in biodiesel production, including cotton (*Gossypium hirsutum*), castor (*Ricinus communis*), Jatropha (*Jatropha curcas*), karanja (*Pongamia pinnata*), mahua (*Madhuca indica*), rubber (*Hevea brasiliensis*), Ethiopian mustard (*Brassica carinata)*, castanhola (*Terminalia catappa*) and milkweed (*Asclepias syriaca*) are not discussed here, as they have been covered in depth in previous works [8, 23, 36, 37].

1.3 Waste oils

Waste cooking or frying oils (WFO), also known as yellow grease (when the FFA content of the grease reaches up to 8-12% wt.) offer a considerable potential to be used as low-cost raw material for biodiesel production. Used oils can be recycled through conversion into soap by saponification, or reused as lubricating oil or hydraulic fluid. Nevertheless, for economic reasons, used frying oil is an interesting feedstock for biodiesel production. In 2008, the total quantity of waste oils produced in

Europe, North America and some Asian countries reached 16.6 Mton [38]. Considering that total fatty acid methyl ester (FAME) production in 2009 was 11 Mton and demand is increasing by about 3.5 Mton/year, the use of WFO could have supplied the total oil demand for FAME production in 2010 [35]. Moreover, the price of waste cooking oil is 2.5–3.5 times cheaper than that of new vegetable oils, thus it has the potential to significantly reduce the final manufacturing cost of biodiesel. Large amounts of waste cooking oil are illegally drop down into rivers and landfills, causing environmental pollution, so recycling is key to reduce environmental contamination [34].

Nye *et al.* [39] described the transesterification of frying margarine and partially hydrogenated soybean oil with excess of alcohol under both acid and alkaline conditions. Later, Mittelbach and Enzelsberger [40] found that the increasing number of polymers during oil heating indicates its unsuitability for edible purposes and subsequently the economical interest to be used as raw material for biodiesel production.

Several parameters can influence conversion from waste oils into biodiesel, including free fatty acid (FFA) composition and water content. Some authors also found that both acidity and peroxide value are key parameters in biodiesel conversion [41]. Heating the oil over a long period of time leads to a significant increase of FFA content, which can reach values up to 10%, with detrimental effects on the common alkaline transesterification process [42, 43]. Alkaline catalyst reacts with FFA and produces soap (saponification reaction), reducing the biodiesel yield and preventing the separation of esters, glycerol and washing water [23]. It has been observed that the yield of biodiesel drop down to 6% when the FFA

content increases up to 5.3 % wt. [10]. Soap formation also increases the viscosity and leads to gel formation [23]. Table 2 shows the maximum levels of FFA recommended for one step homogeneous base catalyst transesterification considering different authors. Acid-catalyzed pretreatment to esterify FFA before alkaline transesterification is proposed when the content of FFA exceeds the maximum level (Table 1.2) [44]. The kinetics of the process has also been studied [45]. A two-step transesterification using ferric sulfate and KOH with methanol has also been developed in order to reduce FFA content [46]. Later, the use of ethanol in a modified two-step transesterification has also been proposed [47].

Table 1.2 Level of FFA recommended for homogeneous base catalyst transesterification

Recommended FFA (% wt.)	Reference
< 3	[41]
≤ 2	[48] [49]
< 1	[50] [51] [52]
< 0.5	[15]

Heterogeneous alkaline catalyzed transesterification of waste frying oils has been studied. Some authors achieved a yield of 79.7 % wt. using Sr/ZrO_2 [53] and 87.28 % wt. using calcined snail shel [54] at laboratory scale; biodiesel properties fulfilled ASTM D 6751. Moreover, a first order kinetic model of synthesis of biodiesel from waste frying oil using calcined snail shell has been developed [54]. To avoid the saponification process when the content of FFA is high, transesterification with heterogeneous acid

catalysts has been investigated. Jacobson *et al.* [55] used a combination of alumina, Al_2O_3 with ZrO_2 and modification of ZrO_2–Al_2O_3 with tungsten oxide (WO_3) at 200 °C and a reaction time of 10 h achieving a FAME yield of 65% wt. Lou *et al.* [56] attained a yield of 92% wt. when waste cooking oil was transesterified at 80 °C, using methanol-to-oil molar ratio of 30:1, 10% wt. catalyst (referred to weight of oil) and 8 h reaction time, at laboratory scale.

Enzyme catalyzed transesterification of waste oils has also been proposed. Charpe and Rathod [12] produced biodiesel from waste sunflower frying oil by transesterification using *Pseudomonas fluorescens* as a catalyst in a batch reactor. In a recent study, Maceira *et al.* [57] used immobilized lipase Novozym 435 as catalyst in waste frying oil transesterification achieving a biodiesel yield of 89.1% wt. at laboratory scale.

In a recent review, Lam *et al*. [58] state that the best solution to produce biodiesel from oil with high FFA, i.e. waste frying oils, is provided by the use of heterogeneous acid catalyst and enzymes. However, authors state that a few latest technological developments such as oscillatory flow reactor (OFR) [59], ultrasonication [60, 61], microwave reactor [62], co-solvent [14, 63] and supercritical methanol [64] have the potential to overcome the mass transfer limitation problem in acid heterogeneous and enzyme catalyzed transesterification of waste frying oils. Using OFR at laboratory scale, Harvey *et al*. [59] achieved a yield of FAME of approx. 99%, although traces of monoglycerides were detected in the biodiesel. Using sonochemical continuous reactors, a yield of FAME higher than 90% was reported [65, 66]. An extensive review on combustion and emission properties of waste oil biodiesel has been carried out [67]. It has been found

that waste oil usually exhibit the same properties as biodiesel from fresh vegetable oil. In fact, Lapuerta *et al*. [68] found that biodiesel from waste cooking oil is at least not inferior to that from refined vegetable oil as a particulate matter emissions (PM)-reducing fuel. Moreover, they did not appreciate important differences on engine performance and emissions between WFO and other non-used oils.

The processing of high FFA raw materials to provide biodiesel is at industrial scale. At present, there are about fourteen biodiesel plants in the EU showing different technologies to convert high FFA feedstocks in biodiesel. Moreover, some American enterprises have built multiple plants to process high FFA feedstocks, i.e. the Renewable Energy Group, BlackGold, Pacific Biodiesel Technologies, JatroDiesel, Mcgyan Biodiesel, Enhanced Biofuels or Cetane Energy. Up to date, the company BioDiesel International (BDI) has implemented a multi-feedstock technology in more than 30 large-scale industrial biodiesel plants in four different continents – Europe (401,000 t/y), China (100,000 t/y), USA (5,000 t/y) and Australia (50,000 t/y)-, using a wide range of raw materials, including high FFA oils and fats. World-wide largest multi-feedstock biodiesel plant employing BDI technology is located in Amsterdam (Netherlands) with a total annual capacity of 100,000 tons, using cooking oil, animal fats and palm fatty acid distillate (PFAD) as raw materials. This plant also produces glycerol (80%) and solid fertilizers as by-products.

1.4 Animal fats

The most common animal fats used to produce biodiesel at industrial scale are tallow [69], lard [70] and chicken fat [71]. Compared to commonly

used edible vegetable oils, animal fats frequently offer an economic advantage because of their low price [69]. The use of refined animal fats for biodiesel production is currently among the most economical options, costing US$0.4–US$0.5 per liter, while transesterification of vegetable oils at present costs around US$0.6–US$0.8 per liter [34].

Generally, the fatty acid profile of animal fats is characterized for a high content of saturated fatty acid, which causes some drawbacks on the physical and chemical properties of biodiesel, such as poor cold properties, while its low unsaturation level has several advantages, including high cetane number, oxidation stability and calorific value [19].

1.4.1 Tallow

Tallow is a rendered form of animal fat, processed from suet. It is characterized for its high melting point. It can be stored for large periods of time without refrigeration without risk of decomposition. The most used animal fats to produce biodiesel derive from beef or mutton and are food-grade and low-grade tallow. Food-grade tallow is quite expensive but has low FFA content, which allows feasible alkaline transesterification reaction; whereas low-grade tallow is cheaper but has high content of FFA, so a more expensive biodiesel conversion is needed (Table 1.1).

Ma *et al*. [72] completed methanolysis of beef tallow with a low content of FFA (0.29% wt.) in 15 min using NaOH as catalyst. In a recent study using food grade tallow, Liu *et al.* [73] reduced the time of reaction to 5 min using radio frequency heating, achieving a FAME yield of 96.3% wt. Araujo *et al*. [74] carried out alkaline transesterification of beef tallow with

a high content of FFA (3.6%) by means of heating and preliminary formation of a microemulsion.

Low-grade tallow, also known as brown grease (or grease with a FFA content above 35% wt.), is less expensive than food-grade tallow, but its high FFA content makes it unsuitable for the one-step base catalyzed methanolysis currently in use in most biodiesel plants in Europe [75].

To convert low-grade tallow with a content of FFA above 15% in biodiesel, three methods have been proposed [75]: a) one step alkali catalyzed methanolysis, although this methodology has been rejected as the conversion yield is lower than 56%; b) two step esterification of FFA followed by methanolysis, with a maximum yield of 93%; c) two step methanolysis followed by esterification, with a maximum yield of 97%.

Nebel and Mittelbach [76] studied the extraction of fats from meat and bone meal and found that n-hexane was the most suitable solvent for the extraction process. The extracted fatty material showed a FFA content of 11% wt. and a fatty acid composition characterized by an high content of saturated and monounsaturated fatty acid (28% wt. of palmitic acid, 20% wt. of stearic acid and 40% wt. of oleic acid) and a low content of polyunsaturated fatty acid. The oil produced was converted into FAME using a two-step reaction achieving a FAME yield of 45.7% wt. [76] Moreover, Mbaraka *et al.* [77] developed a pretreatment step for alkyl ester production using low-grade beef tallow based on propylsulfonic acid-functionalized mesoporous silica materials for methanol esterification of FFA.

1.4.2 Lard

Lard is pork fat with soft grain, commonly used as cooking fat, shortening or as spread similar to butter. Fatty acid composition of lard (mixture of feather meal fat and skin fat) is characterized for an higher content of unsaturated fatty acid (44.4% wt. of C18:1, 10.2% wt. of C18:2 and 0.5% wt. of C18:3) and a lower content of saturated fatty acid (1.4% wt. of C14:0, 23.6% wt. of C16:0 and 14.2% wt. of C18:0) with respect to tallow [78]. Whereas, it has been observed that pigs that have been fed with different diets can produce lard with significantly different fatty acid contents and iodine values [79].

Berrios *et al.* [80] converted lard with low FFA content (0.13%) in biodiesel achieving 90% wt. of FAME yield. Dias *et al*. [18] carried out acid transesterification of waste lard achieving a biodiesel yield of 65% wt. However, the most significant yield (77.8% wt.) was provided when lard was blended with soybean oil, showing that blending might be an interesting alternative for recycling such wastes. Production of biodiesel from waste lard with no pre-treatment using supercritical methanol was also investigated, providing comparable results to biodiesel conversion of refined lard [81].

1.4.3 Chicken fat

Chicken fat is extracted from feather meal, which is prepared from chicken wastes. Feather meal contains substantial amounts of chicken fat, varying from 2% to 12%, depending on feather type [82]. Its fatty acid profile consists of 0.50-0.70% wt. of myristic acid, 24-25% wt. of palmitic

acid, 5.8-7.8% wt. of palmitoleic acid, 5.8-5.9% wt. of stearic acid, 38.2-40.5% wt. of oleic acid, 18.3-23.8% wt. of linolenic acid and 0.7-1.9% wt. of linolenic acid.

Mattingly [83] produced biodiesel from chicken fat with 2.3% FFA. This author concluded that a pretreatment was needed in order to get high biodiesel yields. Schulte [84] achieved biodiesel yields up to 91% wt. using supercritical methanol. Recently, Alptekin and Canacki [85] produced biodiesel from chicken fat with a high FFA content (13.45% wt.), optimizing the pretreatment reaction by using different acid catalysts to reduce the FFA level (below 1% wt.). The maximum methyl ester yield was found to be 87.4% wt. Moreover, Feddern et al. [86] characterized chicken skin fat and found it possesses both a low FFA content and a fatty acid profile suitable for transesterification to biodiesel production.

1.4.4 Other animal fats

In a recent study, recovery of lipids from alligator fat (AF) tissue was studied by solvent extraction as well as by microwave rendering. Microwave rendering resulted in AF oil recovery of 61% by weight of the frozen AF tissue [87]. The fatty acid profile of AF showed that palmitic acid (C16:0), palmitoleic acid (C16:1) and oleic acid (C18:1) were the dominant fatty acid accounting 89-92% of all lipids by mass [87]; these fatty acid are the most suitable constituents to produce the ideal biodiesel properties [23].

In another recent study, the feasibility of the use of animal fat from the leather industry fleshing wastes to produce biodiesel was also investigated [88]. The leather industry is among the considerable polluting industries and produces high quantity of fat-originated solid and liquid

wastes while processing hides and skins [89]. These wastes cause environmental problems. One way to reduce these wastes is to use them as feedstocks for biodiesel production. The most important problem is the higher acid value of the fleshing oil (24.30 mg KOH g^{-1}). For this reason, a pretreatment with sulfuric acid to the fleshing oil is needed. Nevertheless, the properties of straight FAME derived from this feedstock do not meet the EU biodiesel standards for cold filter plugging point (considering each member state requirements), ester and sulfur content [88]. For this reason, blends with diesel fuel constitute a valuable solution.

1.5 Insects

In the category of macro-organisms, insects show a great potential in terms of fat accumulation, in some cases above 25-30%, especially during the immature stages (larva, pupa, nymph) [90]. The fat contents and properties of oleaginous insects varies according to the species (Table 1.3), being Coleoptera and Lepidoptera species the ones that provide the highest amount of fat [91]. Insects have shown a high potential to replace oleaginous seeds as raw material for biodiesel production, due to their high food efficiency, high reproduction rate and short life cycle [92]. Furthermore, biodiesel derived from insect oil fulfills both ASTM D6751 and EN 14214 standards [92, 93].

Table 1.3 Properties of oils from insect (*Chrysomya megacephala* oil, CMO; Black soldiers fly oil, BSFO; Yellow mealworm beetle oil, YMBO)

Properties	CMO [92]	BSFO [94]	BSFO [95]	YMBO [96]
Iodine value (g I/g oil)	73	96	89	96
Saponification number (mg KOH/g oil)	202.11	157.5	157	162
Peroxide value (g/100 g)	n.m	0.03	0.18	0.27
Acid value (mg KOH/g)	1.10	8.7	8.2	7.6
Moisture and volatile materials (% w/w)	0.01	n.m	n.m	n.m
Cloud point (ºC)	n.m	5	6.8	3.7

n.m= not mentioned

In the past few years, biodiesel production from insect oil is gaining interest in the scientific community [93, 94, 97]. This technology is based on the fact that many insects possess a lipid body rich in monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acid [98]. MUFA are among the preferred fatty acid for biodiesel production due to their ability to improve the engine behavior under cold weather conditions, besides biodiesel oxidative stability [99].

The amount of lipids and the fatty acid composition of the insect depend not only on the species but also on the diet used to grow it [90, 100] (Table 1.4). For the selection of suitable insects to produce fats to be used as biodiesel feedstock, the following parameters should be considered: fats content, duration of the life cycle, requirements of space to grow, reproductive capacity and low cost feeding [90]. In the search of more economical nourishment, it is important to select insects that are able to consume waste to both produce oil and recycling purposes. Therefore, the

insect *Hermetia illucens*, also known as black soldier fly (BSF), has been investigated as a source of oil for biodiesel production [94, 95] and also for its capability for waste management [101]. Li *et al*. [94] used BSF larvae for the bioconversion of diary manure on biodiesel and sugar. Results showed a consumption of 78% of the initial value of manure (1248.6 g of fresh manure) in 21 days. They produced 15.8 g of biodiesel and 96.2 g of sugar from 70.8 g dry BSF larvae. Other wastes, i.e. lignocellulosic materials, have been tested. Zheng *et al*. [95] analyzed different mixtures of restaurant solid waste (RSW), rice straw and Rid-X (bacteria that facilitate the break down of the solid organic wastes). Considering a ratio of 7:3 (RSW : rice straw) plus 0.35% v/v Rid-X, they achieved 35.6% of biodiesel per dry insect biomass. Animal waste is another residue that may cause health hazards and environmental pollution. From this group, cattle, pig and chicken manure have been used to grow BSF larvae [97]. The highest BSF larvae growth (327.6 g) resulted in 98.5 g of crude fat and 91.4 g de biodiesel.

Table 1.4 Terrestrial insect species with the most suitable fatty acid composition for biodiesel production

Species	Oil (%)	Culture media	C14:0	C15:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:3	C20:0	Other	Ref
Chrysomya megacephala (Fabricius) larvae	24.40-26.29	Restaurant garbage	3.9	0.3	35.4	13.0	0.3	2.7	24.3	1.2	0.3	2.9	[92]
Tenebrio molitor L.	14.56	Decayed vegetables	8.1	1.5	17.6	9.3	1.7	11.4	1.6	19.7	n.d	3.8	[96]
Hermetia illucens larvae	39.6	Rice straw and restaurant waste	8.1	1.5	14.2	4.5	0.8	7.6	22.5	2.1	n.d	33.3	[95]
Hermetia illucens larvae	23.16	Fresh dairy manure	n.d	1	14.8	3.8	n.d	3.6	23.6	n.d	n.d	49.1	[94]
Termes sp	36.55	Not mentioned	0.13	n.d	2.47	0.19	n.d	31.90	1.86	0.34	n.d	63.11	[102]
Brachytrupes portentosus Lichtenstein	20.6	Not mentioned	n.d	n.d	1.61	0.71	0.13	35.79	3.4	n.d	n.d	58.36	[102]

n.d= not detected

Species	Oil (%)	Culture media	C12:0	C14:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:3	C20:0	Other	Ref
Termes sp	36.55	Not mentioned	n.d	0.13	2.47	0.19	n.d	31.90	1.86	0.34	n.d	63.11	[102]
Brachytrupes portentosus Lichtenstein	20.6	Not mentioned	n.d	n.d	1.61	0.71	0.13	35.79	3.4	n.d	n.d	58.36	[102]
Hermetia illucens prepupae	21.42	Cow manure	20.92	2.85	16.05	n.d	n.d	5.68	32.11	0.19	n.d	22.2	[101]
Hermetia illucens prepupae	30.38	10% fish offal + 90% cow manure	34.10	6.46	14.30	n.d	n.d	2.35	16.52	0.74	n.d	25.43	[101]

n.d= not detected

In another study, *Chrysomya megacephaly*, a necrophagous blowfly, during its larvae development was fed with restaurant garbage for five days and achieved an oil content in a range from 24.40% to 26.29% [92]. But the most important finding is the oil acid value, lower than that of most insects and close to that of vegetable oils (Table 1.3).

Regarding the production of fatty acid methyl esters from insect oil, a two-step process has been implemented in most cases: acid esterification (due to the high acidity of the oil) followed by basic transesterification. Reaction parameters including temperature, amount of catalyst, time and methanol-to-oil molar ratio were optimized (Table 1.5). Results showed that insect oil-based biodiesel properties fulfilled the ASTM D6751 and EN 14214 standards in terms of cetane number, density, flash point, water content, although only a few met the European standard methyl esters content (> 96.5%), kinematic viscosity, alcohol content and both the acid number value and the oxidation stability required by both standards (Table 1.6).

Table 1.5 Optimum parameters during biodiesel production from insect oil

Insect FAME	Acid esterification parameters				Basic transesterification parameters				Ester content (%)
	Methanol to oil ratio	Catalyst (% w/w)	Time (h)	Temperature (ºC)	Methanol to oil ratio	Catalyst (% w/w)	Time (h)	Temperature (ºC)	
Tenebrio molitor [96]	8:1	1	2	73	6:1	0.8	0.5	65	96.8
Hermetia illucens [97]	8:1	1	1	75	6:1	0.8	0.5	65	93
Hermetia illucens [94]	8:1	1	2	73	8:1	0.8	0.5	65	97.2
C. megacephala [92]	not applied				6:1	1.6	0.5	55	87.71

Table 1.6 Properties of biodiesel from different insect oils (*Chrysomya megacephala* larvae, CML; Black soldiers fly larvae, BSFL; Yellow mealworm beetle, YMB). n.m =not mentioned

Property	FAME from CML [92]	FAME from BSFL [95]	FAME from YMB [96]	FAME from BSFL [94]	ASTM D6751	EN 14214
Cetane number	54.8	55	58	n.m	Min. 47	Min.51
Kinematic viscosity at 40 ºC (mm^2/s)	4.048	5.96	5.9	4.5	1.9-6.0	3.5-5.0
Density (kg/m^3)	874.3	895	860	872	n.m	860-900
Flash point (ºC)	170	123	127	121	Min. 93	Min. 101
Cloud point (ºC)	6	4.2	n.m	n.m	-	-
Water content (% v/v)	<0.03	0.03	0.03	0.03	Max. 0.05	Max. 0.05
Acid number (mg KOH/g)	0.35	0.6	0.9	0.8	Max. 0.5	Max. 0.5
Net calorific value (MJ/kg)	38.69	n.m	n.m	n.m	n.m	n.m
Oxidation stability (h)	3.6	n.m	n.m	n.m	Min. 3	Min. 6

1.6 Soapstocks

Soapstock is a by-product from vegetable oil refinement and other low cost raw materials for biodiesel production, such as animal fats. Soapstock main uses include animal feed and raw material for soap and fatty acid production. In 2009, there was a worldwide production of about 36 Mton of soybean, which derived in 2.16 Mton of soybean soapstock [35]. Soapstock contains a significant amount of water (about 50% wt.), which can be emulsified with the lipid constituents and is difficult to remove. For this reason, soapstock is a poor feedstock for biodiesel production. In addition, the presence of both FFA and acylglycerol makes the transesterification reaction more difficult. Alkaline catalysis is not feasible due to its high FFA level [44].

Another alternative is provided by the soapstock produced from the milk transformation industry: residual butter and related fat, named scum, are washed and collected in effluent treatment plants. Scum usually comprises a mixture of fats, lipids, proteins and packing materials. A large dairy, processing 500,000 liters of milk per day, will produce approximately 200–350 kg of effluent scum per day, which makes disposal difficult [103]. Most dairies remove scum to solid waste disposal sites or by incineration. However, this is economically wasteful and generates pollutants. Sivakumar *et al.* [103] transesterified triglycerides from scum using alkaline homogeneous catalyst, achieving an ester yield of 96.7% wt. Biodiesel from this raw material has been deeply analyzed by Moser [10].

1.7 Microorganisms for triglycerides production

Microbial oil or single cell oil proceeds from different oleaginous microorganisms, i.e. bacteria, fungi and microalgae [104]. These microorganisms are able to accumulate intracellular lipids above 20% of their dry cell weight. Besides, they do not require arable land and allow the recycling of residual biomass, as it can be used as a carbon source [35]. The accumulation of lipids depends on the kind of microorganism, culture conditions and the relation C/N, as under nitrogen limitation the accumulation of oil increases. The oleaginous microorganisms are able to consume a variety of carbon substrates following different metabolic pathways [105]. Currently, technologies for the production of microbial oil are still in pilot scale, i.e. Nestea Oil Company uses waste as medium and expects commercial production after 2015 [106].

1.7.1 Microalgae

Microalgae are unicellular photosynthetic microorganisms, living in saline or fresh water environments, which convert sunlight, water and carbon dioxide to biomass [107]. Microalgae are vital for life on earth; they produce approximately half of the atmospheric oxygen and use simultaneously the greenhouse gas carbon dioxide to grow photoautotrophically.

The microalgae biodiversity is estimated as more than 50,000 species of which only around 30,000 have been analyzed and studied [108]. They are classified according to several parameters, i.e. pigmentation, life cycle, morphology and cell structure. There are two main populations: filamentous and phytoplankton algae. Both populations, in particular phytoplankton,

increase in number rapidly to form algae blooms [109]. Like higher plants, microalgae store lipids in the form of triacylglycerides (TAG). Many species, such as *Chlorella spp.* or *Nannochloropsis spp.,* exhibit rapid growth and high fats productivity, and many microalgae species can be induced (by varying the temperature, nitrogen concentration or with CO_2 enrichment during the microalgal culture) to accumulate substantial quantities of lipids exceeding 60% of their dry biomass [110]. In fact, oil productivity of many microalgae species greatly surpasses that of the best producing oil crops [111]. Tsukahara and Sawayama [112] affirmed that a realistic value of microalgae biomass production is between 15 and 25 t/ha/year, considering as microalgal cultivation area the total installation for tubular reactors including a biocoil system. Taking into account a conservative value of 30% lipid content in microalgae cells, it corresponds to a lipid production of 4.5–7.5 t/ha/year [112]. This amount is extremely higher compared to the production of oil from soybean (0.4 t/ha/year), rapeseed (1.4-1.6 t/ha/year), palm (3.6 t/ha/year) and *Jatropha* (4.1 t/ha/year) [113, 114]. In other words, culturing microalgae for biodiesel production involve the least land area and holds an important key feature for effective land utilization [25].

The relative composition of algal lipids greatly depends on the species, medium and environmental conditions in which the cells are cultured and harvested [115]. In general, algal cells synthesize triglycerides when the energy input, through carbon assimilation, exceeds the immediate metabolic needs of the cell. However, it has also been shown that microalgae increase the proportion of triglycerides produced upon nutrient

starvation and other environmental stresses, such as temperature and essential nutrients [115].

Xu *et al.* [116] characterized the oil production after heterotrophic growth of *Chlorella protothecoides*. The oil showed a predominant content of oleic acid methyl ester (60.84%), while the content of C18:1, C18:2 and C18:3 was over 80%. Moreover, the produced biodiesel presented a high heating value of 41 MJ kg^{-1}, density of 0.864 kg L^{-1}, viscosity of 5.2 10^{-4} Pa s (at 40 ºC) and cold filter plugging point of −11 ºC. Generally, microalgae fatty acid range varies from 12 to 22 carbon chain length and can be either saturated and unsaturated. The number of double bonds never exceeds 6 and unsaturated fatty acid are cis isomers [117]. However, Lv *et al*. [118] observed from five different species of microalgae (*Isochrysis sp., Gymnodinium sp., Platymonas subcordiforus, Heterosigma akashiwo Hada and Skeletonema costatum*) that the accumulation of saturated and unsaturated fatty acid varies considering cell growth, stationary phase and intracellular structures (membrane vs. storage component). In fact, the content of saturated fatty acid in the microalgal oil can vary from 30% to 70% wt. depending on the phase (stationary or exponential). In this sense, Sanford *et al*. [119] analyzed the properties of two diverse samples of crude algal oil and observed that the FFA resulted 0.45 and 1.75% wt.

Microalgae are the fastest-growing photosynthesizing organisms, able to complete an entire growth cycle every few days. Especially bred mustard varieties can produce reasonably high oil yields, and have the added benefit that the biomass left over once the oil has been pressed out acts as an effective and biodegradable pesticide [109]. Furthermore, microalgae can be grown almost anywhere, even on sewage or salt water, and does not require fertile land or food crops. Most commercial microalgae

production is based on R-select species such as *Chlorella sp.,* or extremophile species, such as *Arthrospira sp*.

The most important microalgal production systems use suspended cultures in either open ponds (called also raceways) or closed reactors (tubular photobioreactors) [120]. Raceway ponds are open shallow ponds with a paddle wheel to provide circulation of the algae and nutrients [121]. Raceways are relatively inexpensive to build and operate, but often suffer low productivity due to contamination, poor mixing, dark zones and inefficient use of CO_2 [113, 122]. Closed microalgae bioreactors can be used to culture some species heterotrophically at high densities, offering theoretical advantages in terms of avoiding contamination, yielding higher culture densities and providing closer control over growth media [115]. Regardless of the specific advantages and disadvantages of raceways and tubular photobioreactors, both involve significant challenges of biomass recovery. Because of the harvesting challenges associated with suspended microalgae, there is a growing interest in the use of immobilized or attached algal processes, such as Matrix-immobilized microalgae or algal biofilms.

Recent studies [24, 25, 122] agree that the most important challenges in this field include the development of technologies to supply minimum amounts of inputs (including energy consumption) to microalgal cells, less CO_2 released to the atmosphere, the optimization of harvesting, dewatering and lipid extraction from microalgal biomass, the cultivation under higher cell densities and the promotion of chemically induced or auto-flocculation of microalgal cells.

The production cost of microalgal oil depends on many factors, including the biomass yield from the culture system, oil content, scale of

production systems and cost of recovering oil from biomass [34]. However, the production of biodiesel using microalgal biomass has been handicapped by the inability to find a reliable and cost effective method to produce and harvest large quantities of microalgae feedstock [121]. In fact, biodiesel production from microalgae is therefore still far more expensive than that of petroleum–diesel fuels. The production of microalgae-generated oil currently costs US$20–US$30 per gallon (US$5.3–US$8.0 per liter), with some estimates soaring to US$60 [123].

A possibility to reduce the production costs could be the valorization of secondary products; for example, microalgae biomass may also be used to feed an anaerobic digester for methane production [124], or used to produce bioplastic materials [125]. Residual biomass from these processes can potentially be used as fertilizer, soil amendment or feed for fish or livestock [126]. In addition to biodiesel production, large scale methods to produce and harvest microalgae have been used in wastewater treatment [121].

In the last years, microalgae have been promoted by private and public inversion. In an extensive review, Singh and Gu [127] listed the world algae production companies and their cultivation methods. The UK Carbon Trust is responsible of the most impressive algae investment made in the EU, supported by the research program "Algae Biofuels Challenge (ABC)", 2009. The partnership built in 2007 by the Spanish company AURANTIA and the US Green Fuel Tech of Massachusetts aims to scale up to 100 ha of algae greenhouses, producing 25,000 t/yr of algae biomass. A cement plant near Jerez (Spain) provides the necessary CO_2. The Italian company ENI produces algae oil in a 1 ha pilot plant, in Sicily (Italy). This project tests the open ponds photobioreactor facility [127].

1.7.2 Filamentous fungi

Among the oleaginous microorganisms reported in literature, filamentous fungi show the highest lipids accumulation after yeast, besides the capacity to produce a wide range of products, i.e. enzymes, antibiotic and chemicals [128]. Some of the main differences between filamentous fungi and other oleaginous species (yeast, microalgae and bacteria) on the production of oils are based on the capability of filamentous fungi to build pellets in submerged cultures, due to filamentous growth during fermentation. Moreover, the viscosity of the broth is reduced, thus improving the mixing and mass transfer performance. Finally, due to the formation of pellets, they are easy to harvest from broth by simple cell filtration, which reduces the cost compared with traditional methods like centrifugation [129].

To decrease the cost of the process, methanolysis from fungal biomass has been proposed as an alternative to the oil extraction process. Through the use of methanol and a catalyst, usually H_2SO_4 or HCl, some authors reported a yield of FAME conversion of 91%, being the cetane number 56.4, thus making this technique an attractive alternative for the biodiesel industry [130].

The stored lipids in filamentous fungi contain a high percentage of saturated [131] and polyunsaturated fatty acid [132], accumulated during the stationary phase in special organelles, named lipid granules. Like bacteria and yeast, filamentous fungi may also consume a wide range of carbon sources, including lignocellulosic biomass (Table 1.7), thus providing inexpensively raw material for biodiesel. Although lignocellulose comprises hemicellulose, cellulose and lignin, only hemicellulose and cellulose may be

consumed as feedstock for biological conversion. For this purpose, to make carbohydrates accessible to microorganisms, lignocellulose needs a pretreatment before hydrolysis [133]. Zikou *et al*. [134] used a mixture of xylose and glucose, which are abundant sugars from lignocellulosic biomass, to produce γ-linolenic acid (GLA) by *Zygomycetes Thamnidium elegans*. Results showed that the best combination of xylose to glucose is 1:1, achieving 12.6 g/l lipids and 936 mg/l GLA. Instead, when glucose was used as the sole medium, the values were 15 g/l and 1014 mg/l, respectively. *M. isabellina* was also tested and a positive influence of the increment of these sugars separately in the medium over the accumulation of lipids was found [135]. The same filamentous fungus was used for the production of oil when rice hulls hydrolysate, which is a lignocellulosic material, was used as a substrate. Authors proposed a mathematical model to simulate the consumption of sugar and nitrogen, the fat-free biomass formation and the accumulation of lipids [136]. Khot *et al*. [137] isolated fungi of tropical mangrove wetlands, but only 5 out of 14 showed lipid accumulation above 20% dry cell biomass. Fungi from this ecosystem were also used for the production of lignocellulosic enzymes. The oil of three out of the previous five was transesterified, the biodiesel properties predicted and it was found that the most appropriate fungus was IBB M1, known as *Aspergillus terreus* strain. Another important issue to be fixed when lignocellulosic biomass is used consists in the inhibitory effects of the lignocellulose-derived compounds over oil accumulation (lignin aldehydes, furan aldehydes and weak acid). When *Mortierella isabellina* was used to determine the inhibitory effect of these compounds, the lignin derivative was found to be the main inhibitor considering lipid accumulation, while acetic and formic acid doubled the lipid accumulation with respect to the control test [133]. It

was concluded that the most suitable combination of fungus and lignocellulosic material substrate for fungal oil production was provided by the strain *M. isabellina* when it consumed non-detoxified lignocellulosic hydrolysate, due to both the high oil content and the simplified process of fermentation [138].

In terms of environmental preservation, the bioremediation of soils contaminated by hydrocarbons is an important issue. For this purpose, the use of *Aspergillus terreus* has been investigated to transform petroleum hydrocarbons in oils to be used in the biodiesel industry. Results showed that the use of hydrocarbons as carbon source provides sevenfold higher lipid accumulation compared to the use of glucose as substrate [139]. Crude glycerol is a by-product of the biodiesel industry, which has recently been released in high quantities due to the increasing biodiesel demand. It usually comprises residues of alcohol (methanol or ethanol) and a basic catalyst. This by-product has been tested as a carbon source for *Mucor sp*., *Cunninghamella echinulata*, *Mortierella ramanniana*, *Thamnidium elegans, Zygorhynchus moelleri* [140, 141] *and Mortierella isabellina* [141]. Chatzifragkou *et al*. [141] used the fungi mentioned above and compared lipid accumulation with that of yeasts. Results showed that all fungi were able to accumulate higher amount of oil than yeasts under nitrogen-limited conditions. Bellou *et al*. [140] focused their research on the production of polyunsaturated fatty acid (PUFA) produced by filamentous fungi. In the majority of the tested fungi, authors observed that PUFA was mainly accumulated in mycelial membranes during mycelial growth. However, one of the studied filamentous fungi (*Mortierella ramannniana*) depicted the opposite trend. In this sense, PUFA continued decreasing after the end of

the growth phase, thus suggesting PUFA are involved in primary metabolism of this microorganism [140].

Filamentous fungi have been genetically engineered focusing on lipid production, giving relevance to metabolic routes governing fatty acid synthesis and lipid storage. Unique metabolic features have been identified in *Mortierella alpina* and *Mortierella circinelloides*, particularly with respect to NADPH metabolism and sterol biosynthesis, which might be related to differences in fungal lipid phenotype [142]. The gene coding for acetyl-CoA carboxylase (ACC) was isolated from *Mucor rouxii*. This gene is able to increase by 40% the total fatty acid content of non-oleaginous microorganism [143]. Wynn *et al*. [144] studied the significant role of malic enzyme on lipid accumulation. Authors used a fungus with low lipid accumulation (*Mucor circinelloides*) and found out that the enzyme disappeared 15 hours after the depletion of the nitrogen source, which was coincident with the end of lipid accumulation. Instead, when a high lipid accumulation fungus like *Mortierella alpine* was used, the enzyme was held 60 hours after the completion of the nitrogen source, which lasted longer than the lipid accumulation.

The accumulation of lipids from filamentous fungi is increasingly attractive because of the oil high yields, versatility of the microorganisms to use different carbon sources (including wastes like lignocellulosic material) and the possibility to be grown in submerged cultures, which give the opportunity to easily collect the biomass. In this context, genetic engineering may be a magnificent tool to help in the inclusion of these microorganisms to provide an alternative oil to the biodiesel industry. Although most research in this area is focused on the production of high value-added products such as enzymes and polyunsaturated fatty acid,

among many others, the production of microbial oil could provide an extra value to the process.

Table 1.7 Oil content and fatty acid composition from different filamentous fungi

Fungus	Oil content (g/l)	Carbon source	Fatty acid composition									Ref
			C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	
Aspergillus oryzae	3.5	Potato processing wastewater	4	11.6	15.6	19.3	30.3	6.5	5.5	2	2.3	[145]
Mortierella isabellina	18.5	Xylose	n.d	24.9	2.6	2.8	56.2	10.9	2.5	n.d	n.d	[146]
Mortierella isabellina	n.r	Glucose	1.2	28.2	5.8	1	55.5	5.8	2.4	n.d	n.d	[130]
Mortierella isabellina ATCC 42613	10.2	Glucose	n.d	20	2.32	1.74	58.4	12.5	3.21	n.d	n.d	[135]
*Mortierella isabell*ina ATCC 42613	8.8	Xylose	n.d	25.6	3.59	2.44	52.7	10.8	2.87	n.d	n.d	[135]

n.d= not detected

Fungus	Oil content (g/l)	Carbon source	Fatty acid composition									Ref
			C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	
Thamnidium elegans CCF-1465	15	Glucose	n.d	22.3	n.d	7.5	58.3	8.7	3.2	n.d	n.d	[134]
Thamnidium elegans CCF-1465	5.1	Xylose	n.d	27.9	2.3	7.3	50.8	7.2	4.5	n.d	n.d	[134]
Cunninghamella echinulata	1.23	Glycerol	n.d	19.3	1.5	8.6	35.4	18.5	15.3	n.d	n.d	[140]
Mortierella ramanniana	3.18	Glycerol	n.d	21	1.3	5.8	49.1	15.9	4.3	n.d	n.d	[140]
Thamnidium elegans	0.93	Glycerol	n.d	21.7	1.8	11.7	39.3	16.2	7.2	n.d	n.d	[140]

n.d= not detected

Fungus	Oil content (g/l)	Carbon source	Fatty acid composition									Ref
			C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	
Cunninghamella echinulata ATHUM 4411	1.56	Glycerol	n.d	20.3	2.2	4.9	44.5	17.4	8.7	n.d	n.d	[147]
Thamnidium elegans CCF 1465	2.9	Glycerol	n.d	19.2	1.3	11.7	50.4	11.8	3.9	n.d	n.d	[147]
Mortierella ramanniana MUCL 9235	2.71	Glycerol	n.d	25.6	2	4.3	43	16.3	6.1	n.d	n.d	[147]
Mortierella isabellina MUCL 15102	1.86	Glycerol	n.d	20.7	3.4	6	44.9	14.5	4.4	n.d	n.d	[147]
Zygorhynchus moelleri MUCL1430	1.57	Glycerol	n.d	15.1	1.4	5.5	21.9	47.5	3.7	n.d	n.d	[147]

n.d= not detected

Fungus	Oil content (g/l)	Carbon source	Fatty acid composition									
			C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	Ref
Cunninghamella bainieri 2A1	4.18	Glucose	n.d	18.4	n.d	15.2	39.6	10.2	7	n.d	n.d	[147]
Aspergillus terreus	1.52	Hydrolyzate of wheat straw	0.3	17.4	0.6	8.5	57	8.2	0.6	0.7	n.d	[138]
M. isabellina	2.63	Hydrolyzate of wheat straw	0.7	24.3	2.6	3.8	47.8	14.9	2	0.9	n.d	[138]
M. vinacea	2.46	Hydrolyzate of wheat straw	0.4	20.2	2.3	2.8	53.3	14.3	3.7	0.5	n.d	[138]
Mucor circinelloides	9.2	Thin stillage	n.d	15.7	n.r	2.3	29.6	50	1.4	1.2	n.d	[148]

n.d= not detected

Fungus	Oil content (g/l)	Carbon source	Fatty acid composition									Ref
			C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	
Mortierella isabellina NRRL 1757	3.99	Xylose	n.d	22.51	2.42	2.93	50.7	13.77	3.42	n.d	n.d	[133]
Mortierella isabellina NRRL 1757	4.80	Mannose	n.d	23.58	3.00	0.13	54.07	10.94	2.56	n.d	n.d	[133]
Mortierella isabellina NRRL 1757	5.77	Glucose	n.d	20.38	2.12	0.24	56.15	9.96	4.05	n.d	n.d	[133]
Mortierella isabellina NRRL 1757	3.82	Fructose	n.d	20.88	1.49	3.58	55.03	10.85	2.69	n.d	n.d	[133]
Mucor sp. LGAM 365	0.96	Glycerol	n.d	26	2.1	5.5	31.5	21.9	9.9	n.d	n.d	[141]

n.d= not detected

Fungus	Oil content (g/l)	Carbon source	Fatty acid composition									Ref
			C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	
Cunninghamella echinulata ATHUM 4411	1.56	Glycerol	n.d	20.3	2.2	4.9	44.5	17.4	8.7	n.d	n.d	[141]
Thamnidium elegans CCF 1465	2.90	Glycerol	n.d	19.2	1.3	11.7	50.4	11.8	3.9	n.d	n.d	[141]
Mortierella ramanniana MUCL 9235	2.71	Glycerol	n.d	25.6	2.0	4.3	43.0	16.3	6.1	n.d	n.d	[141]
Mortierella isabellina MUCL 15102	1.86	Glycerol	n.d	20.7	3.4	6.0	44.9	14.5	4.4	n.d	n.d	[141]
Zygorhynchus moelleri	1.57	Glycerol	n.d	15.1	1.4	5.5	21.9	47.5	3.7	n.d	n.d	[141]

n.d= not detected

1.7.3 Yeast

Traditionally, yeasts have been used in the food and beverage industry, so the majority of yeasts have been adapted to meet these procedures. The ability to accumulate lipids above 20% of its weight is achieved by only 5% of the known yeasts [149]. Lipid accumulation in oleaginous yeast occurs under excess of carbon sources, being scarce the nitrogen source, so the carbon excess is channeled into triglycerides [150]. Similarly to other microorganisms, yeast is able to consume different sources of carbon and nitrogen, from waste to laboratory pure sources. However, to take advantage of this technology, the use of widely available waste is a key parameter. According to this, the main by-products of the rapeseed oil-based biodiesel industry, glycerol (carbon source) and rapeseed meal (nitrogen source), were used as culture medium for the oleaginous yeast *R. toruloides Y4* and the accumulation of oil was analyzed. Results showed that the accumulation of oil reached up to 19.7 g/l, higher than 16.2 g/l achieved when a medium composed by glycerol and yeast extract as nitrogen source was used. Besides, the oil fatty acid composition comprised a high content of monounsaturated fatty acid, which makes it suitable for biodiesel production [151]. Many authors have proposed the use of glycerol as carbon source to grow different oleaginous yeasts, i.e. *C. curvatus* [152], *Rhodotorula glutinis* [153], *Rhodotorula graminis* [154] and *Rhodosporidium toruloides* [155]. In all cases, it was considered a suitable carbon source for lipogenesis. Also, the hydrolysate from lignocellulosic materials has been considered an interesting substrate due to the availability and economic feasibility [156-158].

The culture conditions, like C/N ratio (close to 100), substrate, culture mode, microelements and inorganic salts are crucial in lipid accumulation [150]. While the ratio C/N plays the most important role in lipid accumulation, the culture mode is also of special interest. For this reason, Zhao *et al*. [159] used different feeding strategies with yeast *R toruloides Y4* and concluded that the fed-batch strategy exhibited the largest oil accumulation potential under large-scale production plant, while keeping the residual glucose concentration to 5 g/l of carbon source and the fed-batch cycles were multiple times repeated. Authors removed the majority of the mature culture at the end of each cycle, keeping 900 ml of the culture in the bioreactor. Then, fresh media were added and a new cultivation cycle was initiated. As a result, the highest amount of lipids reported in the literature, 78.7 g/l, was achieved (Table 1.8).

The main disadvantage of oleaginous yeast is the extraction of the oil, due to the resistance of the cell walls to different solvents. In most cases, a chloroform methanol stream has been used, although this solution is not environmentally friendly because of the toxicity of reagents. An interesting alternative is provided by an enzyme-assisted method, consisting in a microwave-aided heating pre-treatment, further enzymatic treatment with the recombinant β-1,3-glucomannanase, plMAN5C and later oil extraction with ethyl acetate. The percentage of extraction with this method is close to 96.6% of the total oil [133].

Table 1.8 shows the fatty acid composition of yeast oil. Although it varies depending on the species and substrate, it is mostly composed by palmitic and oleic acid, the latter being preferred for the biodiesel industry due to its high unsaturation degree [160]. Wahlen *et al*. [161] compared

biodiesel properties, performance and emissions in a diesel engine; biodiesel being produced from soybean, algae, bacteria and yeast oil. Only small differences in terms of exhaust emissions were detected, as biodiesel from yeast oil emitted lower hydrocarbon but higher NO_x emissions.

Table 1.8 Oleaginous yeast, culture medium, oil content and fatty acid composition.

Yeast	Oil content (g/l)	Substrate	Mode culture	Fatty acid composition						Ref
				C16:0	C18:0	C18:1	C18:2	C18:3	Other acid	
Rhodosporidium toruloides	19.7	Glycerol	Fed-batch	7.2	10.2	64.8	13.6	2.8	1.4	[151]
Pichia kudriavzevii	7.59	Glycerol	Fed-batch	29.3	8.89	41.9	9.22	n.d	6.0	[162]
Candida tropicalis	17.6	Glucose	Batch	24.6	50.2	15.4	n.d	n.d	9.8	[163]
Cryptococcus curvatus	19.0	Glycerol + organic waste from brewery industry	Fed-batch	13.5	12.6	51.1	10.5	n.d	8.6	[164]
Cryptococcus curvatus	6.6	Glucose + corncob hydrolysate	Fed-batch	22.1	7.5	57.2	7.2	0.8	3.5	[132]
Lipomyces starkeyi	13.95	Cellobiose	Batch	38.3	2.9	51.2	1.7	n.d	5.7	[156]

n.d = not detected

Yeast	Oil content (g/l)	Substrate	Mode culture	Fatty acid composition						Ref
				C16:0	C18:0	C18:1	C18:2	C18:3	Other acid	
Lipomyces starkeyi	12.61	Glucose	Batch	34.1	3.2	55.7	1.3	n.d	5.5	[156]
Lipomyces starkeyi	12.71	Xylose	Batch	37.7	3.2	51.4	1.9	n.d	5.6	[156]
R. toruloides Y4	78.7	Glucose	Fed-batch	32.8	2.1	48.8	4.7	1.2	2.1	[159]
R. toruloides Y4	12.6	Glucose + $(NH_4)_2SO_4$	Batch	26.4	5.5	61.5	3.1	n.d	1.8	[165]
Rhodosporidium toruloides	18.5	Glycerol	Batch	28.7	15.3	41.5	10.1	2.6	1.8	[155]
Cryptococcus curvatus	17.4	Glycerol	Fed-batch	23	16.7	39.6	15.2	0.66	0.9	[152]
Rhodosporidium toruloides	12.3	Detoxified biomass hydrolysate	Batch	29.31	9.68	49.36	9.62	2.26	1.64	[166]

n.d= not detected

1.7.4 Bacteria

The synthesis of intracellular lipids in oleaginous bacteria occurs during the logarithmic phase and the beginning of the stationary growth phase [167]. However, only few species of bacteria can accumulate lipids suitable for biodiesel, as they mainly accumulate polyhydroxy alkanoates (PHA) and polyhydroxy butyrate (PHB) [168, 169]. The species that produce more amount of lipids are those belonging to *Streptomyces*, *Nocardia*, *Rhodococcus* and *Mycobacterium* [170]. The amount of triglycerides (TAG) and fatty acid composition differs depending on the species used for fermentation (Table 1.9). Gouda *et al*. [167] tested *R. opacus* and *Gordonia sp* using different agro-industrial wastes (molasses, potato infusion, wheat bran, hydrolyses barley, orange waste, tomato peel waste, artichoke waste, Na-gluconate) as carbon sources. Molasses provided the highest percentage of lipid in cell, 93% and 96% for *R. opacus* and *Gordonia sp.*, respectively, while carob waste offered the best source for TAG accumulation, being 88.9 and 57.8 mg per liter of medium for *R. opacus* and *Gordonia sp.*, respectively and C17:1 the main fatty acid produced (20.7%) by *R. opaccus*. When *Gordonia sp* consumed molasses, they followed the same trend in terms of the accumulation of lipid in cell mass (96%). However, the highest accumulation of TAG (57.8 mg/l) was achieved when orange waste was consumed, being C22:0 the predominant fatty acid, in a percentage close to 35%. Two different strains of bacterium *R. opacus*, DSM 1069 and PD630, were inoculated in lignocellulosic compounds (4-hydroxybenzoic and vanillic acid) [171]. The experiments

showed that both strains can consume these carbon sources and accumulate lipids close to 20% of their own weight.

With regard to bacterial biodiesel properties and subsequent engine testing, only one analysis has been reported [161]. In this study, the bacterium *R. opacus* was grown in sucrose and biodiesel properties were compared with those from microalgae and yeast oil-based biodiesel. Biodiesel bacterial molecular properties differ considerably with the other biofuels in terms of carbon chain length. The physical properties were similar to other microbial biodiesel, with the exception of the heating value, that was lower. When bacterial biodiesel was ran on a diesel engine, it provided the lowest power output, while NO_x and HC emissions were higher and lower than other microbial biodiesel, respectively.

Bacteria that accumulate the highest proportion of triglycerides are providing neither sufficient oil yield under industrial conditions nor an economically sound process. For these reasons, genetic engineering is supporting this biotechnology to be considered a viable alternative for the biodiesel industry. Rucker *et al*. [172] demonstrated the feasibility of the lipid metabolism of *Escherichia coli* for TAG accumulation, but the yield achieved was below the threshold to be considered a viable source for biodiesel production. Authors propose two metabolic engineering steps, to increase either the supply of phosphatidic acid during late exponential and stationary phases growth or the supply of acyl-CoA.

One of the most interesting use of bacteria in the production of biodiesel was described by Kalscheuer *et al*. [173]. In this study, the genetically modified bacteria *E. coli* was recombined with two different enzymes from *Zymomonas mobilis* and *Acinetobacter baylyi*. The target was to produce fatty acid ethyl esters (FAEE) in vivo, called “microdiesel”.

Under fed-batch fermentation using renewable carbon sources, they achieved a FAEE concentration of 1.28 g l^{-1}, corresponding to a FAEE content of the cells of 26% of the cellular dry mass. *Gordonia sp. KTR9* may be considered among the suitable bacteria for in vivo synthesis of fatty acid ethyl esters from short chain alcohols. This species has a large number of genes dedicated to both the formation of fatty acid and lipid biosynthesis. Furthermore, it tolerates the addition of more than 4% methanol, 4% ethanol and 2% propanol in the medium [174].

It may be concluded from above works that biodiesel produced from bacterial oil can be considered as an alternative to first and second generations biodiesel. However, more research is needed to both improve bacterial oil yield and provide economically viable substrates.

Table 1.8 Oil content and fatty acid composition from different bacteria oil

Bacteria	Oil content (%)	C12:1	C12:0	C14:1	C14:0	C16:1	C16:0	C18:1	C18:0	C18:3	Ref
Escherichia coli XL100	2.5	0.64	5.8	12.37	27.60	16.95	24.23	11.28	1.16	n.d	[175]
Gordonia sp. DG	71	n.d	2	12	n.d	n.d	2	n.d	n.d	11	[167]
Bacillus subtilis	33.42	-	-	-	0.7	n.d	26.3	43.6	14.6	n.d	[176]
Pseudomonas spp	42.7	-	-	-	11.1	7	33.3	8.4	28.7	-	[176]
Pseudomonas aeruginosa 44T1	38	n.d	n.d			0.6	17.6	63.7			[177]
Rhodococcus opacus PD630	80	n.d	10	4	n.d	n.d	n.d	4	9	18	[167]
Rhodococcus opacus DSM 1069	17.9					4	19.5	11.9	15.2		[171]
Rhodococcus opacus PD630	38				3.3	4.4	27	5.6	3.6		[171]

n.d=not detected

1.7.5 Cyanobacteria

Cyanobacteria, also called blue-green bacteria, are photoautotrophic bacteria that play a significant role in the global biological carbon sequestration, oxygen production and nitrogen cycle [29]. They have evolved into chloroplasts of algae and green plants [178].

Considering biodiesel production, cyanobacteria have received less attention than other feedstocks, such as microalgae [179]. Cyanobacterial biomass has traditionally been associated with the production of bioethanol [180, 181].

Due to their high photosynthetic levels, growth rates and the simplicity of the culture methods, cyanobacteria could present some advantages for biodiesel production compared with microalgae [182]. Moreover, as mentioned above and on the contrary to eukaryotic algae, prokaryotes can easily be genetically engineered to enhance the production of biofuels [183]. The genome of 41 strains of cyanobacteria has already been completely sequenced [179].

Cyanobacteria can accumulate considerable amounts of lipids in the thylakoid membranes and FFA into extracellular media, simplifying downstream product isolation [179]. For this reason, the cyanobacterium *Synechococcus elongates* PCC7942 has been engineered to increase FFA content [29, 184]. In this sense, Rittmann [183] has genetically engineered a mutant gene of *Synechocystis* that can accumulate up to 50% of dry weight in lipids. Many studies are focused on the dependence of growth conditions and fatty acid composition of lipid production of cyanobacteria [179]. It has been observed that decreasing growth temperature, the degree of

unsaturation increases and the biosynthesis of shorter acyl chains takes place [185]. In wastewater of swine industry, a rise in lipid content and cyanobacterial biomass was found [186]. Light intensity has a direct effect on the increase of the production of monounsaturated fatty acid and the decrease of polyunsaturated ones [187].

Cyanobacteria can produce biofuels (lipid-based biodiesel and ethanol), besides being a potential candidate to generate H_2 [188] and NH_4^+ based fertilizer [179]. Moreover, they have received considerable attention as a promising system for biological CO_2 mitigation, driving down CO_2 emission [179] or as bioremediation agents to remove heavy metals from aquatic ecosystems [189].

1.8 Acknowledgments

This research was supported by the Spanish Ministry of Education and Science (ENE2010-15159) and the Andalusian Economy, Innovation and Enterprise Council, Spain (TEP-4994).

1.9 References

1. *US imports threaten EU biodiesel.* Biofuels Bioproducts & Biorefining, 2008. 2(1): p. 4-4.
2. Rottig, A., et al., *Fatty acid alkyl esters: perspectives for production of alternative biofuels.* Applied Microbiology and Biotechnology, 2011. 85(6): p. 1713-1733.
3. Al-Zuhair, S., *Production of biodiesel: possibilities and challenges.* Biofuels Bioproducts & Biorefining, 2007. 1(1): p. 57-66.
4. EU, *Regulation (EC) No 715/2007 of the European Parliament and of the Council of 20 June 2007 on type approval of motor vehicles with respect to emissions from light passenger and commercial vehicles (Euro 5 and Euro 6)* 2007.
5. Dorado, M.P., et al., *An approach to the economics of two vegetable oil-based biofuels in Spain.* Renewable Energy, 2006. 31(8): p. 1231-1237.
6. Zhang, Y., et al., *Biodiesel production from waste cooking oil: 2. Economic assessment and sensitivity analysis.* Bioresource Technology, 2003. 90(3): p. 229-240.
7. Krawczyk, T., *Biodiesel*, in *International News on Fats, Oils and Related Materials*. 1996, American Oil Chemists Society Press: Champaign, Illinois. p. 801.
8. Dorado, M.P., *Raw materials to produce low cost biodiesel. Chapter 4.*, in *Biofuels refining and performance*, A. Nag, Editor. 2008, McGraw Hill Professional. p. 107-148.
9. Antizar-Ladislao, B. and J.L. Turrion-Gomez, *Second-generation biofuels and local bioenergy systems.* Biofuels Bioproducts & Biorefining, 2008. 2(5): p. 455-469.
10. Moser, B., *Biodiesel production, properties, and feedstocks.* In Vitro Cellular & Developmental Biology - Plant, 2009. 45(3): p. 229-266.

11. Fishinger, M., *Biofuel from waste oils*. 1980, Columbus, Ohio: Ohio State University.

12. Charpe, T.W. and V.K. Rathod, *Biodiesel production using waste frying oil.* Waste Management, 2010. 31(1): p. 85-90.

13. Dorado, M.P., et al., *Testing waste olive oil methyl ester as a fuel in a diesel engine.* Energy & Fuels, 2003. 17(6): p. 1560-1565.

14. Sabudak, T. and M. Yildiz, *Biodiesel production from waste frying oils and its quality control.* Waste Management, 2011. 30(5): p. 799-803.

15. Zhang, Y., et al., *Biodiesel production from waste cooking oil: 1. Process design and technological assessment.* Bioresource Technology, 2003. 89(1): p. 1-16.

16. Ito, T., et al., *Biodiesel production from waste animal fats using pyrolysis method.* Fuel Processing Technology, 2012. 94(1): p. 47-52.

17. Jayasinghe, P. and K. Hawboldt, *A review of bio-oils from waste biomass: Focus on fish processing waste.* Renewable & Sustainable Energy Reviews, 2012. 16(1): p. 798-821.

18. Dias, J.M., M.C.M. Alvim-Ferraz, and M.F. Almeida, *Production of biodiesel from acid waste lard.* Bioresource Technology, 2009. 100(24): p. 6355-6361.

19. Guru, M., et al., *Biodiesel production from waste animal fat and improvement of its characteristics by synthesized nickel and magnesium additive.* Energy Conversion and Management, 2009. 50: p. 498-502.

20. Haas, M.J., S. Bloomer, and K. Scott, *Simple, high-efficiency synthesis of fatty acid methyl esters from soapstock.* Journal of the American Oil Chemists' Society, 2000. 77(4): p. 373-379.

21. Keskin, A., et al., *Using of cotton oil soapstock biodiesel-diesel fuel blends as an alternative diesel fuel.* Renewable & Sustainable Energy Reviews, 2008. 33: p. 553-557.

22. Usta, N., et al., *Combustion of biodiesel fuel produced from hazelnut soapstock/waste sunflower oil mixture in a diesel engine.* Energy Conversion and Management, 2005. 46: p. 741-755.

23. Pinzi, S., et al., *The ideal vegetable oil-based biodiesel composition: a review of social, economical and technical implications.* Energy & Fuels, 2009. 23: p. 2325–2341.

24. Kumar, A., et al., *Enhanced CO(2) fixation and biofuel production via microalgae: recent developments and future directions.* Trends in Biotechnology, 2012. 28(7): p. 371-380.

25. Lam, M.K. and K.T. Lee, *Microalgae biofuels: A critical review of issues, problems and the way forward.* Biotechnology Advances, 2012. 30(3): p. 673-690.

26. Hall, J., et al., *Oil production by a consortium of oleaginous microorganisms grown on primary effluent wastewater.* Journal of Chemical Technology and Biotechnology, 2011. 86(1): p. 54-60.

27. Manzano-Agugliaro, F., et al., *Insects for biodiesel production.* Renewable and Sustainable Energy Reviews, 2012. 16: p. 3744–3753.

28. Vicente, G., et al., *Biodiesel production from biomass of an oleaginous fungus.* Biochemical Engineering Journal, 2009. 48(1): p. 22-27.

29. Parmar, A., et al., *Cyanobacteria and microalgae: A positive prospect for biofuels.* Bioresource Technology, 2012. 102(22): p. 10163-10172.

30. Balasubramanian, L., et al., *Cyanobacteria cultivation in industrial wastewaters and biodiesel production from their biomass: A review.* Biotechnology and Applied Biochemistry, 2012. 58(4): p. 220-225.

31. Rottig, A., et al., *Fatty acid alkyl esters: perspectives for production of alternative biofuels.* Applied Microbiology and Biotechnology, 2010. 85(6): p. 1713-1733.

32. Pramanik, K., *Properties and use of Jatropha curcas oil and diesel fuel blends in compression ignition engine.* Renewable Energy, 2003. 28(2): p. 239-248.

33. Demirbas, A., *Potential resources of non-edible oils for biodiesel.* Energy Sources Part B, 2009. 4: p. 310-314.

34. Balat, M., *Potential alternatives to edible oils for biodiesel production – A review of current work.* Energy Conversion and Management, 2011. 52(2): p. 1479-1492.

35. Azocar, L., et al., *Biotechnological processes for biodiesel production using alternative oils.* Applied Microbiology and Biotechnology, 2010. 88(3): p. 621-636.

36. Pinzi, S. and M.P. Dorado, *Feedstocks for advanced biodiesel production*, in *Advances in biodiesel production: Processes and technologies*, R. Luque and J.A. Melero, Editors. 2012, Woodhead Cambridge, UK.

37. Pinzi, S. and M.P. Dorado, *Vegetable-based feedstocks for biofuels production*, in *Handbook of biofuels production: Processes and technologies*, R. Luque, J.M. Campelo, and J.H. Clark, Editors. 2010, Woodhead Publishing Limited: Cambridge, UK.

38. Gui, M.M., K.T. Lee, and S. Bhatia, *Feasibility of edible oil vs. non-edible oil vs. waste edible oil as biodiesel feedstock.* Energy, 2008. 33(11): p. 1646-1653.

39. Nye, M.J., et al., *Conversion of used frying oil to diesel fuel by transesterification: preliminary tests.* Journal of the American Oil Chemists' Society, 1983. 60(8): p. 1598–1601.

40. Mittelbach, M. and H. Enzelsberger, *Transesterification of heated rapeseed oil for extending diesel fuel.* Journal of the American Oil Chemists' Society, 1999. 76(5): p. 545–550.

41. Dorado, M.P., et al., *An alkali-catalyzed transesterification process for high free fatty acid feedstocks.* Transactions of ASAE, 2002. 45(3): p. 525-529.

42. Mittelbach, M., B. Pokits, and A. Silberholz. *Diesel fuels derived from vegetable oils, IV: Production and fuel properties of fatty acid methyl esters from used frying oil*. in *Liquid Fuels from Renewable Resources. Proceedings of an Alternative Energy Conference*. 1992. Nashville, Tennesse (USA).

43. El Sabagh, S.M., S.T. Keera, and A.R. Taman, *The characterization of biodiesel fuel from waste frying oil.* Energy Sources Part a-Recovery Utilization and Environmental Effects, 2010. 33(5): p. 401-409.

44. Canakci, M. and J. Van Gerpen, *Biodiesel production from oils and fats with high free fatty acids.* Transaction of the ASAE, 2003. 44: p. 1429–1436.

45. Jain, S., M.P. Sharma, and S. Rajvanshi, *Acid base catalyzed transesterification kinetics of waste cooking oil.* Fuel Processing Technology, 2010. 92(1): p. 32-38.

46. Wang, Y., et al., *Comparison of two different processes to synthesize biodiesel by waste cooking oil.* Journal of Molecular Catalysis A: Chemical, 2006. 252: p. 107–112.

47. Guzatto, R., et al., *Transesterification double step process modification for ethyl ester biodiesel production from vegetable and waste oils.* Fuel, 2012. 92(1): p. 197-203.

48. Ramadhas, A.S., S. Jayaraj, and C. Muraleedharan, *Biodiesel production from high FFA rubber seed oil.* Fuel, 2005. 84(4): p. 335-340.

49. Sahoo, P.K., et al., *Biodiesel development from high acid value polanga seed oil and performance evaluation in a CI engine.* Fuel, 2006. 86 p. 448-454.

50. Ma, F.R. and M.A. Hanna, *Biodiesel production: a review.* Bioresource Technology, 1999. 70(1): p. 1-15.

51. Freedman, B., E.H. Pryde, and T.L. Mounts, *Variables affecting the yields of fatty esters from transesterified vegetable oils.* Journal of the American Oil Chemists' Society, 1984. 61: p. 1638–1643.

52. Kumar Tiwari, A., A. Kumar, and H. Raheman, *Biodiesel production from jatropha oil (Jatropha curcas) with high free fatty acids: An optimized process.* Biomass and Bioenergy, 2007. 31(8): p. 569-575.

53. Wan Omar, W.N.N. and N.A.S. Amin, *Biodiesel production from waste cooking oil over alkaline modified zirconia catalyst.* Fuel Processing Technology, 2012. 92(12): p. 2397-2405.

54. Birla, A., et al., *Kinetics studies of synthesis of biodiesel from waste frying oil using a heterogeneous catalyst derived from snail shell.* Bioresource Technology, 2012. 106(0): p. 95-100.

55. Jacobson, K., et al., *Solid acid catalyzed biodiesel production from waste cooking oil.* Applied Catalysis B: Environmental, 2008. 85(1,Äì2): p. 86-91.

56. Lou, W.-Y., M.-H. Zong, and Z.-Q. Duan, *Efficient production of biodiesel from high free fatty acid-containing waste oils using various carbohydrate-derived solid acid catalysts.* Bioresource Technology, 2008. 99(18): p. 8752-8758.

57. Maceiras, R., et al., *Effect of methanol content on enzymatic production of biodiesel from waste frying oil.* Fuel, 2009. 88(11): p. 2130-2134.

58. Lam, M.K., M.T. Lee, and A.R. Mohamed, *Homogeneous, heterogeneous and enzymatic catalysis for transesterification of high free fatty acid oil (waste cooking oil) to biodiesel: A review.* Biotechnology Advances, 2011. 28(4): p. 500-518.

59. Harvey, A.P., M.R. Mackley, and T. Seliger, *Process intensification of biodiesel production using a continuous oscillatory flow reactor.* Journal of Chemical Technology & Biotechnology, 2003. 78(2-3): p. 338-341.

60. Wang, J.-X., et al., *Lipase-catalyzed production of biodiesel from high acid value waste oil using ultrasonic assistant.* Chinese Journal of Biotechnology, 2007. 23(6): p. 1121-1128.

61. Refaat, A.A. and S.T. El-Sheltawy. *Comparing three options for biodiesel production from waste vegetable oil*. in *Waste management and the environment IV. International Conference on Waste Management and the Environment*. 2008. Granada, Spain.

62. Lertsathapornsuk, V., et al., *Microwave assisted in continuous biodiesel production from waste frying palm oil and its performance in a 100-kW diesel generator.* Fuel Processing Technology, 2008. 89(12): p. 1330-1336.

63. Guan, G., K. Kusakabe, and S. Yamasaki, *Tri-potassium phosphate as a solid catalyst for biodiesel production from waste cooking oil.* Fuel Processing Technology, 2009. 90(4): p. 520-524.

64. Lee, S., D. Posarac, and N. Ellis, *An experimental investigation of biodiesel synthesis from waste canola oil using supercritical methanol.* Fuel, 2012. 91(1): p. 229-237.

65. Hingu, S.M., P.R. Gogate, and V.K. Rathod, *Synthesis of biodiesel from waste cooking oil using sonochemical reactors.* Ultrasonics Sonochemistry, 2010. 17(5): p. 827-832.

66. Thanh, L., et al., *A two-step continuous ultrasound assisted production of biodiesel fuel from waste cooking oils: A practical and economical approach to produce high quality biodiesel fuel.* Bioresource Technology, 2010. 101(14): p. 5394-5401.

67. Enweremadu, C.C. and H.L. Rutto, *Combustion, emission and engine performance characteristics of used cooking oil biodiesel-A review.* Renewable and Sustainable Energy Reviews, 2010. 14(9): p. 2863-2873.

68. Lapuerta, M., J. Rodriguez-Fernandez, and J.R. Agudelo, *Diesel particulate emissions from used cooking oil biodiesel.* Bioresource Technology, 2008. 99(4): p. 731-740.

69. Oner, C. and S. Altun, *Biodiesel production from inedible animal tallow and an experimental investigation of its use as alternative fuel in a direct injection diesel engine.* Applied Energy, 2009. 86: p. 2114-2120.

70. Lu, J., et al., *Enzymatic synthesis of fatty acid methyl esters from lard with immobilized Candida sp.* Process Biochemistry, 2007. 42: p. 1367-1370.

71. Guru, M., et al., *Biodiesel production from waste chicken fat based sources and evaluation with Mg based additive in a diesel engine.* Renewable Energy, 2010. 35: p. 637-643.

72. Ma, F., L.D. Clements, and M.A. Hanna, *The effects of catalyst, free fatty acids and water on transesterification of beef tallow.* Transaction of ASAE, 1998. 41: p. 1261–1264.

73. Liu, S.Y., et al., *Fast biodiesel production from beef tallow with radio frequency heating.* Renewable Energy, 2010. 36(3): p. 1003-1007.

74. Araujo, B.Q., et al., *Synthesis and characterization of beef tallow biodiesel.* Energy & Fuels, 2010. 24: p. 4476-4480.

75. Fröhlich, A., B. Rice, and G. Vicente, *The conversion of low grade tallow into biodiesel-grade methyl ester.* Journal of the American Oil Chemists' Society, 2010. 87: p. 825-833.

76. Nebel, B.A. and M. Mittelbach, *Biodiesel from extracted fat out of meat and bone meal.* European Journal of Lipid Science Technology, 2006. 108: p. 398–403.

77. Mbaraka, I.K., K.J. McGuire, and B.H. Shanks, *Acidic mesoporous silica for the catalytic conversion of fatty acids in beef tallow.* Industrial & Engineering Chemistry Research, 2006. 45(9): p. 3022–3028.

78. Mondal, P., M. Basu, and N. Balasubramanian, *Direct use of vegetable oil and animal fat as alternative fuel in internal combustion engine.* Biofuels Bioproducts & Biorefining-Biofpr, 2008. 2(2): p. 155-174.

79. D'Arrigo, M., et al., *Effect of dietary linseed oil and a-tocopherol on selected properties of pig fat.* Canadian Journal of Animal Science, 2002. 82(3): p. 339-346.

80. Berrios, M., et al., *Application of the factorial design of experiments to biodiesel production from lard.* Fuel Processing Technology, 2009. 90(12): p. 1447-1451.

81. Shin, H.Y., et al., *Biodiesel production from waste lard using supercritical methanol.* Journal of Supercritical Fluids, 2012. 61: p. 134-138.

82. Kondamudi, N., et al., *A green process for producing biodiesel from feather meal.* Journal of Agricultural and Food Chemistry, 2009. 57(14): p. 6163-6166.

83. Mattingly, B.G., *Production of biodiesel from chicken fat containing free fatty acids*. 2006, University of Arkansas.

84. Schulte, W.B., *Biodiesel production from tall oil and chicken fat via supercritical methanol treatment*. 2007, University of Arkansas.

85. Alptekin, E. and M. Canakci, *Optimization of pretreatment reaction for methyl ester production from chicken fat.* Fuel, 2010. 89(12): p. 4035-4039.

86. Feddern, V., et al., *Physico-chemical composition, fractionated glycerides and fatty acid profile of chicken skin fat.* European Journal of Lipid Science and Technology, 2010. 112(11): p. 1277-1284.

87. Ayalasomayajula, S., et al., *Potential of alligator fat as source of lipids for biodiesel production.* Industrial & Engineering Chemistry Research, 2012. 51(4): p. 2166-2169.

88. Alptekin, E., M. Canakci, and H. Sanli, *Evaluation of leather industry wastes as a feedstock for biodiesel production.* Fuel, 2012. 95(0): p. 214-220.

89. Ravindran, B. and G. Sekaran, *Bacterial composting of animal fleshing generated from tannery industries.* Waste Management, 2012. 30(12): p. 2622-2630.

90. Manzano-Agugliaro, F., et al., *Insects for biodiesel production.* Renewable and Sustainable Energy Reviews, 2012. 16(6): p. 3744-3753.

91. Ramos-Elorduy, J., *Energy supplied by edible insects from Mexico and their nutritional and ecological importance.* Ecology of Food and Nutrition, 2008. 47(3): p. 280-297.

92. Li, Z., et al., *Chrysomya megacephala (Fabricius) larvae: A new biodiesel resource.* Applied Energy, 2012. 94(0): p. 349-354.

93. Leung, D., et al., *Biodiesel from Zophobas morio Larva Oil: Process Optimization and FAME Characterization.* Industrial & Engineering Chemistry Research, 2012. 51(2): p. 1036-1040.

94. Li, Q., et al., *Bioconversion of dairy manure by black soldier fly (Diptera: Stratiomyidae) for biodiesel and sugar production.* Waste Management, 2011. 31(6): p. 1316-1320.

95. Zheng, L., et al., *Biodiesel production from rice straw and restaurant waste employing black soldier fly assisted by microbes.* Energy, 2012. 47(1): p. 225-229.

96. Zheng, L., et al., *Exploring the potential of grease from yellow mealworm beetle (Tenebrio molitor) as a novel biodiesel feedstock.* Applied Energy, 2013. 101(0): p. 618-621.

97. Li, Q., et al., *From organic waste to biodiesel: Black soldier fly, Hermetia illucens, makes it feasible.* Fuel, 2011. 90(4): p. 1545-1548.

98. Rumpold, B.A. and O.K. Schluter, *Nutritional composition and safety aspects of edible insects.* Molecular Nutrition & Food Research, 2013. 57(5): p. 802-823.

99. Pinzi, S., et al., *The Ideal Vegetable Oil-based Biodiesel Composition: A Review of Social, Economical and Technical Implications.* Energy & Fuels, 2009. 23: p. 2325-2341.

100. Belluco, S., et al., *Edible Insects in a Food Safety and Nutritional Perspective: A Critical Review.* Comprehensive Reviews in Food Science and Food Safety, 2013. 12(3): p. 296-313.

101. St-Hilaire, S., et al., *Fish Offal Recycling by the Black Soldier Fly Produces a Foodstuff High in Omega-3 Fatty Acids.* Journal of the World Aquaculture Society, 2007. 38(2): p. 309-313.

102. Raksakantong, P., et al., *Fatty acids and proximate composition of eight Thai edible terricolous insects.* Food Research International, 2010. 43(1): p. 350-355.

103. Sivakumar, P., K. Anbarasu, and S. Renganathan, *Bio-diesel production by alkali catalyzed transesterification of dairy waste scum.* Fuel, 2010. 90(1): p. 147-151.

104. Li, Q., W. Du, and D. Liu, *Perspectives of microbial oils for biodiesel production.* Applied Microbiology and Biotechnology, 2008. 80(5): p. 749-756.

105. Xu, J.Y., et al., *Microbial oil production from various carbon sources and its use for biodiesel preparation.* Biofuels Bioproducts & Biorefining-Biofpr, 2013. 7(1): p. 65-77.

106. Neste oil, *the only way is forward, annual report 2012*, N. oil, Editor. 2012. p. 131.

107. Demirbas, A. and M. Fatih Demirbas, *Importance of algae oil as a source of biodiesel.* Energy Conversion and Management, 2010. 52(1): p. 163-170.

108. Gonzalez-Delgado, A.-D. and V. Kafarov, *Microalgae based biorefinery: issues to consider.* Ct&F-Ciencia Tecnologia y Futuro, 2012. 4(4): p. 5-21.

109. Demirbas, A., *Use of algae as biofuel sources.* Energy Conversion and Management, 2010. 51(12): p. 2738-2749.

110. Lin, L., et al., *Opportunities and challenges for biodiesel fuel.* Applied Energy, 2011. 88(4): p. 1020-1031.

111. Wijffels, R.H., M.J. Barbosa, and M.H.M. Eppink, *Microalgae for the production of bulk chemicals and biofuels.* Biofuels Bioproducts & Biorefining, 2010. 4(3): p. 287-295.

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

113. Chisti, Y., *Biodiesel from microalgae.* Biotechnology Advances, 2010. 25(3): p. 294-306.

114. Lam, M.K. and K.T. Lee, *Renewable and sustainable bioenergies production from palm oil mill effluent (POME): Win-win strategies toward better environmental protection.* Biotechnology Advances, 2012. 29(1): p. 124-141.

115. Greenwell, H.C., et al., *Placing microalgae on the biofuels priority list: a review of the technological challenges.* Journal of the Royal Society Interface, 2010. 7(46): p. 703-726.

116. Xu, H., X. Miao, and Q. Wu, *High quality biodiesel production from a microalga Chlorella protothecoides by heterotrophic growth in fermenters.* Journal of Biotechnology, 2006. 126(4): p. 499-507.

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

118. Lv, X., et al., *Variations in lipid yields and compositions of marine microalgae during cell growth and respiration, and within intracellular structures.* Journal of Experimental Marine Biology and Ecology, 2010. 391(1-2): p. 73-83.

119. Sanford, S.D., et al., *Feedstock and Biodiesel Characteristics Report*. 2009, Renewable Energy Group.

120. Sheehan, J., et al., *A look back at the US Department of Energy's aquatic species program—biodiesel from algae*. 1998, National Renewable Energy Laboratory: Golden, CO.

121. Christenson, L. and R. Sims, *Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts.* Biotechnology Advances, 2012. 29(6): p. 686-702.

122. Mata, T.M., A.A. Martins, and N.S. Caetano, *Microalgae for biodiesel production and other applications: A review.* Renewable and Sustainable Energy Reviews, 2012. 14(1): p. 217-232.

123. Whipple, T. *Peak oil review*. in *International peak oil conference* 2009. Denver, Colorado: Association for the Study of Peak Oil and Gas (ASPO).

124. Yen, H.-W. and D.E. Brune, *Anaerobic co-digestion of algal sludge and waste paper to produce methane.* Bioresource Technology, 2007. 98(1): p. 130-134.

125. Chiellini, E., et al., *Biodegradable thermoplastic composites based on polyvinyl alcohol and algae.* Biomacromolecules, 2008. 9(3): p. 1007-1013.

126. Mulbry, W., et al., *Recycling of manure nutrients: use of algal biomass from dairy manure treatment as a slow release fertilizer.* Bioresource Technology, 2005. 96(4): p. 451-458.

127. Singh, J. and S. Cu, *Commercialization potential of microalgae for biofuels production.* Renewable & Sustainable Energy Reviews, 2010. 14(9): p. 2596-2610.

128. Karimi, K. and A. Zamani, *Mucor indicus: Biology and industrial application perspectives: A review.* Biotechnology Advances, 2013. 31(4): p. 466-481.

129. Xia, C.J., et al., *A new cultivation method for microbial oil production: cell pelletization and lipid accumulation by Mucor circinelloides.* Biotechnology for Biofuels, 2011. 4.

130. Liu, B. and Z. Zhao, *Biodiesel production by direct methanolysis of oleaginous microbial biomass.* Journal of Chemical Technology and Biotechnology, 2007. 82(8): p. 775-780.

131. Venkata Subhash, G. and S. Venkata Mohan, *Biodiesel production from isolated oleaginous fungi Aspergillus sp. using corncob waste liquor as a substrate.* Bioresource Technology, 2011. 102(19): p. 9286-9290.

132. Mitra, D., et al., *Value-added oil and animal feed production from corn-ethanol stillage using the oleaginous fungus Mucor circinelloides.* Bioresource Technology, 2012. 107(0): p. 368-375.

133. Zeng, J., et al., *Lignocellulosic biomass as a carbohydrate source for lipid production by Mortierella isabellina.* Bioresource Technology, 2013. 128(0): p. 385-391.

134. Zikou, E., et al., *Evaluating glucose and xylose as cosubstrates for lipid accumulation and γ-linolenic acid biosynthesis of Thamnidium elegans.* Journal of Applied Microbiology, 2013. 114(4): p. 1020-1032.

135. Ruan, Z., et al., *Evaluation of lipid accumulation from lignocellulosic sugars by Mortierella isabellina for biodiesel production.* Bioresource Technology, 2012. 110(0): p. 198-205.

136. Economou, C.N., et al., *Single cell oil production from rice hulls hydrolysate.* Bioresource Technology, 2011. 102(20): p. 9737-9742.

137. Khot, M., et al., *Single cell oil of oleaginous fungi from the tropical mangrove wetlands as a potential feedstock for biodiesel.* Microbial Cell Factories, 2012. 11.

138. Zheng, Y.B., et al., *Feasibility of filamentous fungi for biofuel production using hydrolysate from dilute sulfuric acid pretreatment of wheat straw.* Biotechnology for Biofuels, 2012. 5.

139. Kumar, A.K., P. Vatsyayan, and P. Goswami, *Production of Lipid and Fatty Acids during Growth of Aspergillus terreus on Hydrocarbon Substrates.* Applied Biochemistry and Biotechnology, 2010. 160(5): p. 1293-1300.

140. Bellou, S., et al., *Lipids Containing Polyunsaturated Fatty Acids Synthesized by Zygomycetes Grown on Glycerol.* Applied Biochemistry and Biotechnology, 2012. 166(1): p. 146-158.

141. Chatzifragkou, A., et al., *Biotechnological conversions of biodiesel derived waste glycerol by yeast and fungal species.* Energy, 2011. 36(2): p. 1097-1108.

142. Vongsangnak, W., et al., *Genome-scale analysis of the metabolic networks of oleaginous Zygomycete fungi.* Gene, 2013. 521(1): p. 180-190.

143. Ruenwai, R., S. Cheevadhanarak, and K. Laoteng, *Overexpression of Acetyl-CoA Carboxylase Gene of Mucor rouxii Enhanced Fatty Acid Content in Hansenula polymorpha.* Molecular Biotechnology, 2009. 42(3): p. 327-332.

144. Wynn, J.P., A.B.A. Hamid, and C. Ratledge, *The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi.* Microbiology-Uk, 1999. 145: p. 1911-1917.

145. Muniraj, I.K., et al., *Microbial lipid production from potato processing wastewater using oleaginous filamentous fungi Aspergillus oryzae.* Water Research, 2013. 47(10): p. 3477-3483.

146. Gao, D.F., et al., *Microbial lipid production from xylose by Mortierella isabellina.* Bioresource Technology, 2013. 133: p. 315-321.

147. Taha, E.M., et al., *Lipid biosynthesis in Cunninghamella bainieri 2A1 in N-limited and N-excess media.* Annals of Microbiology, 2010. 60(4): p. 615-622.

148. Lunin, V.V., et al., *Biodiesel fuel production from lipids of filamentous fungi.* Applied Biochemistry and Microbiology, 2013. 49(1): p. 46-52.

149. Beopoulos, A., J.M. Nicaud, and C. Gaillardin, *An overview of lipid metabolism in yeasts and its impact on biotechnological processes.* Applied Microbiology and Biotechnology, 2011. 90(4): p. 1193-1206.

150. Ageitos, J.M., et al., *Oily yeasts as oleaginous cell factories.* Applied Microbiology and Biotechnology, 2011. 90(4): p. 1219-1227.

151. Uckun Kiran, E., A. Trzcinski, and C. Webb, *Microbial oil produced from biodiesel by-products could enhance overall production.* Bioresource Technology, 2013. 129(0): p. 650-654.

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

153. Saenge, C., et al., *Potential use of oleaginous red yeast Rhodotorula glutinis for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids.* Process Biochemistry, 2011. 46(1): p. 210-218.

154. Galafassi, S., et al., *Lipid production for second generation biodiesel by the oleaginous yeast Rhodotorula graminis.* Bioresource Technology, 2012. 111(0): p. 398-403.

155. Xu, J.Y., et al., *Microbial conversion of biodiesel byproduct glycerol to triacylglycerols by oleaginous yeast Rhodosporidium toruloides and the individual effect of some impurities on lipid production.* Biochemical Engineering Journal, 2012. 65: p. 30-36.

156. Gong, Z.W., et al., *Co-fermentation of cellobiose and xylose by Lipomyces starkeyi for lipid production.* Bioresource Technology, 2012. 117: p. 20-24.

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

158. Uckun Kiran, E., et al., *Enhancing the value of nitrogen from rapeseed meal for microbial oil production.* Enzyme and Microbial Technology, 2012. 50(6-7): p. 337-42.

159. Zhao, X., et al., *Lipid production by Rhodosporidium toruloides Y4 using different substrate feeding strategies.* Journal of Industrial Microbiology & Biotechnology, 2011. 38(5): p. 627-632.

160. Pinzi, S., et al., *Influence of vegetable oils fatty acid composition on reaction temperature and glycerides conversion to biodiesel during transesterification.* Bioresource Technology, 2011. 102(2): p. 1044-1050.

161. Wahlen, B.D., et al., *Biodiesel from Microalgae, Yeast, and Bacteria: Engine Performance and Exhaust Emissions.* Energy & Fuels, 2012. 27(1): p. 220-228.

162. Sankh, S., et al., *Biodiesel production from a newly isolated Pichia kudriavzevii strain.* Fuel, 2013. 106(0): p. 690-696.

163. Dey, P. and M.K. Maiti, *Molecular characterization of a novel isolate of Candida tropicalis for enhanced lipid production.* Journal of Applied Microbiology, 2013. 114(5): p. 1357-1368.

164. Ryu, B.-G., et al., *High-cell-density cultivation of oleaginous yeast Cryptococcus curvatus for biodiesel production using organic waste from the brewery industry.* Bioresource Technology, 2013. 135(0): p. 357-364.

165. Wu, S.G., et al., *Phosphate-limitation mediated lipid production by Rhodosporidium toruloides.* Bioresource Technology, 2010. 101(15): p. 6124-6129.

166. Zhao, X.B., et al., *Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast Rhodosporidium toruloides and preparation of biodiesel by enzymatic transesterification of the lipid.* Bioprocess and Biosystems Engineering, 2012. 35(6): p. 993-1004.

167. Gouda, M.K., S.H. Omar, and L.M. Aouad, *Single cell oil production by Gordonia sp DG using agro-industrial wastes.* World Journal of Microbiology & Biotechnology, 2008. 24(9): p. 1703-1711.

168. Shi, S.B., et al., *Prospects for microbial biodiesel production.* Biotechnology Journal, 2011. 6(3): p. 277-285.

169. Kosa, M. and A.J. Ragauskas, *Lipids from heterotrophic microbes: advances in metabolism research.* Trends in Biotechnology, 2011. 29(2): p. 53-61.

170. Alvarez, H.M. and A. Steinbuchel, *Triacylglycerols in prokaryotic microorganisms.* Applied Microbiology and Biotechnology, 2002. 60(4): p. 367-376.

171. Kosa, M. and A.J. Ragauskas, *Bioconversion of lignin model compounds with oleaginous Rhodococci.* Applied Microbiology and Biotechnology, 2012. 93(2): p. 891-900.

172. Rucker, J., et al., *Engineering E. coli for triglyceride accumulation through native and heterologous metabolic reactions.* Applied Microbiology and Biotechnology, 2013. 97(6): p. 2753-2759.

173. Kalscheuer, R., T. Stolting, and A. Steinbuchel, *Microdiesel: Escherichia coli engineered for fuel production.* Microbiology-Sgm, 2006. 152: p. 2529-2536.

174. Eberly, J.O., D.B. Ringelberg, and K.J. Indest, *Physiological characterization of lipid accumulation and in vivo ester formation in Gordonia sp KTR9.* Journal of Industrial Microbiology & Biotechnology, 2013. 40(2): p. 201-208.

175. Lu, X., H. Vora, and C. Khosla, *Overproduction of free fatty acids in E. coli: Implications for biodiesel production.* Metabolic Engineering, 2008. 10(6): p. 333-339.

176. Patnayak, S. and A. Sree, *Screening of bacterial associates of marine sponges for single cell oil and PUFA.* Letters in Applied Microbiology, 2005. 40(5): p. 358-363.

177. Deandres, C., et al., *Cellular lipid-accumulation by Pseudomonas aeruginosa 44T1.* Applied Microbiology and Biotechnology, 1991. 35(6): p. 813-816.

178. Tamagnini, P., et al., *Cyanobacterial hydrogenases: diversity, regulation and applications.* FEMS Microbiology Reviews, 2007. 31(6): p. 692-720.

179. Quintana, N., et al., *Renewable energy from cyanobacteria: energy production optimization by metabolic pathway engineering.* Applied Microbiology and Biotechnology, 2011. 91(3): p. 471-490.

180. Deng, M.D. and J.R. Coleman, *Ethanol synthesis by genetic engineering in Cyanobacteria.* Applied and Environmental Microbiology, 1999. 65(2): p. 523-528.

181. Dexter, J. and P. Fu, *Metabolic engineering of cyanobacteria for ethanol production.* Energy & Environmental Science, 2009. 2(8): p. 857-864.

182. Meng, X., et al., *Biodiesel production from oleaginous microorganisms.* Renewable Energy, 2009. 34(1): p. 1-5.

183. Rittmann, B.E., *Opportunities for renewable bioenergy using microorganisms.* Biotechnology and Bioengineering, 2008. 100(2): p. 203-212.

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

185. Liu, X.J., Y. Jiang, and F. Chen, *Fatty acid profile of the edible filamentous Cyanobacterium nostoc flagelliforme at different temperatures and developmental stages in liquid suspension culture.* Process Biochemistry, 2005. 40(1): p. 371-377.

186. Cañizares-Villanueva, R.O., et al., *Chemical composition of Cyanobacteria grown in diluted, aerated swine wastewater.* Bioresource Technology, 1995. 51(2-3): p. 111-116.

187. Walsh, K., G.J. Jones, and R. Hugh Dunstan, *Effect of irradiance on fatty acid, carotenoid, total protein composition and growth of Microcystis aeruginosa.* Phytochemistry, 1997. 44(5): p. 817-824.

188. Schütz, K., et al., *Cyanobacterial H2 production — a comparative analysis.* Planta, 2004. 218(3): p. 350-359.

189. Hall, D.O., et al., *The potential applications of Cyanobacterial photosynthesis for clean technologies.* Photosynthesis Research, 1995. 46(1): p. 159-167.

Chapter 2

"Influence of cultivation practices on biodiesel quality"

Chapter 2. Influence of cultivation practices on biodiesel quality

From "Influence of nitrogen fertilization on physical and chemical properties of fatty acid methyl esters from *Brassica napus* oil" published in Fuel, 2013, 111, pp 865-871

Abstract Biodiesel is a biodegradable fuel that originates from a variety of oleaginous raw materials, although most studies have been traditionally based on vegetable oils. Rapeseed (*Brassica napus*) is the raw material most widely used in the production of biodiesel in temperate climate, i.e. central Europe, due to its physical properties and the content of oleic acid of its oil. However, this plant needs nitrogen fertilization at different stages of growth to achieve an optimal oilseed production, thus increasing costs. For this reason, the aim of this work was to find out the importance of nitrogen fertilization through the study of its influence on the physicochemical properties of both rapeseed oil and its biodiesel. Rapeseed variety "Lucia" was used to carry out this research. The oil from each sample of rapeseed was extracted using mechanical processing (cold pressed) and analyzed according to European standards. Results indicate some differences on the acid value and fatty acid composition among the samples of oil depending on the level of nitrogen fertilization. With respect to the fatty acid composition, the sample 0-0 (no nitrogen fertilization) showed a decrease on oleic acid compared to the rest of samples. With the aid of an experimental design, the optimum transesterification parameters were also

determined. Results indicate that the temperature of the reaction and KOH increased for the sample 0-0, while less amount of methanol-to-oil ratio was needed. Finally the most important physical and chemical properties of biodiesel were analyzed. It may be concluded that nitrogen fertilization influences oil properties. Moreover, despite of the saving during the cultivation, when the crops are not fertilized there is a rise in biodiesel production costs due to an increase of the reaction transesterification temperature and KOH needs, besides the reduction in seeds production.

2.1 Introduction

The consumption of fossil fuels is increasing due to massive use in the transport sector. The dependence on limited petroleum-based fuels forces to search for alternatives that meet this growing demand and the directive over renewable energy. In this way, biofuels, and particularly biodiesel (FAME), which is used in diesel engines without significant engine modifications, represent a viable solution. Biodiesel consists in a renewable alternative with similar characteristics to diesel fuel. It is commonly produced by transesterification of triglycerides with an alcohol (usually methanol, CH_3OH) in the presence of a basic, acid or heterogeneous catalyst [1-3]. Comparing the different oils used for biodiesel production in 2011, sunflower, soybean and rapeseed reached a production of 250,784 t and 8,000,000 t, respectively [4].

As the second largest oil crop in the world, rapeseed (*Brassica napus*) attracts great economic and scientific interest with regard to seed oil [5]. Furthermore, thermal studies using blends of this kind of biodiesel have shown a higher thermal stability than diesel fuel [6]. The maximum seed oil content is close to 40% [7]. This crop requires for its growth macronutrients

such as nitrogen, among some others [8]. To meet these macronutrient needs, fertilization (either organic or inorganic) must be added to the crop. Some authors state that an excess of fertilization may cause a reduction in the seed oil content, while increasing protein content [9]. Moreover, it has been found that nitrogen fertilization can affect the composition of fatty acid of the oil [7].

Several studies agree that biodiesel production and properties are strictly dependent on oil fatty acid composition used as raw material [10-13]. Moreover, a high acidity of the oil may cause the formation of soaps during basic transesterification [14]. It has also been found that the length of the chain (LC) and the degree of unsaturation (number of double bonds, UD) of the fatty acid can have an effect on the physical properties of biodiesel, including kinematic viscosity (μ), cetane number (CN), lower heating value (LHV), cold properties and NO_x emissions [12, 15].

It has been shown that biodiesel emissions of carbon monoxide (CO) and particulate matter (PM) are generally lower compared to those of diesel fuel, although there is an increase in nitrogen oxides (NO_x) [16, 17]. Some studies suggests that NO_x emissions depend on the number of double bounds of fatty acid composition methyl esters [18]. In turn the degree of unsaturation of FAME is strongly related to NO_x and PM [19]. In this regard, it would be appropriate to adjust the physical and chemical properties of biodiesel to minimize these harmful gases.

The aim of this study is to determine the effect of the level of nitrogen fertilization on the quality and the fatty acid composition of rapeseed oil and its suitability to be used as a feedstock for biodiesel production.

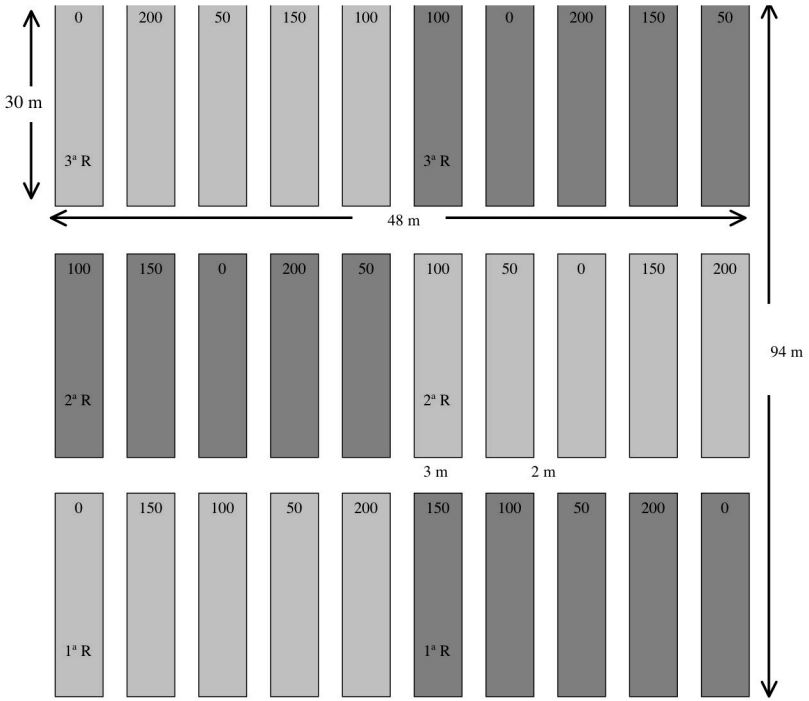
2.2 Materials and Methods

2.2.1 Raw materials

Brassica napus, variety “Lucia”, was selected to carry out this work. Seeds were produced from a trial in a research and training institute for agricultural and fishery (IFAPA) "Alameda del Obispo", Cordoba, Spain, in which plots were arranged with different doses of nitrogen fertilization (Figure 2.1). The nitrogen source was ammonium nitrosulphate (26% N). The main factor was N basal (0 and 50 kg/ha), while N topdressing was considered as the secondary factor (0, 50, 100, 150 and 200 kg/ha). Crops were named x-y, where x indicates basal and y indicates topdressing fertilization.

The soil was sandy loam texture of fluvial origin. According to the soil taxonomy, it corresponds to typic xerofluvent. It presents a basic pH (8-8.7) and high CO_3Ca content. The organic matter is low (0.6-1%), P content is medium-low and K content is moderate. The presence of soluble nitrates can be considered low (47 kg NO_3 in the upper 60 cm).

Figure 2.1 Crop design considering different doses of nitrogen fertilization. Dark plots indicate 0 kg/ha basal nitrogen fertilization; light plots indicate 50 kg/ha basal nitrogen fertilization. Numbers inside the plots indicate the doses of topdressing nitrogen fertilization. Each test was made in triplicate (R: replications)



2.2.2 Reagents

Oil analysis

Absolute ethanol PA, diethyl ether stabilized with 6 ppm BTH PA-ACS, phenolphthalein dissolved in ethanol (10 g/l), methanol PA-ACS-ISO and potassium hydroxide 0.1 N and 0.5 N were acquired from Panreac (Barcelona, Spain) and used to determine the acidity of oil samples.

For the calculation of the peroxide value, glacial acetic acid PA-ACS, PA-ACS water, soluble starch RE, KI-PA ISO, 0.1 N sodium thiosulfate VS and trichloromethane stabilized ethanol PA-ACS-ISO, all supplied by Panreac (Barcelona, Spain), were used.

The preparation of samples for subsequent analysis of fatty acid composition by gas chromatography (UNE-EN 14103) required n-hexane HPLC and sodium methylate solution in methanol 30% PS, supplied by Panreac (Barcelona, Spain). Heptadecanoic acid (C17:0) was used as internal standard and was purchased from Fluka (Steinheim, Germany).

For the coulometric Karl Fischer titration, HYDRANAL-Coulomat oil was used as anolyte and HYDRANAL-Coulomat CG as catholyte, both purchased from Fluka (Steinheim, Germany).

Biodiesel

KOH pellets 85% p.a. CODEX (USP_NF) and methanol ACS-ISO were acquired from PANREAC (Barcelona, Spain). KOH and methanol were used in the transesterification as catalyst and alcohol, respectively. For the drying of biodiesel, sodium sulphate anhydrous powder (99.5%) from

PANREAC (Barcelona, Spain) was used. Heptadecanoic acid from Fluka (Steinheim, Germany) was used as internal standard in FAME determination.

2.3 Statistical analysis software

Statgraphics© centurion XVI (StatPoint Technologies, Warrenton-Virginia, USA) was used for building and analyzing the response surface results, allowing to design the multiple response optimization and graphical responses.

2.4 Methodology and instruments

The oil was extracted by cold pressing using a screw press model KND-FARMET DUO-PV (Česká Skalice, Czech Republic). Then was filtered using a filter plate with fine filter paper 200x200 mm from Filtrox company (St. Gallen, Switzerland). For the storage and preservation of samples, they were placed in opaque containers, inert and watertight, sealed and placed at a temperature of 10 °C. To prepare samples for testing, the standard UNE-EN ISO 661 was followed.

The determination of the acidity or percentage of free fatty acid was carried out using the cold solvent method, as described by the standard UNE-EN ISO 660. The density was determined by a hydrometer, following the standard UNE-EN ISO 3675. The peroxide value was calculated from the iodine released from potassium iodine, following the standard UNE-EN ISO 3960.

To determine the fatty acid content of the oil, samples were prepared as follows: 5 ml of hexane were added to 0.1 g of oil and stirred 30 s with a

vortex. Next, 0.5 ml of sodium methylate was added and stirred for 3 min. Finally, the mixture was centrifuged and the upper phase was selected [20].

The high calorific value was calculated following the stantard ASTM D240, using a bomb calorimeter model C200, IKA (Staufen, Germany). Low calorific value (LCV) was calculated using the following expression:

$$LCV=HCV-(h_{fg}mH_2O)/mC_xH_yO_2 \quad \text{(eq.1)}$$

Where hfg is the standard heat of vaporization of water, in kJ/kg, $mC_xH_yO_2$ is the mass of methyl ester, in kg and mH_2O is the mass of water produced during combustion, in kg, calculated considering the methyl ester chemical reaction [11, 21]:

$$C_xH_yO_2+m(O_2+3.76N_2)\rightarrow xCO_2+(y/2)H_2O+m(3.76N_2) \quad \text{(eq. 2)}$$

Where x and y are the number of atoms of carbon and hydrogen in a fatty acid (or methyl ester molecule), respectively and m corresponds to the stoichiometric oxygen requirement. The chemical formula of each biodiesel was calculated considering methyl esters composition.

Kinematic viscosity (ν) as determined using a glass capillary-type viscometer, following the standard UNE-EN ISO 3104. Density (ρ) of oil and biodiesel were analyzed using a PROTON densimeter at 15 °C. Water content was analyzed according to ASTM D4928-00 using a METTLER TOLEDO tritrator model DL32. Flash point (FP) was calculated by means of a Stanhope-Seta (Chertsey, UK) Setaflash Series 3 flash point tester, following the European standard ISO 3679.

The determination of the fatty acid composition of each biodiesel sample was performed by gas chromatography, following the protocol UNE-EN 14103. A gas chromatograph Perkin Elmer (Waltham, Massachusetts, USA) Clarius 500 coupled to a flame ionization detector (FID) equipped with a Perkin Elmer capillary column 30 m x 0.25 mm Elite 5-ms (0.25 microns particle diameter) was used.

To determine the repeatability and standard error of all measurements, each analysis was performed in triplicate. The Tuckey [22] test was used to determine the variability of each parameter values.

Table 2.1 Factorial design to optimize biodiesel production from rapeseed oil with different doses of nitrogen fertilization; yield A corresponds to the sample without nitrogen fertilizaton (0-0) while yield B indicates the group of samples with similar fatty acid composition

Transesterification reaction parameters									
	Temperature (ºC)			Oil-to-methanol (molar ratio)			Potassium hydroxide (% wt.)		
Level code	-1	0	1	-1	0	1	-1	0	1
Reaction values	40	52.5	65	1:4	1:5	1:6	1	1.5	2

Type of experiment	Run number	Temperature (ºC)	Oil-to-methanol (molar ratio)	Potassium hydroxide (% wt.)	Yield A (% wt.)	Yield B (% wt.)
Linear step						
	1	-1	-1	0	87.42	86.54
	2	1	1	0	97.35	93.45
	3	-1	0	1	95.77	98.36
	4	-1	0	-1	86.73	94.88

Type of experiment	Run number	Temperature (ºC)	Oil-to-methanol (molar ratio)	Potassium hydroxide (% wt.)	Yield A (% wt.)	Yield B (% wt.)
	5	0	1	-1	92.83	92.21
	6	0	1	1	94.70	93.82
	7	0	0	0	93.55	93.63
	8	1	0	-1	91.39	91.42
	9	0	-1	-1	86.44	84.80
	10	0	0	0	96.67	94.06
	11	-1	1	0	92.96	97.83
	12	1	-1	0	89.03	91.10
	13	0	0	0	94.47	96.77
	14	0	-1	1	93.72	96.85
	15	1	0	1	93.46	96.00

During the transesterification reaction, each sample was prepared in a 250 ml flask immersed in a water bath equipped with a temperature controller (± 0.2 ºC of accuracy). A magnetic stirrer provided agitation at 1100 rpm. Initially, each flask was filled with 100 g of vegetable oil and heated to the reaction temperature. Then, the solution of methanol and potassium hydroxide was added to each flask- reactor.

The samples were centrifuged at 3500 rpm for 10 min to separate biodiesel from free glycerol. Then, the mixture was washed with distilled water and subsequently centrifuged to remove the aqueous layer composed mainly by methanol, residual catalyst and glycerol. Finally, the samples were dried with anhydrous sodium sulphate powder.

The selected design of experiments was "Box Behnken", included into Statgraphics centurion XVI, which allow the optimization of the main factors that influence the transesterification of biodiesel, such as temperature, methanol and catalyst. Fifteen experiments (Table 2.1) were performed with three central points and two degrees of freedom. The design goal was to maximize the yield of FAME.

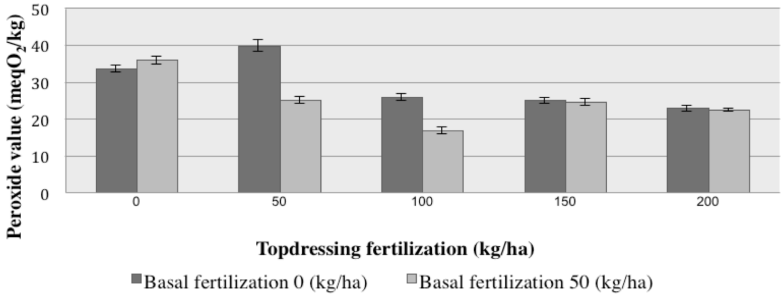
Table 2.2 Oil yield and seed yield for each test

Fertilization		Seed yield (kg/ha)	Oil yield (%)
Basal	Topdressing		
0	0	2111	40
50	0	2833	40.3
0	50	2907	39.7
50	50	2759	38.7
0	100	2870	41
50	100	3167	39.3
0	150	3037	40.3
50	150	3093	39
0	200	2907	39.7
50	200	2926	38

2.5 Results and discussion

2.5.1 Oil properties

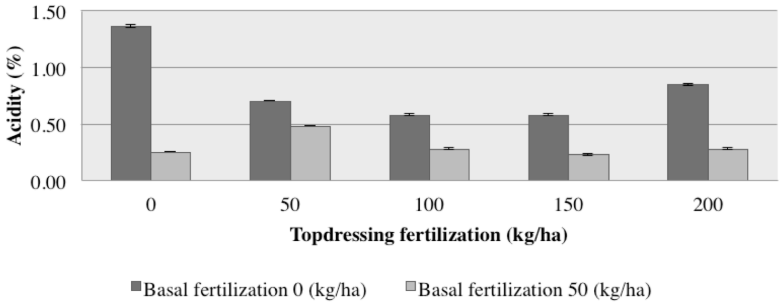
Figure 2.2 Peroxide value of rapeseed oil produced from crops with different nitrogen fertilization



Information concerning oil yield (%) and seed yield (kg/ha) for each fertilization trial is provided in Table 2.2. As may be seen, there is a slight difference in oil content between samples, mainly due to the nitrogen fertilization. In fact, the higher the amount of nitrogen, the higher the protein content and the lower the seed oil content. In this sense, best results concerning oil content were achieved when fertilization was low. In any case, ANOVA found no significant differences between samples in terms of both seed yield and oil content. Considering the fertilization trials, oil properties are shown in Table 2.3. Peroxide value (PV) is a parameter related to oil oxidation, which may affect biodiesel properties. In this sense, several works have shown that high PV may reduce ignition delay [23]. Although a no clear trend between oil PV and crop nitrogen fertilization can

be appreciated (Figure 2.2), when no basal fertilization was applied, both topdressing fertilization up to 50 kg/ha provided a significant increase of PV, while crops with topdressing higher than 100 kg/ha exhibited an almost constant lower peroxide value. However, a decrease of PV as a general trend may be seen, if the essays for topdressing fertilization 50 kg/ha and 100 kg/ha were excluded, when basal fertilization was 0 kg/ha and 50 kg/ha, respectively. The lowest value of PV was achieved with basal and topdressing of 50-100.

Figure 2.3 Acid value of rapeseed oil produced from crops with different nitrogen fertilization



Acid value may affect the transesterification process. In this sense, a content of free fatty acid (FFA) higher than 3% (wt.) is not recommended in homogenous alkaline transesterification because saponification reaction may occur [16]. In the present work, results indicate that the presence of basal fertilization of nitrogen causes a decrease of the oil acid value compared to those crops without basal fertilization (Figure 2.3), no matter the value of topdressing. This result suggests that the acidity of the oil could be affected by nitrogen fertilization at the beginning of their growth. Also, when no fertilization is used (neither topdressing nor basal fertilization), acidity significantly increases. Moreover, when basal fertilization is used, the acidity is almost constant, while when no basal fertilization is used the higher the topdressing fertilization the lower the acidity (with the exclusion of the use of 200 kg/ha of topdressing).

Table 2.3 Properties of rapeseed oil samples

Properties	0-0*	0-50*	0-100*	0-150*	0-200*
Acidity (%)	1.35 (0.01)	0.70 (0.00)	0.58 (0.00)	0.70 (0.01)	0.85 (0.00)
Peroxide value ($meqO_2/kg$)	33.64 (0.80)	39.72 (1.57)	25.80 (0.87)	24.99 (0.68)	22.97 (0.67)
Density at 15ºC (kg/m^3)	919.33 (0.47)	919.67 (0.47)	919.33 (0.94)	919.67 (0.94)	920.00 (0.82)
Viscosity at 40ºC (mm^2/s)	28.82 (2.34)	28.60 (0.58)	26.33 (0.10)	30.08 (1.52)	27.35 (0.07)
Water content (mg/kg)	673.51 (38.12)	684.84 (37.20)	649.59 (97.73)	680.02 (63.84)	620.93 (122.66)
Low calorific value (kJ/g)	38.81 (0.02)	38.89 (0.03)	38.88 (0.02)	38.88 (0.04)	38.90 (0.03)

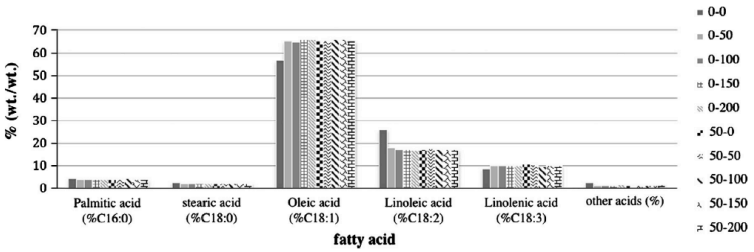
*Error in brackets, expressed as relative standard deviation, in percentage (n=3 replicates); samples are named x-y, where x indicates basal and y indicates topdressing fertilization.

Properties	50-0*	50-50*	50-100*	50-150*	50-200*
Acidity (%)	0.25 (0.00)	0.48 (0.00)	0.27 (0.01)	0.23 (0.01)	0.27 (0.01)
Peroxide value ($meqO_2/kg$)	36.01 (0.97)	25.13 (0.83)	16.88 (0.82)	24.55 (0.89)	22.52 (0.33)
Density at 15ºC (kg/m^3)	920.33 (0.94)	920.33 (0.47)	921.00 (0.82)	919.67 (1.25)	920.33 (0.94)
Viscosity at 40ºC (mm^2/s)	25.38 (0.84)	27.79 (0.70)	26.13 (1.73)	25.85 (0.20)	29.28 (2.52)
Water content (mg/kg)	599.72 (53.79)	584.91 (28.89)	565.32 (82.87)	573.45 (68.84)	545.91 (71.62)
Low calorific value (kJ/g)	38.87 (0.04)	38.86 (0.01)	38.89 (0.00)	38.91 (0.02)	38.91 (0.02)

*Error in brackets, expressed as relative standard deviation, in percentage (n=3 replicates); samples are named x-y, where x indicates basal and y indicates topdressing fertilization.

As previously mentioned, several studies indicate that fatty acid composition influences the quality of fatty acid methyl esters (FAME) produced through the transesterification process [13, 24]. In the present work, results indicate that fertilization produces an increase of monounsaturated fatty acid (oleic acid) content from around 57% to approximately 65% and a decrease on polyunsaturated fatty acid (linoleic acid) content from around 26% to approximately 17% (Figure 2.4). As a result, it may be inferred that when no fertilization is used, oleic and linolenic acid depict the lowest values (although with no significant differences between linolenic acid values), while linoleic acid exhibits the highest one. The rest of fatty acid do not seem to be altered by the use of fertilization. This is of special interest, provided that biodiesel produced from oils with a high content of oleic acid are highly recommended [12]. Moreover, the European standard EN 14214 limits the content of linolenic acid to a maximum of 12%, in any case below the results from this research.

Figure 2.4 Fatty acid composition of rapeseed oil produced from crops with different nitrogen fertilization



No significant differences in kinematic viscosity, density, moisture content and low calorific value of the different samples of rapeseed oil were appreciated.

2.5.2 Transesterification optimization

As crop 0-0 depicted some different property values (i.e. oleic acid content) compared to the rest of the crops, in order to find out the optimal transesterification conditions, the design of experiments was focused on two groups, the first group (A) including the oil with the lowest content of oleic acid (sample 0-0) and the second one (B) comprising the remaining samples. As shown in Table 2.4, results indicate there is a decrease of the transesterification optimal temperature when any N fertilization is used (group B) compared to group A, varying from 56.17 °C to 40.03 °C. This result is in agreement with a previous work where it was found that the degree of unsaturation affects the optimum temperature of transesterification [10]. The opposite trend between the two groups for the optimal oil-to-methanol ratio (when N is used a higher amount of methanol is required) and amount of catalyst (when N is used, less KOH is needed) was found. The predictive model to determine the conversion of methyl esters obtained from the experimental design can be seen in Table 2.4 according to the reaction temperature (°C), amount of catalyst (% wt.) and the molar ratio of oil-to-methanol.

Table 2.4 Confirmatory run of transesterification on optimal parameters and fatty acid composition of oil, [1]: not determined

Samples	Optimal parameters			Fatty acid						
	Oil-to-methanol (molar ratio)	Temperature (ºC)	Potassium hydroxide (% wt.)	C16:0 (% wt.)	C18:0 (% wt.)	C18:1 (% wt.)	C18:2 (% wt.)	C18:3 (% wt.)	n.d[1] (% wt.)	FAME yield (% wt.)
0-0	1:5.35	56.17	1.88	4.33	2.36	56.51	25.99	8.47	2.35	97.41
0-50	1:6.00	40.03	1.66	4.04	2.17	65.18	17.66	9.85	1.09	98.09
0-100	1:6.00	40.03	1.66	3.87	2.12	64.92	17.19	10.09	1.85	98.19
0-150	1:6.00	40.03	1.66	3.87	2.14	65.85	16.94	10.01	1.10	98.08
0-200	1:6.00	40.03	1.66	3.85	2.15	65.78	16.54	9.80	1.88	97.97
50-0	1:6.00	40.03	1.66	3.91	2.10	65.22	17.18	10.47	1.12	98.64
50-50	1:6.00	40.03	1.66	3.94	2.10	65.35	17.25	10.29	1.05	98.22
50-100	1:6.00	40.03	1.66	4.07	2.15	65.83	17.23	9.66	1.05	98.20
50-150	1:6.00	40.03	1.66	3.95	2.14	65.88	17.05	9.86	1.13	98.90
50-200	1:6.00	40.03	1.66	3.89	2.14	65.13	17.14	9.79	1.91	97.90

To validate results provided by the design of experiments, a confirmatory run was carried out and reaction optimal condition results are shown in Table 2.5. As may be seen, no significant differences on yield conversion for group B samples is observed. Moreover, every confirmatory run allows achieving a FAME content that fulfil the EN 14214 standard.

Considering the analyzed biodiesel properties (Table 2.6), no significant differences depending on nitrogen fertilization were found. Each sample of FAME complies with the European Standard EN 14214.

Table 2.5 Models to predict fatty acid methyl ester conversion (T: temperature; C: KOH; M: oil-to-methanol molar ratio)

Sample	Model expression	R^2 (%)	Standard error
0-0	Yield of FAME = -56.6472 + 1.7197 T + 31.7245 C + 28.0418 M – 0.0105 T2 + 0.1112 T C – 0.1394 T M – 6.2433 C2 – 2.7050 C M – 1.4133 M2	92.75	1.6032
0-50	Yield FAME = -4.1304 + 0.1499 T + 84.3890 C + 9.2150 M + 0.0021 T2 – 0.3576 T C + 0.022 T M – 11.6700 C2 – 5.2200 C M + 0.0175 M2	88.05	1.7073
0-100	Yield FAME = -4.1304 + 0.1499 T + 84.3890 C + 9.2150 M + 0.0021 T2 – 0.3576 T C + 0.022 T M – 11.6700 C2 – 5.2200 C M + 0.0175 M2	88.05	1.7073
0-150	Yield FAME = -4.1304 + 0.1499 T + 84.3890 C + 9.2150 M + 0.0021 T2 – 0.3576 T C + 0.022 T M – 11.6700 C2 – 5.2200 C M + 0.0175 M2	88.05	1.7073
0-200	Yield FAME = -4.1304 + 0.1499 T + 84.3890 C + 9.2150 M + 0.0021 T2 – 0.3576 T C + 0.022 T M – 11.6700 C2 – 5.2200 C M + 0.0175 M2	88.05	1.7073

Sample	Model expression	R^2 (%)	Standard error
50-0	Yield FAME = -4.1304 + 0.1499 T + 84.3890 C + 9.2150 M + 0.0021 T2 – 0.3576 T C + 0.022 T M – 11.6700 C2 – 5.2200 C M + 0.0175 M2	88.05	1.7073
50-50	Yield FAME = -4.1304 + 0.1499 T + 84.3890 C + 9.2150 M + 0.0021 T2 – 0.3576 T C + 0.022 T M – 11.6700 C2 – 5.2200 C M + 0.0175 M2	88.05	1.7073
50-100	Yield FAME = -4.1304 + 0.1499 T + 84.3890 C + 9.2150 M + 0.0021 T2 – 0.3576 T C + 0.022 T M – 11.6700 C2 – 5.2200 C M + 0.0175 M2	88.05	1.7073
50-150	Yield FAME = -4.1304 + 0.1499 T + 84.3890 C + 9.2150 M + 0.0021 T2 – 0.3576 T C + 0.022 T M – 11.6700 C2 – 5.2200 C M + 0.0175 M2	88.05	1.7073
50-200	Yield FAME = -4.1304 + 0.1499 T + 84.3890 C + 9.2150 M + 0.0021 T2 – 0.3576 T C + 0.022 T M – 11.6700 C2 – 5.2200 C M + 0.0175 M2	88.05	1.7073

Table 2.6 Properties of rapeseed oil methyl esters

Properties	0-0*	0-50*	0-100*	0-150*	0-200*
Flash point (ºC)	171.5 (1.08)	175.17 (2.10)	166.17 (8.37)	170.50 (5.67)	178.17 (1.84)
Density at 15ºC (kg/m^3)	882.00 (1.41)	880.67 (0.94)	880.67 (0.47)	880.33 (2.87)	880.33 (1.25)
Viscosity at 40ºC (mm^2/s)	4.67 (0.04)	4.54 (0.06)	4.37 (0.03)	4.67 (0.04)	4.25 (0.04)
Water content (mg/kg)	379.73 (7.56)	345.46 (12.63)	355.96 (24.41)	350.45 (12.23)	377.11 (12.96)
Low calorific value (kJ/g)	38.53 (0.37)	38.74 (0.08)	38.68 (0.16)	38.68 (0.05)	38.78 (0.05)
Properties	50-0*	50-50*	50-100*	50-150*	50-200*
Flash point (ºC)	175.00 (2.94)	172.67 (6.60)	177.67 (0.47)	179.33 (1.89)	178.33 (4.09)
Density at 15ºC (kg/m^3)	881.00 (2.16)	880.00 (0.82)	881.33 (1.25)	880.00 (0.00)	881.63 (1.29)
Viscosity at 40ºC (mm^2/s)	4.67 (0.04)	4.18 (0.04)	4.52 (0.14)	4.20 (0.06)	4.10 (0.02)
Water content (mg/kg)	350.11 (13.84)	360.50 (18.67)	346.08 (18.42)	329.44 (15.99)	337.12 (16.68)
Low calorific value (kJ/g)	38.55 (0.06)	38.64 (0.07)	38.71 (0.03)	38.51 (0.28)	38.73 (0.04)

*Error in brackets, expressed as relative standard deviation, in percentage (n=3 replicates).

2.6 Conclusions

In this study, the effect of basal and topdressing fertilization in rapeseed crops considering oil properties, biodiesel conversion and quality has been studied. The nitrogen fertilization affects some rapeseed oil properties, mainly acidity, peroxide value and fatty acid composition. Higher acidity and peroxide value have been observed when not basal fertilization was applied. Moreover, no fertilization provokes acidity increase. Oleic and linolenic acid content increase, while linoleic acid content decreases when no fertilization during the growth of the oilseed is applied. For the rest of oil and biodiesel properties, no significant changes were observed. Finally, if no fertilization is applied during the crop growth, a rise of the optimal transesterification temperature and amount of KOH is reached, thereby increasing production costs. In any case, all biodiesel samples met the EU 14214 standard.

2.7 Acknowledgements

Authors gratefully thanks to the Spanish Ministry of Education and Science (ENE2010-15159) and the Andalusian Research, Innovation and Enterprise Council, Spain (TEP-4994) for the financial aid and generous assistance and valuable information provided by professor Vidal Barrón.

2.8 Reference

1. Dorado, M.P., et al., *Optimization of alkali-catalyzed transesterification of Brassica carinata oil for biodiesel production.* Energy & Fuels, 2004. 18(1): p. 77-83.
2. Melero, J.A., et al., *Zr-SBA-15 acid catalyst: Optimization of the synthesis and reaction conditions for biodiesel production from low-grade oils and fats.* Catalysis Today, 2012. 195(1): p. 44-53.
3. Vyas, A.P., J.L. Verma, and N. Subrahmanyam, *A review on FAME production processes.* Fuel, 2010. 89(1): p. 1-9.
4. Beghin, J.C., et al. *Food and agricultural policy research institute U.S. and world agricultural outlook*. Staff Reports 2006 [cited 2011 June]; Available from: fapri.iastate.edu/outlook/.
5. Weselake, R.J., et al., *Increasing the flow of carbon into seed oil.* Biotechnology Advances, 2009. 27(6): p. 866-878.
6. Tutunea, D., *Thermal investigation of biodiesel blends derived from rapeseed oil.* Journal of Thermal Analysis and Calorimetry, 2013. 111(1): p. 869-875.
7. Gao, J., et al., *Effects of manure and fertilizer applications on canola oil content and fatty acid composition.* Agronomy Journal, 2010. 102(2): p. 790-797.
8. Egesel, C.O., M.K. Gul, and F. Kahriman, *Changes in yield and seed quality traits in rapeseed genotypes by sulphur fertilization* European Food Research and Technology, 2009. 229(5): p. 841-841.
9. Brennan, R.F., M.G. Mason, and G.H. Walton, *Effect of nitrogen fertilizer on the concentrations of oil and protein in canola (Brassica napus) seed.* Journal of Plant Nutrition, 2000. 23(3): p. 339-348.
10. Pinzi, S., et al., *Influence of vegetable oils fatty acid composition on reaction temperature and glycerides conversion to biodiesel during transesterification.* Bioresource Technology, 2011. 102(2): p. 1044-1050.

11. Pinzi, S., et al., *Multiple response optimization of vegetable oils fatty acid composition to improve biodiesel physical properties.* Bioresource Technology, 2011. 102(15): p. 7280-7288.

12. Pinzi, S., et al., *The ideal vegetable oil-based biodiesel composition: A review of social, economical and technical implications.* Energy & Fuels, 2009. 23: p. 2325-2341.

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

14. Canakci, M. and J. Van Gerpen, *Biodiesel production from oils and fats with high free fatty acids.* Transactions of the Asae, 2001. 44(6): p. 1429-1436.

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

16. Dorado, M.P., et al., *Exhaust emissions from a diesel engine fueled with transesterified waste olive oil.* Fuel, 2003. 82(11): p. 1311-1315.

17. Lapuerta, M., O. Armas, and J. Rodriguez-Fernandez, *Effect of biodiesel fuels on diesel engine emissions* Progress in Energy and Combustion Science, 2009. 35(4): p. 383-383.

18. Knothe, G., C.A. Sharp, and T.W. Ryan, *Exhaust emissions of biodiesel, petrodiesel, neat methyl esters, and alkanes in a new technology engine.* Energy & Fuels, 2006. 20(1): p. 403-408.

19. Pinzi, S., et al., *The effect of biodiesel fatty acid composition on combustion and diesel engine exhaust emissions.* Fuel, 2013. 104(0): p. 170-182.

20. Priego-Capote, F., J. Ruiz-Jimenez, and M.D. Luque de Castro, *Identification and quantification of trans fatty acids in bakery products by gas chromatography-mass spectrometry after focused microwave Soxhlet extraction.* Food Chemistry, 2007. 100(2): p. 859-867.

21. Mehta, P.S. and K. Anand, *Estimation of a lower heating value of vegetable oil and biodiesel fuel.* Energy & Fuels, 2009. 23(8): p. 3893-3898.

22. Keselman, H.J. and J.C. Rogan, *A comparison of the modified-Tukey and Scheffe methods of multiple comparisons for pairwise contrasts.* Journal of the American Statistical Association, 1978. 73(361): p. 47-52.

23. Pattamaprom, C., W. Pakdee, and S. Ngamjaroen, *Storage degradation of palm-derived biodiesels: Its effects on chemical properties and engine performance.* Renewable Energy, 2012. 37(1): p. 412-418.

24. Pinzi, S., et al., *Influence of vegetable oils fatty-acid composition on biodiesel optimization.* Bioresource Technology, 2011. 102(2): p. 1059-1065.

Chapter 3

"The potential of agro-industrial waste and oleaginous yeast utilization on biodiesel production"

Chapter 3. The potential of agro-industrial waste and oleaginous yeast utilization on biodiesel production

Abstract Environmental problems are making it necessary to find renewable and sustainable alternatives to fossil fuels in the field of transport. Biodiesel may replace diesel fuel under an environmentally friendly and economically sound process, as long as the raw material employed is of low cost and can be derived from sustainable sources. Such an alternative feedstock from non-vegetable sources could be provided by microbial oil produced by oleaginous microorganisms that may possess similar chemical composition to that of the most commonly used vegetable oils in the biodiesel industry. Moreover, oleaginous yeasts could accumulate intracellular lipids through cultivation on various agro-industrial wastes. Oleaginous yeasts may accumulate different amounts of microbial oil with varying fatty acid profiles, depending on the substrate or growing conditions. This review presents the most relevant aspects regarding yeast oil production using agro-industrial waste as culture media and evaluates the potential of this microbial oil as feedstock for biodiesel production.

3.1 Introduction

The global need for energy supply in the field of transport indicates the urgent need to find renewable-based alternatives to fossil fuels. Although all resources of conventional oil have not been discovered yet,

they are unlikely to be easily accessed and will make little difference to the depletion timing of petroleum reserves [1]. To address this issue, current research focuses on the development of economically sound technologies for the production of biofuels [2]. In this way, biodiesel is a renewable, safe and non-toxic biofuel that can be produced from a wide range of edible and non-edible oleaginous feedstocks [3]. The conversion of oleaginous raw materials into biodiesel is carried out through the homogeneously or heterogeneously catalysed transesterification of large branched triglycerides (TG) into smaller, straight-chain esters [4]. The high cost of feedstocks to produce biodiesel is the major obstacle in the commercialization of the product [5]. Furthermore, if the feedstocks originate from agricultural crops, it is often asserted that this type of biodiesel may contribute to the rise of food prices [6]. For this reason, research is focused on the selection of renewable raw materials that do not compete with the food supply chain.

Microbial oil showing similar chemical composition and energy value to plant and animal oils may fulfill the requirements mentioned above [7]. Moreover, agro-industrial waste could be used as substrate to cultivate oleaginous microorganisms. A number of microorganisms belonging to the genera of algae, bacteria, yeast and fungi have the ability to accumulate lipids under specific cultivation conditions [8]. Among them, utilization of oleaginous yeast is advantageous due to fast growth rate and high oil content compared to algae [9]. - Some oleaginous yeast strains, i.e. *Cryptococcus sp., Lipomyces sp*., *Rhodosporidium sp*. and *Rhodotorula sp*., can accumulate intracellular lipids up to 60% of their dry cell weight when glucose is used as carbon source [10]. However, they represent a minor

proportion of the total yeast population and only 5% of yeasts have been reported to be able to accumulate more than 25% lipids [11].

Lipid accumulation in oleaginous microorganisms typically occurs under nitrogen limited conditions and in the presence of a high sugar content [12]. During the growth phase, nitrogen is necessary for synthesis of proteins and nucleic acids, while the carbon source is used for both energetic and anabolic processes yielding carbohydrates, lipids, nucleic acids and proteins. When the nitrogen source is depleted, the growth rate slows down and the synthesis of proteins and nucleic acids ceases; in oleaginous species, the carbon source is then channeled toward lipid synthesis, leading to the accumulation of TG within intracellular lipid bodies [13]. Since microbial lipids can be produced using various waste streams (e.g. whey, lignocellulosic hydrolysates) as substrate, it has been considered a potential feedstock to support a sustainable biodiesel industry. In fact, industrial waste and lignocellulosic material may be used to provide sugar and nitrogen sources to produce lipids, thus showing good characteristics such as short life cycle and low affection by climate [13]. Currently, the production of microbial oil is more expensive than that of vegetable oils [14]. Koutinas *et al.* (2014) reported that the unitary cost of purified microbial oil production from glucose is $3.4/kg for an annual production capacity of 10,000 t microbial oil and zero cost of glucose. The cost of microbial oil production and conversion to biodiesel was estimated at $5.5/kg oil and $5.9/kg biodiesel, accordingly, when a cost of glucose of $400/t was assumed. Koutinas *et al.* [14] projected that if the cost of manufacture of microbial oil is reduced by 50% until the next decade then microbial oil could potentially compete with vegetable oils regarding biodiesel production cost. In most cases, the cost of microbial oil production

from oleaginous yeast is lower than the cost of oil production from algae. However, oil production from algae and yeast should be developed and compared further in order to evaluate their potential for biodiesel production [14]. Therefore, the production of microbial oil using cheap substrates, such as waste streams, is a key parameter in order to develop an economically and environmentally viable biodiesel production process [15].

3.2 Utilization of non-pretreated industrial waste and by-product streams as feedstock for microbial oil production

Table 3.1 presents total dry weight and microbial oil content achieved via cultivation of various oleaginous yeasts on different industrial wastes (Table 3.1). Crude glycerol is considered one of the most interesting carbon sources, due to its low cost and its increasing availability. It is produced as a by-product of the biodiesel industry, saponification of fats and oils and alcoholic beverage production industry; the high excess of glycerol results in an unbalanced glycerol market [16]. Raw glycerol derived from biodiesel production is contaminated with alkali/acid catalyst and alcohol, and thus, unless purified, it is not useful for conventional applications such as toothpaste, drugs, paints and cosmetics [17]. Moreover, the process of refining crude glycerol into a high purity product is costly and energy consuming [18].

Chatzifragkou *et al*. [19] demonstrated the ability of fifteen strains of fungi and yeast to grow on waste glycerol derived from the biodiesel industry and yeast extract as nitrogen sources. It was concluded that oleaginous yeasts depict higher biomass production compared to

filamentous fungi, however only one strain of yeast, *Rhodotorula* sp. LFMB 22, was able to accumulate lipids. In contrast, Zygomycetes strains accumulated large amounts of lipids. The lipid fraction of the oil obtained from the yeast *Y. lipolytica* LFMB19 comprised more than 80% of neutral lipids that are suitable for the production of biodiesel. Total lipids included glycolipids, waxes, phospholipids, etc. which cannot be converted into methyl esters through transesterification. This would make necessary the inclusion of pretreatments.

Rhodosporidium toruloides, a well known oleaginous yeast, has been proposed for microbial conversion of crude glycerol to triacylglycerols [20]. Crude glycerol samples derived from lipase and alkaline-catalysed transesterification were analysed. Results showed that the use of crude glycerol from alkaline transesterification as carbon source led to the production of higher total dry weight compared to that of glycerol from lipase-catalysed transesterification. However, lipid accumulation was lower when crude glycerol from alkaline transesterification was used. Refined glycerol provided lower biomass concentration and lipid accumulation compared to crude glycerol. The utilization of crude glycerol as carbon source in 24 h batch cultivations employing the strain *Rodhotorula glutinis* BCRC 22360 resulted in higher lipid content and lower total dry weight than refined glycerol [18]. These examples demonstrate that different oleaginous yeast strains present different metabolic responses depending on the origin of the crude glycerol employed as carbon source.

Ammonium chloride and crude glycerol generated from a biodiesel production process using yellow grease as feedstock have been tested as nitrogen and carbon sources in fed-batch cultivations of the oleaginous yeast strain *Cryptococcus curvatus* ATCC 20509 [21]. A one-stage fed-batch was

run for 12 days providing 31.2 g/l of final biomass with 44% lipid content. Residual methanol in the crude glycerol did not inhibit the growth of *C. curvatus*. Yeast extract could also be used as nitrogen source and nutrient supplement, although ammonium sulphate could be considered as an alternative source as it is cheaper than yeast extract. Other oleaginous yeasts, such as *Kodamaea ohmeri, Trichosporonoides spathulata* [22] and *Rhodotorula glutinis* [23], have demonstrated the ability to accumulate lipids when crude glycerol and ammonium sulphate are used as carbon and nitrogen sources. In the case of *R. glutinis,* besides lipid accumulation this strain can also produce carotenoids. The addition of surfactant Tween 20 in the medium increased lipid accumulation and yeast growth.

Lipid accumulation can be influenced by the nitrogen source employed during fermentation. When the oleaginous yeast *R. glutinis* was cultivated on a mixture of crude glycerol and thin stillage (a residue from the brewing industry) it achieved a 27% increase in the production of total biomass compared to that provided using crude glycerol with a standard medium [18]. *C. curvatus* was grown in a basal medium containing crude glycerol, corn steep liquor, baker's yeast autolysate and malt extract using fed-batch fermentation. It resulted in a production of 58.9 g/l dry cell weight and 43% oil content. To reduce the fermentation cost, the baker's yeast autolysate and malt extract were replaced by de-oiled yeast cake from previous fermentation batches. This time, it resulted in an increase of both biomass production and oil content, providing 69.2 g/l and 48%, respectively; the novel medium reduced the cost by 60% [15].

Takakuwa *et al.* [24] produced FAME from *C. curvatus* TYC-19 grown in beet molasses and cheese whey as culture media. The culture medium was mainly composed of sucrose (due to the presence of beet molasses) and lactose (due to cheese whey) and was suitable to produce FAME. Results provided 14.3 g/l dry cell weight and 68.3 mg/l FAME using beet molasses. When cheese whey was used, 13.9 g/l dry cell weight and 78.8 mg/l FAME were achieved. In another study, molasses was successfully employed for the cultivation of *Candida tropicalis*, *Candida lipolytica* and *Rhodotorula mucilaginosa* resulting in an acceptable intracellular lipid accumulation for the production of biodiesel [25].

Palm oil mill effluent (POME), a waste from palm oil wet milling, is suitable to grow *R. glutinis* TISTR 5159 due to its extremely high content of degradable organic matter, partly due to the presence of unrecovered palm oil [26],[27]. Results showed that lipid accumulation improved when additional nitrogen sources, such as ammonium sulphate, and Tween 20 were added to the medium. Furthermore, considering cell growth, *R. glutinis* required relatively high chemical oxygen demand (COD) and low C/N ratio, while higher COD and higher C/N ratio favoured lipid production.

Oil waste from fast food derived from fish, meat like chicken and fried vegetables has been tested as substrate for lipid accumulation using fifteen different yeasts. When vegetable frying oil was used as substrate, *Yarrowia lipolytica* provided the highest accumulation of lipids, in terms of dry cell weight. However, when glucose was added to the medium, a variation of the fatty acid composition was verified [28].

Organic carbon from municipal wastewater has also been tested to produce microbial oil for the biodiesel industry. The oleaginous yeasts *C. curvatus, Y. lypolitica* and *R. glutinis* were inoculated into wastewater

without any nutrient supplementation. Results showed that nutrients were insufficient and the yeast could not compete with the indigenous microorganism [29]. However, when an organic carbon source was added to the medium and inoculated with *C. curvatus*, promising results were achieved. When the medium was supplemented with organic carbon and nitrogen sources, the three strains presented acceptable cell growth. The study concludes that wastewater can be used as water source for yeast cultivation, reducing the cost associated with the use of clean water [30]. Similar conclusions were found when fishmeal wastewater [31] and sewage sludge [32] were used and inoculated with *L. starkeyi*. Supplementing fishmeal wastewater with glucose provided higher cell growth and oil yield [31].

Another source of carbon for the production of lipids are volatile fatty acids (VFA), which are present in sludge and a variety of biodegradable organic wastes [33]. Fei *et al*. [34] tested the use of VFA (acetic, propionic and butyric acids) as carbon source for the cultivation of the oleaginous yeast *C. albidus*. The effect of culture temperature, initial pH, nitrogen source and initial concentration of VFA was investigated. The preferred nitrogen source for optimum accumulation of lipids was ammonium chloride, besides a proportion of VFA (acetic, propionic and butyric acids) of 8:1:1, which demonstrated the yeast preference for acetic acid. This study also stated that the higher the initial concentration of VFA the higher the accumulation of lipids. In a two-stage fed-batch strategy, *Y. lipolytica* MUCL 28849 was initially grown on glucose or glycerol and subsequently VFA were added under nitrogen limitation and after glucose

or glycerol exhaustion. Results showed that the use of glucose and a mixture of VFA provided the best concentration of biomass (41.02 g/l) and lipids (16.50 g/l). Furthermore, when glycerol was used, similar results were achieved, thus demonstrating the suitability of these cheap industrial by-products [35].

C. curvatus ATCC 20509 has been used to produce lipids from two kinds of waste, shrimp processing waste [36] and hydrogen production effluent from food waste (FW-HPE) [37]. N-acetylglucosamine (GlcNAc), the major carbohydrate of the hydrolysate from shrimp processing waste, proved its suitability as substrate to produce microbial lipids, providing up to 28.4% after 167.7 h using shake flask fermentation. The exclusive use of FW-HPE did not result in sufficient lipid accumulation with this yeast strain of *C. curvatus*.

From the results presented in this section, it can be concluded that when non-pretreated industrial waste and by-product streams are used as sources of carbon, nitrogen or other nutrients, the selection of the oleaginous yeast that can grow in this medium is crucial to provide a cost-effective process with high total dry cell weight production and lipid content. Process and media component optimization is necessary in order to develop a sustainable and economically viable process for microbial oil production.

Table 3. 1 Total dry weight production and lipid accumulation using oleaginous yeasts and different industrial waste streams that do not require any pretreatment

Yeast strain	Carbon source	Nitrogen source	Lipid content (%,w/w)	Total dry weight (g/l)	Culture mode	Ref.
Candida lipolytica	Molasses	Ammonium sulphate	59.9	n.m	Flask	[25]
Candida tropicalis	Molasses	Ammonium sulphate	46.8	n.m	Flask	[25]
Cryptococcus albidus	Volatile fatty acids	Ammonium chloride	25.6	1.05	Flask	[38]
Cryptococcus curvatus ATCC 20509	Crude glycerol	Ammonium chloride	52.9	32.9	Fed-batch	[21]
Cryptococcus curvatus	N-acetylglucosamine	Ammonium chloride	34.6	11.32	Flask	[36]
Cryptococcus curvatus	Municipal wastewater + glucose	Peptone + yeast extract	n.m	17.0	Flask	[30]

Yeast strain	Carbon source	Nitrogen source	Lipid content (%,w/w)	Total dry weight (g/l)	Culture mode	Ref.
Cryptococcus curvatus ATCC 20508	Crude glycerol	Baker's yeast autolysate ;malt extract	43	58.9	Fed-batch 6 l fermentor	[15]
Cryptococcus curvatus ATCC 20508	Crude glycerol	Baker's yeast autolysate	43	52.3	Fed-batch 6 l fermentor	[15]
Cryptococcus curvatus ATCC 20508	Crude glycerol	Deoiled Cryptococcus lysate	48	69.2	Fed-batch 6 l fermentor	[15]
Cryptococcus curvatus ATCC 20509	Crude glycerol from biodiesel production using yellow grease	Yeast extract; peptone	52.9	32.9	two-stage fed-batch	[21]
Cryptococcus curvatus TYC-19	Beet molasses	Yeast extract; polypeptone	n.m	14.3	Flask	[24]
Cryptococcus curvatus TYC-19	Cheese Whey	Yeast extract; polypeptone	n.m	13.9	Flask	[24]
Kodamaea Ohmeri	Crude glycerol	Ammonium sulphate	30.33	10.45	Flask	[22]
Lipomyces starkeyi HL	Fishmeal wastewater + glucose	Fishmeal wastewater	15	17.6	Flask	[31]

Yeast strain	Carbon source	Nitrogen source	Lipid content (%, w/w)	Total dry weight (g/l)	Culture mode	Ref.
Rhodotorula sp. LFMB 6	Waste glycerol	Yeast extract	3.4	5.5	Flask	[19]
Rhodotorula sp. LFMB 22	Waste glycerol	Yeast extract	22	8.0	Flask	[19]
Rhodotorula glutinis BCRC 22360	Crude glycerol	Thin stillage	36.5	14.8	Batch fermentor	[18]
Rhodotorula glutinis BCRC 22360	Crude glycerol	Yeast extract	21	11.5	Batch fermentor	[18]
Rhodotorula glutinis TISTR 5159	Crude glycerol	Ammonium sulfate	35.22	5.47	Flask + Tween 20	[23]
Rhodotorula glutinis TISTR 5159	Crude glycerol	Ammonium sulfate	23.05	4.53	Flask	[23]
Rhodotorula glutinis TISTR 5159	Crude glycerol	Ammonium sulfate	33.33	5.26	Flask + Tween 80	[23]
Rhodotorula glutinis TISTR 5159	Crude glycerol	Ammonium sulfate	31.18	5.22	Flask + Gum arabic	[23]

Yeast strain	Carbon source	Nitrogen source	Lipid content (%, w/w)	Total dry weight (g/l)	Culture mode	Ref.
Rhodotorula glutinis TISTR 5159	Palm oil mill effluent	Palm oil mill effluent	20.97	4.15	Batch fermentor	[27]
Rhodotorula glutinis TISTR 5159	Palm oil mill effluent	Yeast extract	32.63	6.33	Batch fermentor	[27]
Rhodotorula glutinis TISTR 5159	Palm oil mill effluent	Ammonium sulfate	38.15	7.07	Batch fermentor	[27]
Rhodotorula glutinis	Municipal wastewater + glucose	Peptone+ yeast extract	n.m	21.4	Flask	[30]
Rhodosporidium toruloides AS2.1389	Crude glycerol from alkaline-catalyzed biodiesel production process	Yeast extract	47.7	19.2	Flask	[20]
Rhodosporidium toruloides AS2.1389	Crude glycerol from an enzyme-catalyzed biodiesel production process	Yeast extract	42.9	20.1	Flask	[20]
Rhodosporidium toruloides AS2.1389	Crude glycerol from alkaline-catalyzed biodiesel production process	Yeast extract	69.5	26.7	Batch fermentor	[20]

Yeast strain	Carbon source	Nitrogen source	Lipid content (%, w/w)	Total dry weight (g/l)	Culture mode	Ref.
Rhodosporidium toruloides AS2.1389	Crude glycerol from an enzyme-catalyzed biodiesel production process	Yeast extract	74.1	18.0	Batch fermentor	[20]
Rhodotorula mucilaginosa	Molasses	Ammonium sulphate	69.5	n.m	Flask	[25]
Trichosporonoides spathulata	Crude glycerol	Ammonium sulphate	40.69	10.15	Flask	[22]
Yarrowia lipolytica	Waste oil from chicken products fat		37.70	9.00	Flask	[28]
Yarrowia lipolytica	Waste oil from frying fish		45.49	8.40	Flask	[28]
Yarrowia lipolytica	Meat products fat		34.02	8.04	Flask	[28]
Yarrowia lipolytica	Waste oil from frying vegetables		57.89	7.56	Flask	[28]

Yeast strain	Carbon source	Nitrogen source	Lipid content (%,w/w)	Total dry weight (g/l)	Culture mode	Ref.
Yarrowia lipolytica	Municipal wastewater + glucose	Peptone + yeast extract	n.m	15.3	Flask	[30]
Yarrowia lipolytica *MUCL 28849*	Glucose + VFA	Ammonium sulfate	40.22	41.02	7 L bioreactor	[35]
Yarrowia lipolytica *MUCL 28849*	Glycerol + VFA	Ammonium sulfate	34.59	41.02	7 L bioreactor	[35]

3.3 Pretreated agricultural products and wastes as substrates for microbial oil production

Non-food grains and cheap substrates have been investigated as potentially low-cost sources of nutrients. Cassava starch [39], a cheap substrate, was pretreated with amylase and used as substrate to grow *R. toruloides* Y4. Microbial oil was accumulated at a lipid content of 63.4% and contained a suitable fatty acid composition comprising above 53.3% of oleic acid. Jerusalem artichoke [40] another cheap source, was tested using both a thermal pretreatment to produce extract and an acid pretreatment to produce hydrolysate. Both hydrolysate and extract showed their feasibility to produce lipids using the same yeast strain.

Various agro-industrial waste and by-product streams, such as lignocellulosic raw materials, are not directly assimilable by oleaginous yeasts and for this reason they should be pretreated in order to produce fermentable monomers [41]. Pretreatment methodologies can be categorised into acidic, basic, thermal, enzymatic or any combination of the above (Table 3.2). Lignocellulosic waste has been studied for microbial oil production. Generally, lignocellulosic waste is composed of 35-55% cellulose, 20-40% hemicellulose and 10-25% lignin [42]. To separate the components of lignocellulosic waste, reduce the crystallinity, remove the lignin and hydrolyse the cellulose into fermentable sugars, pretreatment of is required [43, 44]. Research to determine the consumption of sugars that are present in the hydrolysed lignocellulosic waste, i.e. cellobiose, C5 and C6 sugars, has been conducted. It has been found that *L. starkeyi* can consume a mixture of xylose/cellobiose during fermentation, giving a sugar

to lipid conversion yield of 0.19 g lipid/g sugar, which makes this microorganism suitable for lipid production using lignocellulosic waste [45].

Another important parameter that should be dealt with when lignocellulosic residues are used as feedstock is the yeast tolerance to inhibitors (5-hydroxymethyl-furfural, furfural, acetate, etc.) that are generated during pretreatment of lignocellulosic biomass. The potential effect of these inhibitors has been investigated in the case of *R. toruloides* [10] and *R. graminis* [46]. Results have shown that these microorganisms have a considerable ability to accumulate lipid in the presence of the inhibitors mentioned above, although the toxicity of these compounds is strongly dependent on their concentrations [47]. Yu *et al.* [48] tested the effect of the detoxification of wheat straw hydrolysate pretreated with sulphuric acid from these inhibitors. . Results showed that the culture medium without detoxification generates higher cellular growth and accumulation of lipids, which may be due to the higher amount of sugars compared to the detoxified medium.

The oleaginous yeast *C. curvatus* ATCC 20509 is tolerant to inhibitors contained in the non-detoxified medium when grown on sweet sorghum bagasse [49]. The feedstock was pretreated by microwave with lime followed by an enzymatic hydrolysis. Results were compared with the hydrolysate without pretreatment, showing that pretreatment promotes the increase in total sugars, thus achieving higher lipid accumulation [49]. The potential use of sugarcane bagasse as a source for lipid production was also tested [50]. Detoxified sugarcane bagasse hydrolysate provides an economically sound alternative carbon source for the growth of *Y. lipolytica Po1g* [51]. Galafassi *et al.* [46] compared two different substrates for lipid

production (corncob and crude glycerol) when *R. graminis* was used as oleaginous yeast. The comparison showed that non-detoxified lignocellulosic material (corncob) provides similar lipid productivity to crude glycerol. Non-detoxified hydrolysed corncob has been used to produce oil by fermentation with the yeast strain *Trichosporon dermatis*, providing a lipid content of 40% w/w [52]. Li *et al*. [53] hydrolysed rice straw by a two-step process, including diluted acid pretreatment followed by enzymatic hydrolysis, in order to achieve improved utilization of hemicellulose and cellulose. The hydrolysate without detoxification was employed as substrate in cultivations of *T. fermentans* leading to a lipid concentration of 13.8 g/l, which was higher than that produced by the detoxified substrate (11.9 g/l).

The utilization of protein-rich agro-industrial by-products as nitrogen sources has also been investigated for microbial oil production. Uçkun Kiran *et al.* [54] carried out different pretreatments to rapeseed meal for the production of a high free amino nitrogen (FAN) concentration medium that was subsequently used for microbial oil production using the oleaginous yeast strain *R. toruloides* leading to the production of a microbial oil content of 48% (w/w) when glucose was used as carbon source.

Table 3. 2 Production of dry cell weight and lipid accumulation when different pretreated agricultural products and waste streams are employed

Strains	Carbon sources	Nitrogen sources	Lipid content (%, w/w)	Total dry weight (g/l)	Biomass pre-treatment	Mode culture	Reference
Cryptococcus curvatus ATCC 20509	Sweet sorghum bagasse		63.98	15.50	Pretreated by microwave	Flask	[49]
Cryptococcus curvatus ATCC 20509	Sweet sorghum bagasse		73.26	10.83	Pretreated by microwave with lime	Flask	[49]
Cryptococcus curvatus ATCC 20509	Detoxified liquid wheat straw hydrolysate	Yeast extract	27.1	15.6	Dilute acid pretreated	Flask	[48]
Cryptococcus curvatus ATCC 20509	Non-detoxified liquid wheat straw hydrolysate	Yeast extract	33.5	17.2	Dilute acid pretreated	Flask	[48]
Lipomyces starkeyi ATCC 12659	Detoxified liquid wheat straw hydrolysate	Yeast extract	29.1	12.7	Dilute acid pretreated	Flask	[48]
Lipomyces starkeyi ATCC 12659	Non-detoxified liquid wheat straw hydrolysate	Yeast extract	31.2	14.7	Dilute acid pretreated	Flask	[48]

Strains	Carbon sources	Nitrogen sources	Lipid content (%, w/w)	Total dry weight (g/l)	Biomass pre-treatment	Mode culture	Reference
Rhodotorula glutinis ATCC 204091	Detoxified liquid wheat straw hydrolysate	Yeast extract	20.7	11.8	Dilute acid pretreated	Flask	[48]
Rhodotorula glutinis ATCC 204091	Non-detoxified liquid wheat straw hydrolysate	Yeast extract	25.0	13.8	Dilute acid pretreated	Flask	[48]
Rhodotorula graminis	Corn steep solids	Yeast extract	52.18	15.14	Dilute acid pretreatment followed by an enzymatic hydrolysis step	Flask	[46]
Rhodotorula mucilaginosa TJY15a	Hydrolysate of cassava starch + yeast extract		45.9	10.9	Crude amylase preparation	Batch	[55]
Rhodotorula mucilaginosa TJY15a	Hydrolysate of cassava starch + yeast extract		67.5	106.5	Crude amylase preparation	Mode fed-batch 15-l stirred-tank fermenter	[55]

Strains	Carbon sources	Nitrogen sources	Lipid content (%, w/w)	Total dry weight (g/l)	Biomass pre-treatment	Mode culture	Reference
Rhodosporidium toruloides Y4	Glucose	Rapeseed meal	18.3	62.2	Commercial enzyme pre-treatment	1 L bioreactor	[54]
Rhodosporidium toruloides Y4	Glucose	Rapeseed meal	44.2	34.8	Liquid state fungal pre-treatment	1 L bioreactor	[54]
Strains	Carbon sources	Nitrogen sources	Lipid content (%, w/w)	Total dry weight (g/l)	Biomass pre-treatment	Mode culture	Reference
Rhodosporidium toruloides Y4	Glucose	Rapeseed meal	54.4	77.7	Liquid state fungal pre-treatment followed by fungal autolisis	1 L bioreactor	[54]
Rhodosporidium toruloides Y4	Glucose	Rapeseed meal	41.3	31.2	Liquid state pre-treatment using enzymatic broth	1 L bioreactor	[54]
Rhodosporidium toruloides Y4	Glucose	Rapeseed meal	48.1	82.3	Solid state fungal pre-treatment followed by fungal autolisis	1 L bioreactor	[54]
Rhodosporidium toruloides 21167	Cassava starch	Yeast extract	63.2	18.5	Hydrolysis of cassava starch by the crude amylase preparation	Flask	[39]

Strains	Carbon sources	Nitrogen sources	Lipid content (%, w/w)	Total dry weight (g/l)	Biomass pre-treatment	Mode culture	Reference
Rhodosporidium toruloides 21167	Cassava starch	Yeast extract	63.4	22	Hydrolysis of cassava starch by the crude amylase preparation	2 L bioreactor	[39]
Rhodosporidium toruloides Y4	Jerusalem artichoke extract		39.5	25.5		Flask	[40]
Rhodosporidium toruloides Y4	Jerusalem artichoke extract		43	40		Batch 15 l bioreactor	[40]
Rhodosporidium toruloides Y4	Jerusalem artichoke extract		40	113		Fed-batch 15 l bioreactor	[40]
Rhodosporidium toruloides Y4	Jerusalem artichoke hydrolysates		56.5	70	Anthrone-sulfuric acid method	Fed-batch 15 l bioreactor	[40]

Strains	Carbon sources	Nitrogen sources	Lipid content (%, w/w)	Total dry weight (g/l)	Biomass pre-treatment	Mode culture	Reference
Rhodosporidium toruloides ATCC 10788	Wheat straw hydrolysate	Yeast extract	24.6	9.9	Dilute acid pretreated wheat straw hydrolysate	Flask	[48]
Trichosporon dermatis CH007	Corncob enzymatic hydrolysate		40.1	24.4		Flask	[52]
Trichosporon fermentans HWZ004	Rice straw hydrolysate		52.2	26.4	Dilute acid pretreatment and then enzymatic hydrolysis		[56]
Yarrowia lipolytica (ATCC 20460)	Detoxified liquid wheat straw hydrolysate	Yeast extract	4.4	7.2	Dilute acid pretreated	Flask	[48]
Yarrowia lipolytica (ATCC 20460)	Non-detoxified liquid wheat straw hydrolysate	Yeast extract	4.6	7.8	Dilute acid pretreated	Flask	[48]
Yarrowia lipolytica Po1g	Detoxified sugarcane bagasse hydrolysate + peptone		58.5	11.42	Hydrochloric acid and the inhibitor was subjected to neutralization with $Ca(OH)_2$	Flask	[51]

3.4 Potential use of yeast derived microbial oil for biodiesel production

It has been demonstrated that the selection of the substrate depends on the oleaginous microorganism used for microbial oil production as it influences significantly cell growth and lipid accumulation. Moreover, the combination of both substrate and microorganism has an important effect on the fatty acid composition of the produced microbial oil. In fact, the fatty acid profile of the microbial oil is not only influenced by the selection of the substrate, but also by fermentation conditions. For this reason, it is important to study the effect of each parameter, as they influence both the amount of oil and, more importantly, the optimum oil quality to produce biodiesel.

3.4.1 Microbial oil fatty acid composition linked to optimal biodiesel properties

Several authors state that the oil or fatty acid composition influences biodiesel properties and hence its quality [57-59]. In turn, some of the most significant biodiesel properties are determined by the chemical structure of its fatty acids; among the most significant parameters are the chain length and the degree of unsaturation [58]. For this reason, the analysis of the fatty acid profile of microbial oil makes it possible to predict the quality properties of biodiesel [60]. Moreover, it may allow the "ideal" fatty acid composition to be defined for the microbial oil used to provide a biodiesel with optimal chemical and physical properties.

The influence of the chemical structure of biodiesel fatty acids on fuel physical and chemical properties has been studied [4, 57, 59-63]. While heating value [64, 65], cetane number [66, 67] and oxidation stability [68, 69] increase with the chain length and decrease with the degree of unsaturation, low temperature behaviour properties [70] and viscosity [71] improve with shorter and more unsaturated fatty acid chains. For this reason, the fatty acid profile that provides a fuel with all optimal parameters does not exist [61, 72]. In this sense, it has been suggested that biodiesel with a high level of methyl oleate (mono-unsaturated fatty acid) may exhibit good behaviour regarding ignition quality, fuel stability, cold flow properties and iodine number, considering the European biodiesel standard EN 14214 [60, 61]. Recently, some authors have found the suitability of esters of saturated medium chain acids to produce biodiesel, especially esters of decanoic or capric acid [61, 73]. This is because they show reasonably good cold flow properties and constitute an alternative to the long-chain saturated fatty acid esters with high melting points. In addition, saturated medium chain acids show excellent oxidative stability due to the absence of double bonds. Finally, they are also preferred to polyunsaturated fatty acids (e.g. C18:3), that have a negative effect on the auto-oxidation of biodiesel [74].

Some studies have also shown a direct correlation between the chemical structure of biodiesel fatty acids and fuel exhaust emissions [75-77]. It has been found that exhaust NO_x emissions increase with the reduction of the mean carbon chain length and increasing degree of unsaturation [77, 78]. All these studies may help in the search for the ideal composition of the microbial oil.

In a recent study, Whalen *et al.* [79] analysed the physical properties of biodiesel from microalgae *Chaetoceros gracilis* oil, yeast *Cryptococcus curvatus* oil and bacteria *Rhodococcus opacus* oil, exhibiting different fatty acid composition. They also analysed engine performance and exhaust emissions of a diesel engine fuelled with those biodiesels. When *Cryptococcus curvatus* oil was produced using glucose as substrate, it provided an optimal fatty acid composition for biodiesel production (60% of mono-unsaturated fatty acids and a chain length similar to vegetable oils). Moreover, the biodiesel derived from this microorganism showed the highest biodiesel cetane index (BCI) [80], as well as the lowest CO emissions and similar properties compared to biodiesel from the other microorganisms.

3.4.2 Biodiesel predicted properties based on the fatty acid composition of yeast oil

Although research concerning yeast oil biodiesel properties is missing, statistical models may provide a useful tool to predict biodiesel properties based on yeast oil fatty acid composition [81]. In this sense, Table 3.3 summarises the potential values for yeast oil biodiesel cold filter plugging point (*CFPP*), flash point (*FP*), cetane number (*CN*), low calorific value (*LCV*) and kinematic viscosity (*n*) based on the previous statistical models. For comparison purposes, properties from most commonly used vegetable oil-based biodiesel (rapeseed and palm oil biodiesel) have been included. As may be seen from Table 3.3, no significant differences in terms

of *LCV* and *FP* between the oils were found. According to cetane number, that indicates the ignition quality of the fuel, most samples showed a higher value compared to rapeseed oil biodiesel *CN*, although the value was slightly lower or similar compared to that of palm oil biodiesel. In any case, all samples fulfilled European standard for biodiesel EN 14214. Concerning *CFPP* and *n* values, predictive models for yeast oil biodiesel showed large differences among them and compared to traditional biodiesel. With respect to kinematic viscosity predicted values, only a few oils would meet the limits set by EN 14214 (3.5-5 mm^2/s). The same problem is experienced by the widely used palm oil biodiesel. Eventually, we can conclude that the analysed yeast oil-based biodiesel could be used as fuel form diesel engines only when they are blended with diesel fuel. As most yeast oils show similar fatty acid composition to palm oil, that is directly related to cold weather behaviour, *CFPP* indicates most of them would not be advisable for cold climates, unless biodiesel is blended with diesel fuel.

Table 3.3 Predicted properties for yeast oil biodiesel

Oil origin	Reference	Substrate	Chain length	Unsaturation degree	Low calorific value, LCV (kJ/kg)	Cetane number, CN	Kinematic viscosity, (mm^2/s)	Flash point, FP (ºC)	Cold filter plugging point, CFPP (ºC)
EN 14214					-	51	3.5-5.0	120	-
Rapeseed	[82]		17.72	1.30	37442.56	56.93	4.89	166.88	-3.16
Palm	[63]		17.03	0.61	37442.56	69.87	5.46	162.16	8.23
Cryptococcus curvatus TYC-19	[24]	Beet molasses	17.93	1.59	37498.36	52.23	4.66	167.41	-5.94
Rhodotorula mucilaginosa TJY15a	[83]	Cassava starch	17.25	0.77	37485.45	66.88	5.35	164.80	5.39
Lipomyces starkeyi AS2. 1560	[45]	Cellobiose	17.09	0.59	37474.44	70.55	5.52	164.36	9.18
Cryptococcus curvatus TYC-19	[24]	Cheese whey	17.92	1.54	37510.35	52.99	4.70	167.95	-5.53
Yarrowia lipolytica NC-1	[28]	Chicken product fat	17.07	0.91	37344.29	62.71	5.02	155.62	0.52
Cryptococcus curvatus ATCC 20509	[37]	Hydrogen production effluent	17.04	0.70	37410.75	67.55	5.32	159.89	5.58

Oil origin	Reference	Substrate	Chain length	Unsaturation degree	Low calorific value, LCV (kJ/kg)	Cetane number, CN	Kinematic viscosity, (mm^2/s)	Flash point, FP (ºC)	Cold filter plugging point, CFPP (ºC)
Rhodotorula mucilaginosa	[25]	Molasses	16.09	0.35	37082.19	71.75	5.24	141.12	7.72
Candida curvata NRRL-Y 151	[19]	Raw glycerol	17.46	0.72	37607.47	69.16	5.57	173.55	8.59
Candida oleophila ATCC 20177	[19]	Raw glycerol	17.44	0.90	37526.97	64.51	5.27	167.56	3.32
Zygosaccharomyces rouxii LFMB 3	[19]	Raw glycerol	17.09	0.96	37332.90	61.75	4.96	154.97	-0.39
Rhodotorula sp. LFMB 22	[19]	Raw glycerol	17.27	0.82	37478.33	65.76	5.29	164.27	4.22
Yarrowia lipolytica LFMB 19	[19]	Raw glycerol	17.37	0.93	37480.87	63.49	5.18	164.41	2.07
Pichia membranifaciens LFMB 8	[19]	Raw glycerol	17.72	0.97	37637.70	64.07	5.35	175.47	3.59

Oil origin	Reference	Substrate	Chain length	Unsaturation degree	Low calorific value, LCV (kJ/kg)	Cetane number, CN	Kinematic viscosity, (mm^2/s)	Flash point, FP (ºC)	Cold filter plugging point, CFPP (ºC)
Yarrowia lipolytica ACA-YC 5033	[84]	Raw glycerol	17.59	0.94	37584.68	64.28	5.31	171.62	3.46
Yarrowia lipolytica LFMB 19	[84]	Raw glycerol	17.48	1.03	37497.00	61.80	5.11	165.55	0.64
Yarrowia lipolytica LFMB 20	[84]	Raw glycerol	17.46	0.92	37530.89	64.25	5.26	167.83	3.09
R. toruloides AS21389	[20]	Raw glycerol	17.09	0.66	37449.83	68.75	5.41	162.54	7.08
R. toruloides AS21389	[20]	Raw glycerol	16.89	0.67	37349.35	67.59	5.23	155.99	5.21
Lipomyces starkeyi	[85]	Olive oil mill wastewater	17.52	0.98	37535.47	63.08	5.21	168.15	2.02
Lipomyces starkeyi DSM 70295	[32]	Sewage sludge	16.43	0.28	37274.19	75.45	5.59	152.32	13.29
Cryptococcus albidus ATCC 10672	[34]	Volatile fatty acids	17.69	1.40	37453.24	54.91	4.74	163.61	-4.74

3.5 Effect of the substrate on microbial oil fatty acid composition

The effect of the substrate on the fatty acid composition of microbial oil is shown in Table 3.3. Most substrates lead to the production of a higher content of saturated fatty acids compared with rapeseed oil, the most commonly used oil in Europe for the production of biodiesel. The increase in saturated fatty acids may lead to a cetane number improvement and a greater oxidation stability, although it may be detrimental to cold behaviour properties and viscosity. Most microbial oils shown in Table 3.4 present a similar fatty acid composition to palm oil, which has a higher saturation degree than rapeseed oil. The most similar composition is provided by *L. starkeyi* grown in cellobiose as substrate [45]. A different fatty acid profile is produced through the fermentation of *C. curvatus* grown on cheese whey and beet molasses as substrate. The fatty acid composition of this oil is close to that of rapeseed oil.

Table 3. 4 Fatty acid composition considering different culture media and oleaginous yeast

Strains	Substrate	Fatty acids composition (%, wt/wt)							Reference
		C16:0	C18:0	C16:1	C18:1	C18:2	C18:3	Others	
Candida boidinii ATCC 32195	Raw glycerol	17.9	12.0	6.8	47.7	15.6		-	[19]
Candida curvata NRRL-Y 151	Raw glycerol	28.0	12.5	-	47.6	12.0		-	[19]
Candida. Lipolytica	Molasses	21.6	27.1	5.2	19.0	12.0	-	15.1	[25]
Candida oleophila ATCC 20177	Raw glycerol	12.9	6.6	2.5	65.6	11.0		1.4	[19]
Candida pulcherrima LFMB 1	Raw glycerol	24.0	4.7	4.6	48.0	15.3		3.4	[19]
Candida. Tropicalis	Molasses	29.7	56.2	5.0	2.3	-	-	5.3	[25]
Cryptococcus albidus ATCC 10672	Volatile fatty acids	16.1	5.14	-	17.7	61.1	-	-	[38]
Cryptococcus curvatus ATCC 20509	Crude glycerol from yellow grease	23	16.7	0.9	39.6	15.2	15.2	0.66	[21]
Cryptococcus curvatus ATCC 20509	Hydrogen production effluent+ acetic acids	22.4	17.7	0.3	44.9	10.8	1.1	0.8	[37]
Cryptococcus curvatus MUCL 29819	Acetic Acid	24	6	-	36	15	13	-	[86]

Strains	Substrate	Fatty acids composition (%, wt/wt)							Reference
		C16:0	C18:0	C16:1	C18:1	C18:2	C18:3	Others	
Cryptococcus curvatus TYC-19	Beet molasses	3.5	<0.1	<0.1	41.2	48.1	7.2	-	[24]
Cryptococcus curvatus TYC-19	Cheese whey	3.4	0.4	0.7	43.2	46.4	5.9	-	[24]
Lipomyces starkeyi	Olive oil mill wastewaters	19.1	8.5	0.5	49.1	18.8	3.5	<1.3	[85]
Lipomyces starkeyi AS 2.1560	Cellobiose	38.3	5.4	2.9	51.2	-	1.7	0.3	[45]
Lipomyces starkeyi AS 2.1560	Cellobiose/glucose/xylose	38.0	4.2	3.7	51.7	-	1.3	0.9	[45]
Lipomyces starkeyi DSM 70295	Sewage sludge	55.93	13.8	1.85	25.89	0.1	0.12	2.41	[32]
Yarrowia lipolytica	Glycerol + Volatile fatty acids mixture of acetic, propionic and butyric acid.	14.9	28.1	5.2	25.1	17.6		9.1	[35]
Yarrowia lipolytica	Glucose + Volatile fatty acids mixture of acetic, propionic and butyric acid.	22.6	39.0	4.4	23.1	10.9		-	[35]
Zygosaccharomyces rouxii LFMB 3	Raw glycerol	12.6	4.7	11.5	53.4	15.4		2.4	[19]

Strains	Substrate	Fatty acids composition (%, wt/wt)							Reference
		C16:0	C18:0	C16:1	C18:1	C18:2	C18:3	Others	
Rhodotorula sp. LFMB 22	Raw glycerol	21.7	7.4	1.1	55.9	12.4		1.5	[19]
Rhodotorula sp. LFMB 6	Raw glycerol	20.4	6.7	1.12	50.6	16.0		5.18	[19]
Yarrowia lipolytica LFMB 19	Raw glycerol	21.2	11.2	10.3	31.7	25.6		-	[19]
Pichia membranifaciens LFMB 8	Raw glycerol	12.6	4.1	1.2	68.2	13.9		-	[19]
Yarrowia lipolytica	Chicken products fat	23.40	5.83	-	45.31	22.91	-	2.55	[28]
Yarrowia lipolytica	Waste oil from frying fish	22.87	5.15	-	35.46	18.90	-	17.62	[28]
Yarrowia lipolytica	Meat Products fat	14.82	3.89		36.72	15.76		28.81	[28]
Yarrowia lipolytica	Waste oil from frying vegetables	19.87	5.52		50.48	16.63		7.5	[28]
Rhodotorula glutinis TISTR 5159	Palm oil mill effluent	20.37	10.33	0.83	47.88	7.31	0.85	12.43	[27]
Rhodotorula glutinis TISTR 5159	Crude glycerol	16.80	3.68	0.81	45.75	17.92	4.33	10.71	[23]

Strains	Substrate	Fatty acids composition (%, wt/wt)							Reference
		C16:0	C18:0	C16:1	C18:1	C18:2	C18:3	Others	
Rhodotorula graminis	Corn stover hydrolysate	20.51	7.16	-	42.12	17.15	2.89	10.17	[46]
Yarrowia lipolytica ACA-YC 5033	Crude glycerol	9.9	7.3	10.7	61.1	11.0	-	-	[84]
Yarrowia lipolytica LFMB 19	Crude glycerol	17.8	8.9	8.3	35.7	29.3	-	-	[84]
Yarrowia lipolytica LFMB 20	Crude glycerol	16.4	9.0	10.6	47.0	17.0	-	-	[84]
Rhodotorula mucilaginosaTJY15a	Hydrolysate of cassava starch	22.3	5.2	1.8	63.5	5.7	-	1.5	[55]
Rhodotorula mucilaginosa	Molasses	26.2	37.3	-	22.3	6.5		2.8	[25]
Rhodosporidium toruloides	Crude glycerol from alkaline-catalysed biodiesel production process	29.1	17.8	1.0	38.1	9.7	2.6	1.6	[20]
Rhodosporidium toruloides	Crude glycerol from enzyme-catalysed biodiesel production process	29.2	13.9	1.0	41.4	10.4	2.9	1.3	[20]

3.6 Conclusion

The use of different agro-industrial residues to produce microbial oil from oleaginous yeast could lead to the production of a sustainable raw material for the biodiesel industry. In this way, two purposes are fulfilled, the valorisation of agro-industrial waste and the production of a completely renewable oil for the production of biodiesel. Glycerol, a by-product from biodiesel production, has been shown to be suitable as a carbon source for microbial oil production by many oleaginous yeasts. Crude glycerol combined with oilseed meal hydrolysates derived as by-products from current oilseed-based biodiesel plants could be used efficiently as raw material for microbial oil production that could be subsequently employed for biodiesel production. An advantage of microbial oil compared to vegetable oil is that the fatty acid composition may be modified depending on the nutrient source.

Concerning microbial oil fatty acid composition, it has been shown that this is similar to the main vegetable oils used in biodiesel production, e.g. palm oil. Thus, their physicochemical characteristics might also be expected to be similar. It has been found that the selection of an appropriate oleaginous microorganism depends on the particular agro-industrial waste to be used as substrate, as its composition is crucial in microbial oil production. As future prospects, an economic study including oil production and further transesterification into biodiesel could help to establish whether this alternative may compete with traditional biodiesel industry. If microorganism genetic modification is combined with nutrients and growth conditions selection, microbial oil may constitute a realistic economically

sound alternative to vegetable oil for biodiesel industry. We can conclude, therefore, that microbial oil produced from oleaginous yeasts grown in agro-industrial waste opens a new path to a potentially sustainable and renewable biodiesel industry.

3.7 Acknowledgments

Authors gratefully thanks to the Spanish Ministry of Education and Science (ENE2010-15159) and the Andalusian Research, Innovation and Enterprise Council, Spain (TEP-4994) for the financial support.

3.8 References

1. Sorrell, S., et al. *An assessment of the evidence for a near-term peak in global oil production*. 2009 [cited 2012; Available from: http://www.ukerc.ac.uk.
2. Barnard, D., et al., *Extremophiles in biofuel synthesis.* Environmental Technology, 2010. 31(8-9): p. 871-888.
3. Vyas, A.P., J.L. Verma, and N. Subrahmanyam, *A review on FAME production processes.* Fuel, 2010. 89(1): p. 1-9.
4. Pinzi, S., et al., *Influence of vegetable oils fatty acid composition on reaction temperature and glycerides conversion to biodiesel during transesterification.* Bioresource Technology, 2011. 102: p. 1044-1050.
5. Birla, A., et al., *Kinetics studies of synthesis of biodiesel from waste frying oil using a heterogeneous catalyst derived from snail shell.* Bioresource Technology, 2012. 106(0): p. 95-100.
6. Deng, X., J. Han, and F. Yin, *Net energy, CO2 emission and land-based cost-benefit analyses of jatropha biodiesel: A case study of the Panzhihua region of Sichuan province in China.* Energies, 2012. 5(7): p. 2150-2164.
7. Beopoulos, A., J.M. Nicaud, and C. Gaillardin, *An overview of lipid metabolism in yeasts and its impact on biotechnological processes.* Applied Microbiology and Biotechnology, 2011. 90(4): p. 1193-1206.
8. Subramaniam, R., et al., *Microbial lipids from renewable resources: production and characterization.* Journal of Industrial Microbiology & Biotechnology, 2010. 37(12): p. 1271-1287.
9. Li, Q., W. Du, and D. Liu, *Perspectives of microbial oils for biodiesel production.* Applied Microbiology and Biotechnology, 2008. 80(5): p. 749-756.
10. Hu, C.M., et al., *Effects of biomass hydrolysis by-products on oleaginous yeast Rhodosporidium toruloides.* Bioresource Technology, 2009. 100(20): p. 4843-4847.

11. Ageitos, J.M., et al., *Oily yeasts as oleaginous cell factories.* Applied Microbiology and Biotechnology, 2011. 90(4): p. 1219-1227.

12. Economou, C.N., et al., *Semi-solid state fermentation of sweet sorghum for the biotechnological production of single cell oil.* Bioresource Technology, 2010. 101(4): p. 1385-1388.

13. Amaretti, A., et al., *Single cell oils of the cold-adapted oleaginous yeast Rhodotorula glacialis DBVPG 4785.* Microbial Cell Factories, 2010. 9.

14. Koutinas, A.A., et al., *Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production.* Fuel, 2014. 116(0): p. 566-577.

15. Thiru, M., S. Sankh, and V. Rangaswamy, *Process for biodiesel production from Cryptococcus curvatus.* Bioresource Technology, 2011. 102(22): p. 10436-10440.

16. Andre, A., et al., *Biotechnological conversions of bio-diesel derived crude glycerol by Yarrowia lipolytica strains.* Engineering in Life Sciences, 2009. 9(6): p. 468-478.

17. Khanna, S., A. Goyal, and V.S. Moholkar, *Microbial conversion of glycerol: present status and future prospects.* Critical Reviews in Biotechnology, 2012. 32(3): p. 235-262.

18. Yen, H.-W., Y.-C. Yang, and Y.-H. Yu, *Using crude glycerol and thin stillage for the production of microbial lipids through the cultivation of Rhodotorula glutinis.* Journal of Bioscience and Bioengineering, 2012. 114(4): p. 453-6.

19. Chatzifragkou, A., et al., *Biotechnological conversions of biodiesel derived waste glycerol by yeast and fungal species.* Energy, 2011. 36(2): p. 1097-1108.

20. Xu, J.Y., et al., *Microbial conversion of biodiesel byproduct glycerol to triacylglycerols by oleaginous yeast Rhodosporidium toruloides and the individual effect of some impurities on lipid production.* Biochemical Engineering Journal, 2012. 65: p. 30-36.

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

22. Kitcha, S. and B. Cheirsilp, *Screening of oleaginous yeasts and optimization for lipid production using crude glycerol as a carbon source*, in *9th Eco-Energy and Materials Science and Engineering Symposium*, P.P. Yupapin, S. PivsaArt, and H. Ohgaki, Editors. 2011, Elsevier Science Bv: Amsterdam.

23. Saenge, C., et al., *Potential use of oleaginous red yeast Rhodotorula glutinis for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids.* Process Biochemistry, 2011. 46(1): p. 210-218.

24. Takakuwa, N. and K. Saito, *Conversion of beet molasses and cheese whey into fatty acid methyl esters by the yeast Cryptococcus curvatus.* Journal of Oleo Science, 2010. 59(5): p. 255-260.

25. Karatay, S.E. and G. Donmez, *Improving the lipid accumulation properties of the yeast cells for biodiesel production using molasses.* Bioresource Technology, 2010. 101(20): p. 7988-7990.

26. Latif Ahmad, A., S. Ismail, and S. Bhatia, *Water recycling from palm oil mill effluent (POME) using membrane technology.* Desalination, 2003. 157(1,Äì3): p. 87-95.

27. Saenge, C., et al., *Efficient concomitant production of lipids and carotenoids by oleaginous red yeast Rhodotorula glutinis cultured in palm oil mill effluent and application of lipids for biodiesel production.* Biotechnology and Bioprocess Engineering, 2011. 16(1): p. 23-33.

28. El Bialy, H., O.M. Gomaa, and K.S. Azab, *Conversion of oil waste to valuable fatty acids using Oleaginous yeast.* World Journal of Microbiology & Biotechnology, 2011. 27(12): p. 2791-2798.

29. Hall, J., et al., *Oil production by a consortium of oleaginous microorganisms grown on primary effluent wastewater.* Journal of Chemical Technology and Biotechnology, 2011. 86(1): p. 54-60.

30. Chi, Z.Y., et al., *Lipid production by culturing oleaginous yeast and algae with food waste and municipal wastewater in an integrated process.* Applied Biochemistry and Biotechnology, 2011. 165(2): p. 442-453.

31. Huang, L.H., et al., *Application of fishmeal wastewater as a potential low-cost medium for lipid production by Lipomyces starkeyi HL.* Environmental Technology, 2011. 32(16): p. 1975-1981.

32. Angerbauer, C., et al., *Conversion of sewage sludge into lipids by Lipomyces starkeyi for biodiesel production.* Bioresource Technology, 2008. 99(8): p. 3051-3056.

33. Lim, S.J., et al., *Biological nutrient removal with volatile fatty acids from food wastes in sequencing batch reactor.* Korean Journal of Chemical Engineering, 2008. 25(1): p. 129-133.

34. Fei, Q., et al., *The effect of volatile fatty acids as a sole carbon source on lipid accumulation by Cryptococcus albidus for biodiesel production.* Bioresource Technology, 2011. 102(3): p. 2695-2701.

35. Fontanille, P., et al., *Bioconversion of volatile fatty acids into lipids by the oleaginous yeast Yarrowia lipolytica.* Bioresource Technology, 2012. 114: p. 443-9.

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

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

38. Fei, Q., et al., *The effect of volatile fatty acids as a sole carbon source on lipid accumulation by Cryptococcus albidus for biodiesel production.* Bioresource Technology, 2011. 102(3): p. 2695-2701.

39. Wang, Q., et al., *Lipid production from hydrolysate of cassava starch by Rhodosporidium toruloides 21167 for biodiesel making.* Renewable Energy, 2012. 46: p. 164-168.

40. Zhao, X., et al., *Lipid production from Jerusalem artichoke by Rhodosporidium toruloides Y4.* Journal of Industrial Microbiology & Biotechnology, 2010. 37(6): p. 581-585.

41. Wang, R.H., et al., *Bioconversion of rapeseed meal for the production of a generic microbial feedstock.* Enzyme and Microbial Technology, 2010. 47(3): p. 77-83.

42. Ghosh, P. and T.K. Ghose, *Bioethanol in India: recent past and emerging future.* Advances in biochemical engineering/biotechnology, 2003. 85: p. 1-27.

43. Mosier, N., et al., *Features of promising technologies for pretreatment of lignocellulosic biomass.* Bioresource Technology, 2005. 96(6): p. 673-686.

44. Yousuf, A., *Biodiesel from lignocellulosic biomass-prospects and challenges.* Waste Management, 2012. 32(11): p. 2061-2067.

45. Gong, Z.W., et al., *Co-fermentation of cellobiose and xylose by Lipomyces starkeyi for lipid production.* Bioresource Technology, 2012. 117: p. 20-24.

46. Galafassi, S., et al., *Lipid production for second generation biodiesel by the oleaginous yeast Rhodotorula graminis.* Bioresource Technology, 2012. 111(0): p. 398-403.

47. Zhao, X.B., et al., *Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast Rhodosporidium toruloides and preparation of biodiesel by enzymatic transesterification of the lipid.* Bioprocess and Biosystems Engineering, 2012. 35(6): p. 993-1004.

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

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

50. Wang, W., et al., *Effect of structural changes on enzymatic hydrolysis of eucalyptus, sweet sorghum bagasse, and sugarcane bagasse after liquid hot water pretreatment.* Bioresources, 2012. 7(2): p. 2469-2482.

51. Tsigie, Y.A., et al., *Lipid production from Yarrowia lipolytica Po1g grown in sugarcane bagasse hydrolysate.* Bioresource Technology, 2011. 102(19): p. 9216-9222.

52. Huang, C., et al., *Oil production by the yeast Trichosporon dermatis cultured in enzymatic hydrolysates of corncobs.* Bioresource Technology, 2012. 110: p. 711-4.

53. Li, Y., et al., *Using rice straw hydrolysate for microbial oil production by Trichosporon fermentans HWZ004.* Chinese journal of biotechnology, 2011. 27(9): p. 1309-16.

54. Uckun Kiran, E., et al., *Enhancing the value of nitrogen from rapeseed meal for microbial oil production.* Enzyme and Microbial Technology, 2012. 50(6-7): p. 337-42.

55. Andre, A., et al., *Biotechnological conversions of bio-diesel derived waste glycerol into added-value compounds by higher fungi: production of biomass, single cell oil and oxalic acid.* Industrial Crops and Products, 2010. 31(2): p. 407-416.

56. Li, Y., et al., *Using rice straw hydrolysate for microbial oil production by Trichosporon fermentans HWZ004.* Sheng wu gong cheng xue bao = Chinese journal of biotechnology, 2011. 27(9): p. 1309-16.

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

58. Pinzi, S., et al., *The ideal vegetable oil-based biodiesel composition: a review of social, economical and technical implications.* Energy & Fuels, 2009. 23: p. 2325–2341.

59. Canakci, M., Sanli, H., *Biodiesel production from various feedstocks and their effects on the fuel properties.* Journal of Industrial Microbiological and Biotechnology, 2008. 35: p. 431-441.

60. Ramos, M.J., et al., *Influence of fatty acid composition of raw materials on biodiesel properties.* Bioresource Technology, 2009. 100(1): p. 261-268.

61. Knothe, G., *"Designer" biodiesel: optimizing fatty ester composition to improve fuel properties.* Energy & Fuels, 2008. 22: p. 1358-1364.

62. Harrington, K.J., *Chemical and physical properties of vegetable oil esters and their effect on diesel fuel performance.* Biomass, 1986. 9: p. 1-17.

63. Pinzi, S., et al., *Influence of vegetable oils fatty-acid composition on biodiesel optimization.* Bioresource Technology, 2011. 102(2): p. 1059-1065.

64. Mehta, P.S. and K. Anand, *Estimation of a lower heating value of vegetable oil and biodiesel fuel.* Energy & Fuels, 2009. 23: p. 3893–3898.

65. Freedman, B. and M. Bagby, *Heats of combustion of fatty esters and triglycerides.* Journal of the American Oil Chemists Society, 1989. 66(11): p. 1601-1605.

66. Knothe, G., A.C. Matheaus, and T.W. Ryan, *Cetane numbers of branched and straight-chain fatty esters determined in an ignition quality tester.* Fuel, 2003. 82(8): p. 971-975.

67. Lapuerta, M., J. Rodríguez-Fernández, and E.F. de Mora, *Correlation for the estimation of the cetane number of biodiesel fuels and implications on the iodine number.* Energy Policy, 2009. 37(11): p. 4337-4344.

68. Dunn, R.O., *Effect of oxidation under accelerated conditions on fuel properties of methyl soyate (biodiesel).* Journal of the American Oil Chemists Society, 2002. 79(9): p. 915-920.

69. Knothe, G., *Some aspects of biodiesel oxidative stability.* Fuel Processing Technology, 2007. 88(7): p. 669-677.

70. Sarin, A., et al., *Blends of biodiesels synthesized from non-edible and edible oils: Effects on the cold filter plugging point.* Energy & Fuels, 2010. 24: p. 1996–2001.

71. Knothe, G. and K.R. Steidley, *Kinematic viscosity of biodiesel fuel components and related compounds. Influence of compound*

structure and comparison to petrodiesel fuel components. Fuel, 2005. 84(9): p. 1059-1065.

72. Durrett, T.P., C. Benning, and J. Ohlrogge, *Plant triacylglycerols as feedstocks for the production of biofuels* The Plant Journal, 2008. 54 (4): p. 593–607

73. Knothe, G., S.C. Cermak, and R.L. Evangelista, *Cuphea oil as source of biodiesel with improved fuel properties caused by high content of methyl decanoate* Energy & Fuels, 2009. 23(3): p. 1743-1747.

74. Knothe, G. and R.O. Dunn, *Dependence of oil stability index of fatty compounds on their structure and concentration and presence of metals* Journal of the American Oil Chemists Society, 2003. 72: p. 1155-1160.

75. Knothe, G., C.A. Sharp, and T.W. Ryan, *Exhaust emissions of biodiesel, petrodiesel, neat methyl esters, and alkanes in a new technology engine* Energy & Fuels, 2006. 20: p. 403–408.

76. Graboski, M.S., et al., *The effect of biodiesel composition on engine emissions from a DDC series60 diesel engine*, 2003, National Renewable Energy Laboratory: Golden, Colorado.

77. Schönborn, A., et al., *The influence of molecular structure of fatty acid monoalkyl esters on diesel combustion.* Combustion and Flame, 2009. 156(7): p. 1396-1412.

78. Pinzi, S., et al., *The effect of biodiesel fatty acid composition on combustion and diesel engine exhaust emissions.* Fuel, 2013. 104: p. 170-182.

79. Wahlen, B.D., et al., *Biodiesel from microalgae, yeast, and bacteria: engine performance and exhaust emissions.* Energy & Fuels, 2013. 27(1): p. 220-228.

80. Lapuerta, M., J. Rodríguez-Fernández, and F. Oliva, *Determination of enthalpy of formation of methyl and ethyl esters of fatty acids.* Chemistry and Physics of Lipids, 2010. 163(2): p. 172-181.

81. Pinzi, S., et al., *Multiple response optimization of vegetable oils fatty acid composition to improve biodiesel physical properties.* Bioresource Technology, 2011. 102(15): p. 7280-7288.

82. Leiva-Candia, D.E., et al., *Influence of nitrogen fertilization on physical and chemical properties of fatty acid methyl esters from Brassica napus oil.* Fuel, 2013. 111(0): p. 865-871.

83. Andre, A., et al., *Biotechnological conversions of biodiesel derived waste glycerol into added-value compounds by higher fungi: production of biomass, single cell oil and oxalic acid.* Industrial Crops and Products, 2010. 31(2): p. 407-416.

84. Andre, A., et al., *Biotechnological conversions of bio-diesel-derived crude glycerol by Yarrowia lipolytica strains.* Engineering in Life Sciences, 2009. 9(6): p. 468-478.

85. Yousuf, A., et al., *Microbial conversion of olive oil mill wastewaters into lipids suitable for biodiesel production.* Journal of Agricultural and Food Chemistry, 2010. 58(15): p. 8630-8635.

86. Christophe, G., et al., *Production of oils from acetic acid by the oleaginous yeast Cryptococcus curvatus.* Applied Biochemistry and Biotechnology, 2012. 167(5): p. 1270-1279.

Chapter 4

"Agro-industrial waste used as substrate to produce microbial oil by yeast fermentation"

Chapter 4. Agro-industrial waste used as substrate to produce microbial oil by yeast fermentation

Abstract Biodiesel is a renewable fuel, mainly produced from vegetable oils, that can be used in diesel engines to reduce the consumption of fossil fuels. Current research is focused on the identification of new feedstocks that could be used for biodiesel production and do not compete with food applications. Microbial oil produced from oleaginous yeast constitutes a novel source of triglycerides that can be produced from various renewable resources, including industrial waste and by-product streams. This study focuses on the design of a new concept of biodiesel industry, where the main target is microbial oil production using a combination of commercial sources of nutrients and by-product streams from a sunflower-based biodiesel industry. Microbial oil production has been investigated using three oleaginous yeast strains (i.e. *Rhodosporidium toruloides* DSM 4444, *Lipomyces starkeyi* DSM 70296 and *Cryptococcus curvatus* ATCC 20509) cultivated in both shake flask and bioreactor, using crude glycerol as carbon source and three different sources of nutrient supplements. Sunflower meal generated after oil extraction has been used as a fermentation nutrient-rich supplement, while two other material fractions (e.g. protein isolate, antioxidants). may be used for value-added applications. Experimental results show that this novel biodiesel-industry approach could lead to an

efficient production of microbial oil. The principal fatty acids present in microbial oil are oleic acid (C18:1), palmitic acid (C16:0) and linoleic acid (C18:2). The total saturated fatty acid content is above 34%, being oleic acid content higher than 45%. The fatty acid content of the microbial oil produced in this study is similar to that of palm oil. This study demonstrates that valorization of agro-industrial waste and by-product streams from biodiesel industry could lead to the development of technologically viable new concept of biodiesel industry.

4.1 Introduction

Biodiesel is a renewable fuel that can be used in diesel engines to reduce the consumption of fossil fuels. Biodiesel production is based on the transesterification or esterification of fatty acids with an alcohol in the presence of an acid or basic catalyst. Currently, fatty acids to produce biodiesel are based on vegetable oils, thus increasing biodiesel production costs, because the raw material price represents up to 60-70% of total cost [1]. For this reason, it is necessary to finance this industry through government subsidies. Biodiesel environmental benefits include the reduction of greenhouse gases, so reducing processing cost is key to extend its use, thus providing a sustainable technology.

Microbial oil is a raw material that is being investigated in the production of biodiesel due to similar physical and chemical characteristics to vegetable oils. This raw material is produced from oleaginous microorganisms that are able to accumulate intracellular lipid bodies in quantities above 20% of dry cells weight. The lipid bodies are formed when the oleaginous yeast grows in a medium with excess of carbon source and limited nitrogen source. Under these conditions, the excess of carbon is

transformed into lipids in the endoplasmic reticulum, providing an energy source for the microorganism [2]. Different genera of microorganisms (i.e. bacteria, filamentous fungi, algae and yeasts) show the ability to accumulate lipid. Among them, yeast depicts a better suitability for industrial processes, due to its fast growth rate and high oil content compared to other oleaginous species [3, 4]. However, only 5% of known yeast may accumulate more than 20% of oil in dry cells weight, mainly including *Cryptococcus*, *Rhodosporidium* and *Lipomyces* species.

To provide a economically vial be biotechnology, the use of cheap substrates, i.e. industrial waste, lignocellulosic materials or food waste is needed. Moreover, glycerol (by-product of the traditional biodiesel industry) constitutes an interesting raw material to be used as carbon source for the production of microbial oil [5-8], due to its wide availability. Crude glycerol obtained from the biodiesel industry typically has a purity among 85-95%, including residual methanol and salts of reaction of the catalyst [8]. Another interesting residue is provided by lignocellulosic materials derived from industrial and agricultural processes, i.e. oilseed cake from the oil industry, because it has a high content of proteins and nutrients that may be sufficient for the growth of oil-producing yeasts. So, the use of a medium composed by by-products of both the biodiesel industry and the oil industry may help to reduce the production costs, because the origin of the raw materials is the industry itself, thus leading to a renewable and self-sufficient industry.

The aim of this research is to study the behavior of three oleaginous yeasts (*R. toruloides* DSM 4444, *C. curvatus* ATCC 20509, *L. starkeyi* DSM 70296) in a culture medium including glycerol as the carbon source and three different nutrient supplements, namely sunflower meal

hydrolysate (SFM), pre-extracted protein and antioxidant sunflower meal hydrolysate (PSFM).

4.2 Materials and method

4.2.1 Microorganisms and culture media

Cryptococcus curvatus (ATCC 20509), *Lipomyces starkeyi* (DSM 70296) and *Rhodosporidium toruloides* (DSM 4444) were selected. *R. toruloides* was kept at 4°C in agar slant containing 10 g/l peptone, 48 g/l malt extract, 10 g/l glucose and 20 g/l agar. *C. curvatus* and *L. starkeyi* was kept at 4°C in agar slant compose for 10 g/l glucose, 10 g/l peptone, 10 g/l yeast extract and 20 g/l agar. The inoculum of *R. toruloides* was grown in liquid medium (10 g/l glucose, 48 g/l malt extract, 10 g/l peptone) at 28°C and incubated in rotary shaker (180 rpm) for 24 h. Inoculum of *L. starkeyi* and *C. curvatus* was grown in liquid medium (10 g/l glucose, 10 g/l peptone, 10 g/l yeast extract) at 30°C and incubated in rotary shaker (180 rpm) for 48 and 24 h, respectively.

4.2.2 Batch fermentation

To study the behavior of the three oleaginous yeasts mentioned above, experiments were conducted in 250 ml flasks with cotton cap, initial pH 6-6.3, 10% of inoculum and 50 ml of culture medium composed for two different C/N relations (82 and 135) corresponding to 60 g/l and 100 g/l of glycerol. The commercial medium comprised crude glycerol 95% purity, 2 g/l yeast extract powder (from Lab. M limited, United kingdom) as nitrogen source and 1 g/l bacteriological peptone (from Biolife, Italy). The salt composition of the basal medium was made of 7 g/l sodium phosphate

monobasic (from Riedel-de Haën assay 99.5%, Germany), 2.5 g/l di-Natriumhydrogenphosphat (from MERCK assay 99%, Germany), 1.5 g/l magnesium sulfate heptahydrate (from Sigma Aldrich, Germany), 0.15 g/l iron (III) chloride hexahydrate (stock solution), 0.02 g/l zinc sulfate heptahydrate (stock solution), 0.06 g/l manganese sulfate monohydrate (stock solution) and 0.15 g/l calcium chloride dihydrate (stock solution) [6].

The SFM hydrolysate medium contained 7 g/l sodium phosphate monobasic (from Riedel-de Haën assay 99.5%, Germany) and 2.5 g/l di-Natriumhydrogenphosphat (from MERCK assay 99%, Germany). The SFM hydrolysate was previously analyzed to determine the free aminoacids nitrogen (Ninhydrin colorimetric method) and then diluted with crude glycerol and distilled water to obtain the total volume of work for the experiments with the same C/N ratios of synthetic medium. Subsequently, to avoid the presence of undesired microorganisms in the medium, the culture medium was passed two times, with the help of a peristaltic pump, through filter polycap 36 S pore size 0.2 µm (Whatman). Finally, the sterilized medium was separated aseptically in each flask with total volume of 45 ml of inoculate. The same procedure was performed using medium consisting of PSFM hydrolysate. Each experiment was monitored during 8 days or until complete consumption of the carbon source. pH was kept at 6 by adding a sterile solution of NaOH (2.5N). The culture temperature for each microorganism was: 28°C for *R. toruloides*, 30°C for *L. starkeyi* and 30°C for *C. curvatus*. Finally, the yeasts were incubated in a rotary shaker at 180 rpm.

4.2.3 Fed batch fermentation

It was carried out in a 3.6 l bioreactor (Infors 4, Switzerland) with a working volume of 2.3 l. The composition was the same as for SFM and PSFM hydrolysate described in batch fermentation. The pO_2 concentration was controlled via sequential cascade of two parameters (stirring and air/flow) and maintained in 3 vvm. The pH was adjusted to 6 by automatic control using a pH-meter and adding an autoclaved solution of 2.5 N NaOH with a peristaltic pump. The initial C/N ratio was 135 and when the glycerol concentration decreased close to 20 g/l, an autoclaved solution of glycerol was added aseptically until a concentration of 60 g/l in the medium was reached.

4.2.4 Analysis

Glycerol concentration was determined by HPLC (Waters 600E) using Aminex HPX-87H colum (Biorad), RI detector (waters 410) and the same operating conditions described by Koutinas *et al*. [9]. Free aminoacid nitrogen (FAN) was determined by nihydrin colorimetric method [10]. For dry cell weight calculation, the exact volume of the sample was first measured using a test tube, then centrifuged at 12000g for 15 min (Hettich universal centrifuge, model 320-R, United Kingdom). The supernatant was collected to further analyze the consumption of glycerol and FAN. Wet biomass was washed twice with distilled water and placed in an oven at 100°C until constant weight. The lipids extraction was carried out using chloroform and methanol (2:1 v/v), then filtered and concentrated for rotary evaporator. To analyze the fatty acid composition of the oil, a two-step reaction following the method described by Papanikolaou *et al*. [11] was carried out. Subsequently, fatty acid composition was analyzed by GC.

Samples were gathered by Aseptic sampling system (Infors HT, Switzerland).

4.3 Results and discussions

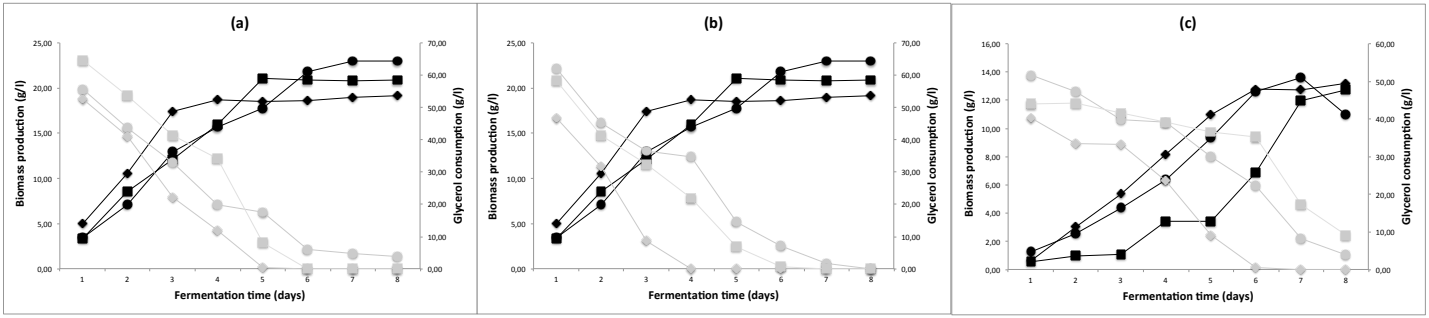
4.3.1 Batch fermentation

According to the literature, different yeast species have been tested for oil production using glycerol as a carbon source and alternative nitrogen sources and nutrients (Table 4.1). In this way, Saenge *et al*. [5] used crude glycerol, ammonium sulphate and no nutrient supplemented medium and fermented using *R. glutinis* which provided a lipid accumulation close to 2.42 g/l in flask fermentation for a C/N of 85. Chatzifragkou *et al* [6] used the same salt medium reported in this study, but different nitrogen source (ammonium sulphate and yeast extract) and C/N ratio (66). Results showed there were two yeasts (*Rhodotorula* sp. LFMB 22 and *Candida oleophila* ATCC 20177) that may accumulate lipids up to 1.76 and 1.44 g/l, respectively. Authors demonstrated that the analyzed species may accumulate more lipids than those previously reported and higher biomass production. For this reason, in the present study the same oleaginous yeast (*R. toruloides* DSM 4444, *C. curvatus* ATCC 20509 and *L. starkeyi* DSM 70296) will be selected to produce lipids using glycerol as substrate.

Table 4.1: Comparison of biomass production and lipid accumulation from different strains of yeasts grown on glycerol in flask fermentation

Strain	Nitrogen sources	C/N	Biomass (g/l)	Lipid (g/l)	Ref.
Candida oleophila ATCC 20177	$(NH_4)_2SO_4$ and yeast extract	66	9.4	1.44	[6]
Candida curvata NRRL-Y 1511	$(NH_4)_2SO_4$ and yeast extract	66	7.9	0.52	[6]
Rhodotorula sp. LFMB 22	$(NH_4)_2SO_4$ and yeast extract	66	8	1.76	[6]
Rhodotorula graminis DBVPG 4620	Corn stover hydrolysate	50	13	2.86	[12]
Rhodotorula glutinis TISTR 5159	$(NH_4)_2SO_4$	60	5.34	1.96	[5]
		85	5.65	2.42	
C. curvatus ATCC 20509	yeast extract and peptone	82	22.96	4.32	This study
		135	26.76	7.72	
R. toruloides DSM 4444	yeast extract and peptone	82	13.78	3.43	This study
		135	20.14	7.61	
L. starkeyi DSM 70296	yeast extract and peptone	82	12.58	3.10	This study
		135	13.12	2.75	

Figure 4.1: Glycerol consumption and biomass production by a) *R. toruloides* b) *C. curvatus* c) *L. starkeyi* during batch fermentation for initial glycerol concentration of 60 g/l



Black line means biomass production; grey line means glycerol consumption; square symbol: PSFM hydrolysate medium; diamond symbol: SFM hydrolysate medium; circle symbol: synthetic medium

Previous and current results are shown in Table 4.1. Once the feasibility of the use of glycerol as substrate has been demonstrated, next step consists in the production of oil using a medium only composed by agro-industrial waste. In this case, the nutrient supply was changed by agro-industrial waste hydrolysate originated from SFM hydrolysate. Hydrolysate was used either directly or including a pre-extraction of proteins and antioxidants before hydrolysis (PSFM hydrolysate). Results show that the consumption of glycerol as carbon source in the SFM hydrolysate medium is faster compared to the commercial medium used as control medium and produce more biomass in the initial phase of fermentation, for all the analyzed strains. However, in the stationary phase the trend is channeled due to the exhaustion of the carbon source, contrary to the control medium (Figure 4.1). The use of the bio-waste medium decreased the lipid accumulation over 50% for all tested yeasts, when the initial concentration of glycerol in the medium was 60 g/l. In terms of biomass production, no significant differences with any culture medium were observed, exception made with the yeast *C. curvatus*, which showed a biomass production increase close to 7 g/l, when both bio-waste culture media were used.

An improvement in lipid production was achieved when the amount of glycerol was increased in all culture media to an initial concentration of 100 g/l (Table 4.2). Results disagree with those reported by Liang et al. [7], that grown *C. curvatus* ATCC 20509 in media with different initial glycerol concentrations and no additional nutrient source. Authors conclude that high initial crude glycerol concentrations (60 and 80 g/l) inhibit cell growth. According to this and results derived from the current study, we can conclude that the addition of nutrients plays an important role in the production of biomass. This conclusion can be extended to all analyzed

strains. For initial concentrations of 100 g/l of glycerol in either SFM or PSFM hydrolysates, in both cases triglycerides production exceeds that from commercial medium used as control in this study.

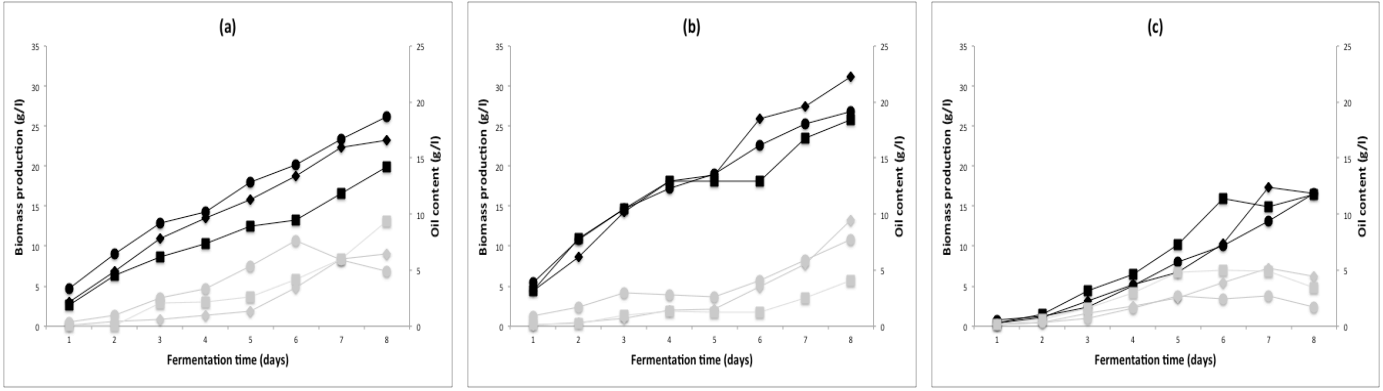
Finally, the medium where the fraction of proteins and antioxidants was extracted showed slightly lower lipid accumulation for all strains compared to the medium where this fraction was not removed (Figure 4.2). However, the possibility of extracting proteins and antioxidants from the substrate, in the first medium, may counterbalance this effect because may be used for value-added applications.

Table 4.2: Biomass production, lipid accumulation, C/N and culture media used in this study considering different yeast strains

Strain	Medium	C/N	Biomass (g/L)	Lipid (g/l)
C. curvatus ATCC 20509	Control medium	82	22.96	4.32
		135	26.76	7.72
	SFM hydrolysate	82	19.01	1.39
		135	31.22	9.51
	PSFM hydrolysate	82	21.10	2.76
		135	30.83	6.01
R toruloides DSM 4444	Control medium	82	13.78	3.43
		135	20.14	7.61
	SFM hydrolysate	82	19.01	1.39
		135	27.4	12.6
	PSFM hydrolysate	82	18.57	1.49
		135	19.9	9.36

Strain	Medium	C/N	Biomass (g/L)	Lipid (g/l)
L. starkeyi DSM 70296	Control medium	82	12.58	3.10
		135	13.12	2.75
	SFM hydrolysate	82	12.79	2.36
		135	17.40	5.14
	PSFM hydrolysate	82	11.98	1.59
		135	15.96	4.98

Figure 4.2: Dry cell weight production and oil content for each fermentation medium used in the study including 100 g/l of initial glycerol concentration in flask mode. (a) *R. toruloides* (b) *C. curvatus* (c) *L. starkeyi*. Black line means dry cells weight; grey line means oil content; square symbol: PSFM hydrolysate medium; diamond symbol: SFM hydrolysate medium; circle symbol: synthetic medium



4.3.2 Fed batch fermentation

In order to increase lipid production, fermentation was performed in a reactor under the conditions described in Materials and methods section. The yeast was selected considering the amount of biomass produced in the flask fermentations. In this sense, as *C. curvatus* ATCC 20509 provided the highest yield of biomass, it was selected for fed-batch fermentation. Results considering SFM, PSFM hydrolysate and crude glycerol as culture media were higher in terms to biomass production but slightly lower considering oil content compared to previous results reported by Liang *et al*. [7] using the same strain of oleaginous yeasts with crude glycerol but different nitrogen sources. Moreover, other strains used the same carbon source and showed lower biomass yield and oil content (Table 4.3).

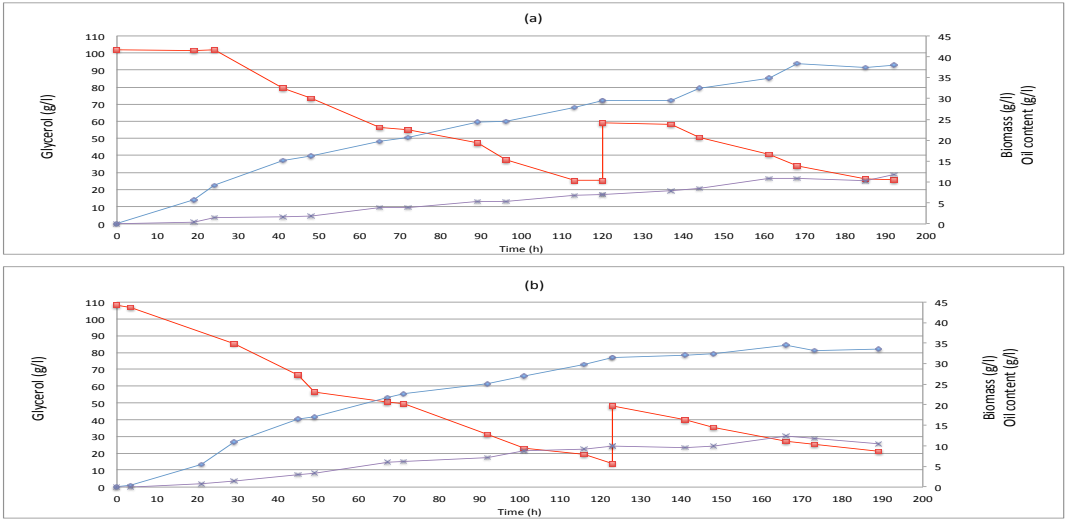
Table 4.3: Comparative of biomass production and lipid concentration for different oleaginous yeasts using glycerol as carbon source and different nitrogen sources in fed batch mode

Strain	Nitrogen sources	dry biomass (g/l)	lipids conc. (g/l)	reference
R. toruloides Y4	hydrolysate from rapeseed meal	31.1	13.00	[13]
C. curvatus ATCC 20509	NH_4CL	32.9	17.40	[7]
R. glutinis TISTR 5159	ammonium sulphate	10.05	6.10	[5]
C. curvatus ATCC 20509	SFM hydrolysate	37.60	16.44	This study
C. curvatus ATCC 20509	PSFM hydrolysate	34.60	12.46	This study

Using fed batch mode, the yeast increased the biomass and lipid accumulation in more than 10% and 40%, respectively, compared to flask fermentation experiments with both cuture media, SFM and PSFM hydrolysates. Regarding the use of hydrolysed SFM and PSFM in fed batch mode, the yeast *C. curvatus* depicted the same trend for both media in terms of biomass production, although concerning lipid accumulation a difference was observed. In this sense, when SFM hydrolysate was used and the fermentation was stopped after 190 h, lipids were still accumulating.. Instead, PSFM hydrolysate reached a peak of lipid accumulation at 170 h of fermentation. This fact demonstrates that the nutrients extracted previously from the hydrolisate contribute to the lipid accumulation (Figure 4.3).

In sum, both media derived from agro-industrial waste used in fed batch fermentation showed good accumulation of lipids when the yeast C. curvatus was used. Even though, more studies regarding new fed batch strategies are necessary to increase the production of intracellular lipids.

Figure 4.3: Dry cells weight and lipid production of *C. curvatus* ATCC 20509 using glycerol and a) SFM hydrolysate and b) PSFM hydrolysate in fed batch mode. Red line means glycerol concentration; blue line means biomass production; purple line means oil content



4.3.3 Fatty acid composition

Fatty acid composition is an important parameter influencing the conversion of triglycerides into fatty acid methyl esters (FAME) [14, 15]. Generally, fatty acid composition of microbial oil obtained from yeast fermentation is very similar to that of palm oil, even though it presents more oleic acid and less palmitic acid (Table 4.4). As a result, microbial oil depicts a higher unsaturation degree (UD), which is recommended because it improves the economic viability of the transesterification process, reducing the optimal reaction temperature [16] and increasing glycerides conversion [15]. To gain knowledge about the influence of the fermentation time over the oil fatty acid composition, samples from each yeast and culture media considering different fermentation phase (growth and stationary phase) were analyzed. Results showed different behavior depending on the yeast. *C. curvatus* oil varied the presence of monounsaturated fatty acid. It may be inferred that the consumption of nutrients to increase the biomass leads this yeast to the production of shorter chain monounsaturated fatty acid (C16:1) instead of oleic acid (C18:1). On the other hand, when *L. starkeyi* reached the stationary phase of fermentation showed an increase in the production of monounsaturated fatty acids (oleic and palmitoleic acids), thus reducing the presence of saturated fatty acids (palmitic and stearic acids). Furthermore, for all tested yeasts, the amount of palmitoleic and oleic acids significantly increased. *R. toruloides* was the only yeast to show a decrease in oleic acid between growth and stationary phases, thus increasing palmitic and linoleic acids. Regarding the effect of each medium in fatty acid composition, differences in the use of the sources of nutrients with no clear trend was found.

Table 4.4: Comparison of fatty acid composition between microbial oils obtained in this study and palm oil

Vegetable oil									
	Reference	%C14:0	%C16:0	%C16:1	%C18:0	%C18:1	%C18:2	%C18:3	n.i
Palm oil	[17]	1.03	44.48	n.d	4.25	39.62	10.09	0.33	-
				C. curvatus					
	Phase								
Synthetic medium	Growth	0	27.59	3.48	4.09	44.62	12.79	7.44	0
	Stationary	0	28.64	3.26	4.99	49.94	13.18	0	0
SFM hydrolysate medium	Growth	0.44	30.34	3.12	6.26	46.09	12.77	0.98	0
	Stationary	0.44	30.21	2.51	5.76	48.44	10.36	0.65	1.5
PSFM hydrolysate medium	Growth	0	32.34	4.42	4.14	43.68	15.42	0	0
	Stationary	0	30.17	4.07	4.07	45.75	14.90	1.04	0
				L. starkeyi					
	Phase								
Synthetic medium	Growth	0	29.89	4.52	6.51	54.79	4.31	0	0
	Stationary	0	29.44	5.73	5.41	56.02	3.40	0	0

		%C14:0	%C16:0	%C16:1	%C18:0	%C18:1	%C18:2	%C18:3	n.i
				L. starkeyi					
	Phase								
	Stationary	0	29.29	4.84	7.20	56.22	2.46	0	0
PSFM hydrolysate medium	Growth	0	29.18	4.33	7.07	55.90	3.51	0	0
	Stationary	0	28.95	5.04	6.23	57.01	2.76	0	0
				R. toruloides					
	Phase								
Synthetic medium	Growth	1.84	32.01	1.42	6.78	49.57	7.78	0.60	0
	Stationary	1.92	32.33	1.27	6.86	48.52	8.34	0.77	0
SFM hydrolysate medium	Growth	1.73	32.07	1.67	6.48	52.76	5.29	0	0
	Stationary	1.94	32.87	1.74	8.98	48.68	5.79	0	0
PSFM hydrolysate medium	Growth	1.89	32.54	1.66	7.93	50.38	5.60	0	0
	Stationary	1.77	33.31	1.24	7.98	46.80	8.09	0.82	0

To analyze the influence of microbial oils on methyl esters properties, models based on fatty acid composition to predict cetane number [18], low calorific value [19], cold filter plugging point, CFPP [19], flash point [19] and viscosity [20] were used (Table 4.5). Cetane number is an important parameter related to the quality of the ignition. High cetane number values improve the combustion, resulting in low exhaust emissions, low noise and high engine performance. In this sense, NOx depict a negative correlation with cetane number. Provided that NOx emissions increase with the use of biodiesel, while the rest of exhaust emissions decrease, it is important to increase cetane number when biodiesel is used. Prediction of methyl esters properties indicate that all microbial oil samples depict high cetane number, similar to that of palm oil methyl esters, with the exception of *C. curvatus*, that showed lower value compared to other yeasts. This negative effect is compensated with improved cold properties behavior (CFPP), low viscosity and similar low calorific value compared to other methyl esters. Subsequently, the prediction of properties indicate that the proposed microbial oil biodiesel complies with the regulation EN 14214 concerning these properties, thus providing a better biodiesel compared to palm oil biodiesel in terms of lower viscosity.

Table 4.5: Predicted biodiesel properties based on fatty acid composition of the microbial oil produced by fermentation

Properties	Cetane number [18]	Low calorific value (kJ/kg) [19]	Cold filter plugging point (ºC) [19]	Flash point (ºC) [19]	Viscosity at 40ºC (mm^2/s) [20]
Synthetic medium					
R. toruloides	68.38	37517.86	7.691	167.160	4.35
C. curvatus	66.14	37529.95	5.507	167.841	4.24
L. starkeyi	68.17	37540.71	8.141	168.777	4.18
SFM hydrolysate medium					
R. toruloides	69.74	37533.72	9.581	168.432	4.37
C. curvatus	66.13	37402.81	4.650	159.330	4.20
L. starkeyi	68.88	37560.91	9.107	170.280	4.26
PSFM hydrolysate medium					
R. toruloides	68.88	37519.67	8.21	167.330	4.37
C. curvatus	65.45	37494.71	4.29	165.374	4.15
L. starkeyi	68.48	37533.90	9.29	168.415	4.23

Properties	Cetane number [18]	Low calorific value (kJ/kg) [19]	Cold filter plugging point (ºC) [19]	Flash point (ºC) [19]	Viscosity at 40ºC (mm^2/s) [20]
			EN 14214		
Palm oil	69.68	37442.56	8.23	162.159	4.40
	>51	-	-	<120	3.5-5.0

4.4 Conclusion

The revalorization of agro-industrial waste in the biodiesel industry has been demonstrated in this research. Appreciable amounts of oil can be obtained from oleaginous yeast fermentations, although each studied strain behaved differently under the same culture conditions, thus indicating the optimization of culture conditions (culture mode, temperature, etc.) for each yeast may improve lipid accumulation. Not only C/N ratio during the fermentation must be adequate for the culture medium, but the nutrient source from either waste or non-waste origin. The effect of isolation of antioxidants and proteins from the hydrolysate plays a significant role, because decreasing the biomass production and modifying the lipids content and the fatty acid composition of the oil. According to this study, the properties of the selected raw material for biodiesel production provide suitable fatty acid methyl esters that even improve palm oil biodiesel properties, currently being used in the biodiesel industry.

4.5 Reference

1. Dorado, M.P., et al., *An approach to the economics of two vegetable oil-based biofuels in Spain.* Renewable Energy, 2006. 31(8): p. 1231-1237.
2. Ageitos, J.M., et al., *Oily yeasts as oleaginous cell factories.* Applied Microbiology and Biotechnology, 2011. 90(4): p. 1219-1227.
3. Beopoulos, A., J.M. Nicaud, and C. Gaillardin, *An overview of lipid metabolism in yeasts and its impact on biotechnological processes.* Applied Microbiology and Biotechnology, 2011. 90(4): p. 1193-1206.
4. Li, Q., W. Du, and D. Liu, *Perspectives of microbial oils for biodiesel production.* Applied Microbiology and Biotechnology, 2008. 80(5): p. 749-756.
5. Saenge, C., et al., *Potential use of oleaginous red yeast Rhodotorula glutinis for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids.* Process Biochemistry, 2011. 46(1): p. 210-218.
6. Chatzifragkou, A., et al., *Biotechnological conversions of biodiesel derived waste glycerol by yeast and fungal species.* Energy, 2011. 36(2): p. 1097-1108.
7. Liang, Y.N., et al., *Converting crude glycerol derived from yellow grease to lipids through yeast fermentation.* Bioresource Technology, 2010. 101(19): p. 7581-7586.
8. Uckun Kiran, E., et al., *Enhancing the value of nitrogen from rapeseed meal for microbial oil production.* Enzyme and Microbial Technology, 2012. 50(6-7): p. 337-42.
9. Koutinas, A., et al., *Production of Fermentation Feedstock from Jerusalem Artichoke Tubers and its Potential for Polyhydroxybutyrate Synthesis.* Waste and Biomass Valorization, 2013. 4(2): p. 359-370.
10. Lie, S., *The EBC-ninhydrin method for determination of free alpha amino nitrogen* J. Inst. Brewery, 1973. 79: p. 37-41.
11. Papanikolaou, S., et al., *Kinetic profile of the cellular lipid composition in an oleaginous Yarrowia lipolytica capable of producing a cocoa-butter substitute*

from industrial fats. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 2001. 80(3-4): p. 215-224.

12. Galafassi, S., et al., *Lipid production for second generation biodiesel by the oleaginous yeast Rhodotorula graminis.* Bioresource Technology, 2012. 111(0): p. 398-403.

13. Uckun Kiran, E., A. Trzcinski, and C. Webb, *Microbial oil produced from biodiesel by-products could enhance overall production.* Bioresource Technology, 2013. 129(0): p. 650-654.

14. Knothe, G., *"Designer" Biodiesel: Optimizing Fatty Ester Composition to Improve Fuel Properties.* Energy & Fuels, 2008. 22(2): p. 1358-1364.

15. Pinzi, S., et al., *Influence of vegetable oils fatty acid composition on reaction temperature and glycerides conversion to biodiesel during transesterification.* Bioresource Technology, 2011. 102(2): p. 1044-1050.

16. Leiva-Candia, D.E., et al., *Influence of nitrogen fertilization on physical and chemical properties of fatty acid methyl esters from Brassica napus oil.* Fuel, 2013. 111: p. 865-871.

17. Pinzi, S., et al., *Response surface modeling to predict biodiesel yield in a multi-feedstock biodiesel production plant.* Bioresource Technology, 2010. 101(24): p. 9587-9593.

18. Ramos, M.J., et al., *Influence of fatty acid composition of raw materials on biodiesel properties.* Bioresource Technology, 2009. 100(1): p. 261-268.

19. Pinzi, S., et al., *Multiple response optimization of vegetable oils fatty acid composition to improve biodiesel physical properties.* Bioresource Technology, 2011. 102(15): p. 7280-7288.

20. Krisnangkura, K., T. Yimsuwan, and R. Pairintra, *An empirical approach in predicting biodiesel viscosity at various temperatures.* Fuel, 2006. 85(1): p. 107-113.

Conclusion and Future works

General conclusions and future lines of research

In this PhD thesis, results were obtained according to the technical feasibility of new raw materials for the production of biodiesel. Besides, the possibility of cost reduction in raw materials that are currently being used in the biodiesel industry was also studied. The most important conclusions are listed below.

Chapter 1:

- Biodiesel from non-edible sources such as animal fat, used oil, insect or single cell oil is an alternative biofuel, which omits the disadvantages caused by the use of vegetable oils as feedstock for the biodiesel industry.
- Microbial oil (also single cells oil), insect oil or microdiesel are promising feedstock for biodiesel production.
- The use of different agro-industrial residues to produce microbial oil from oleaginous yeast could lead to the production of a sustainable raw material for the biodiesel industry.

Chapter 2:

- The nitrogen fertilization affects some rapeseed oil properties, mainly acidity, peroxide value and fatty acid composition. Higher acidity and peroxide value have been observed when not basal fertilization was applied.
- No nitrogen fertilization provokes acidity increase in oil.

- Oleic and linolenic acid content increase, while linoleic acid content decreases when no fertilization during the growth of the oilseed is applied.
- No fertilization is applied during the crop growth, a rise of the optimal transesterification temperature and amount of KOH is reached, thereby increasing production costs.

Chapter 3:

- Glycerol, a by-product from biodiesel production, has been shown to be suitable as a carbon source for microbial oil production by many oleaginous yeasts.
- Crude glycerol combined with oilseed meal hydrolysate derived as by-products from current oilseed-based biodiesel plants could be used efficiently as raw material.
- An advantage of microbial oil compared to vegetable oil is that the fatty acid composition may be modified depending on the nutrient source.
- Concerning microbial oil fatty acid composition, it has been shown that it is similar to that of the main vegetable oils used in biodiesel production, e.g. palm oil.
- It has been found that the selection of the appropriate oleaginous microorganism depends on the particular agro-industrial waste to be used as substrate, as its composition is crucial in microbial oil production.

Chapter 4:

- Appreciable amounts of oil can be obtained from oleaginous yeast fermentations, although each studied strain behave differently under the same culture conditions, thus indicating the optimization of

culture conditions (culture mode, temperature, etc.) for each yeast may improve lipid accumulation.

- Not only the C/N ratio during the fermentation, but the nutrient source from either waste or non-waste origin, must be adequate for the culture medium.
- The effect of isolation of antioxidants and proteins from the hydrolysate plays a significant role in the production of lipids and fatty acid composition of the oil.

Future line of research:

As future prospects, an economic study including microbial oil production and further transesterification into biodiesel could help to establish whether this alternative may compete with traditional biodiesel industry. If microorganism genetic modification is combined with nutrients and growth conditions selection, microbial oil may constitute a realistic economically sound alternative to vegetable oil for biodiesel industry. In that sense, it is necessary to perform an optimization of culture conditions parameters for each microorganism used in this PhD thesis. Furthermore, in the study of cost reduction on nitrogen fertilization of rapeseed oil as feedstock to produce biodiesel, it is necessary to analyse the influence on exhaust emissions and engine performance for each dose of nitrogen fertilization.

References

References

A

- Ageitos, J.M., et al., Oily yeasts as oleaginous cell factories. Applied Microbiology and Biotechnology, 2011. 90(4): p. 1219-1227.
- Al-Zuhair, S., Production of biodiesel: possibilities and challenges. Biofuels Bioproducts & Biorefining, 2007. 1(1): p. 57-66.
- Alptekin, E. and M. Canakci, Optimization of pretreatment reaction for methyl ester production from chicken fat. Fuel, 2010. 89(12): p. 4035-4039.
- Alptekin, E., M. Canakci, and H. Sanli, Evaluation of leather industry wastes as a feedstock for biodiesel production. Fuel, 2012. 95(0): p. 214-220.
- Alvarez, H.M. and A. Steinbuchel, Triacylglycerols in prokaryotic microorganisms. Applied Microbiology and Biotechnology, 2002. 60(4): p. 367-376.
- Amaretti, A., et al., Single cell oils of the cold-adapted oleaginous yeast *Rhodotorula glacialis DBVPG 4785*. Microbial Cell Factories, 2010. 9.
- Andre, A., et al., Biotechnological conversions of bio-diesel derived crude glycerol by *Yarrowia lipolytica* strains. Engineering in Life Sciences, 2009. 9(6): p. 468-478.
- Andre, A., et al., Biotechnological conversions of bio-diesel derived waste glycerol into added-value compounds by higher fungi: production of biomass, single cell oil and oxalic acid. Industrial Crops and Products, 2010. 31(2): p. 407-416.
- Andre, A., et al., Biotechnological conversions of bio-diesel-derived crude glycerol by *Yarrowia lipolytica* strains. Engineering in Life Sciences, 2009. 9(6): p. 468-478.

- Angerbauer, C., et al., Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production. Bioresource Technology, 2008. 99(8): p. 3051-3056.
- Antizar-Ladislao, B. and J.L. Turrion-Gomez, Second-generation biofuels and local bioenergy systems. Biofuels Bioproducts & Biorefining, 2008. 2(5): p. 455-469.
- Araujo, B.Q., et al., Synthesis and characterization of beef tallow biodiesel. Energy & Fuels, 2010. 24: p. 4476-4480.
- Ayalasomayajula, S., et al., Potential of alligator fat as source of lipids for biodiesel production. Industrial & Engineering Chemistry Research, 2012. 51(4): p. 2166-2169.
- Azocar, L., et al., Biotechnological processes for biodiesel production using alternative oils. Applied Microbiology and Biotechnology, 2010. 88(3): p. 621-636.

B

- Balasubramanian, L., et al., Cyanobacteria cultivation in industrial wastewaters and biodiesel production from their biomass: A review. Biotechnology and Applied Biochemistry, 2012. 58(4): p. 220-225.
- Balat, M., Potential alternatives to edible oils for biodiesel production – A review of current work. Energy Conversion and Management, 2011. 52(2): p. 1479-1492.
- Barnard, D., et al., Extremophiles in biofuel synthesis. Environmental Technology, 2010. 31(8-9): p. 871-888.
- Beghin, J.C., et al. Food and agricultural policy research institute U.S. and world agricultural outlook. Staff Reports 2006.
- Bellou, S., et al., Lipids Containing Polyunsaturated Fatty Acids Synthesized by Zygomycetes Grown on Glycerol. Applied Biochemistry and Biotechnology, 2012. 166(1): p. 146-158.
- Belluco, S., et al., Edible Insects in a Food Safety and Nutritional Perspective: A Critical Review. Comprehensive

Reviews in Food Science and Food Safety, 2013. 12(3): p. 296-313.
- Beopoulos, A., J.M. Nicaud, and C. Gaillardin, An overview of lipid metabolism in yeasts and its impact on biotechnological processes. Applied Microbiology and Biotechnology, 2011. 90(4): p. 1193-1206.
- Berrios, M., et al., Application of the factorial design of experiments to biodiesel production from lard. Fuel Processing Technology, 2009. 90(12): p. 1447-1451.
- Birla, A., et al., Kinetics studies of synthesis of biodiesel from waste frying oil using a heterogeneous catalyst derived from snail shell. Bioresource Technology, 2012. 106(0): p. 95-100.
- Brennan, R.F., M.G. Mason, and G.H. Walton, Effect of nitrogen fertilizer on the concentrations of oil and protein in canola (*Brassica napus*) seed. Journal of Plant Nutrition, 2000. 23(3): p. 339-348.

C

- Canakci, M. and J. Van Gerpen, Biodiesel production from oils and fats with high free fatty acids. Transaction of the ASAE, 2003. 44: p. 1429–1436.
- Canakci, M., Sanli, H., Biodiesel production from various feedstocks and their effects on the fuel properties. Journal of Industrial Microbiological and Biotechnology, 2008. 35: p. 431-441.
- Cañizares-Villanueva, R.O., et al., Chemical composition of Cyanobacteria grown in diluted, aerated swine wastewater. Bioresource Technology, 1995. 51(2-3): p. 111-116.
- Charpe, T.W. and V.K. Rathod, Biodiesel production using waste frying oil. Waste Management, 2010. 31(1): p. 85-90.
- Chatzifragkou, A., et al., Biotechnological conversions of biodiesel derived waste glycerol by yeast and fungal species. Energy, 2011. 36(2): p. 1097-1108.
- Chi, Z.Y., et al., Lipid production by culturing oleaginous yeast and algae with food waste and municipal wastewater in an

integrated process. Applied Biochemistry and Biotechnology, 2011. 165(2): p. 442-453.
- Chi, Z.Y., et al., Oleaginous yeast *Cryptococcus curvatus* culture with dark fermentation hydrogen production effluent as feedstock for microbial lipid production. International Journal of Hydrogen Energy, 2011. 36(16): p. 9542-9550.
- Chiellini, E., et al., Biodegradable thermoplastic composites based on polyvinyl alcohol and algae. Biomacromolecules, 2008. 9(3): p. 1007-1013.
- Chisti, Y., Biodiesel from microalgae. Biotechnology Advances, 2010. 25(3): p. 294-306.
- Christenson, L. and R. Sims, Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. Biotechnology Advances, 2012. 29(6): p. 686-702.
- Christophe, G., et al., Production of oils from acetic acid by the oleaginous yeast *Cryptococcus curvatus.* Applied Biochemistry and Biotechnology, 2012. 167(5): p. 1270-1279.

D

- D'Arrigo, M., et al., Effect of dietary linseed oil and a-tocopherol on selected properties of pig fat. Canadian Journal of Animal Science, 2002. 82(3): p. 339-346.
- Deandres, C., et al., Cellular lipid-accumulation by *Pseudomonas aeruginosa* 44T1. Applied Microbiology and Biotechnology, 1991. 35(6): p. 813-816.
- Demirbas, A. and M. Fatih Demirbas, Importance of algae oil as a source of biodiesel. Energy Conversion and Management, 2010. 52(1): p. 163-170.
- Demirbas, A., Potential resources of non-edible oils for biodiesel. Energy Sources Part B, 2009. 4: p. 310-314.
- Demirbas, A., Use of algae as biofuel sources. Energy Conversion and Management, 2010. 51(12): p. 2738-2749.
- Deng, M.D. and J.R. Coleman, Ethanol synthesis by genetic engineering in Cyanobacteria. Applied and Environmental Microbiology, 1999. 65(2): p. 523-528.

- Deng, X., J. Han, and F. Yin, Net energy, CO2 emission and land-based cost-benefit analyses of *jatropha* biodiesel: A case study of the Panzhihua region of Sichuan province in China. Energies, 2012. 5(7): p. 2150-2164.
- Dexter, J. and P. Fu, Metabolic engineering of cyanobacteria for ethanol production. Energy & Environmental Science, 2009. 2(8): p. 857-864.
- Dey, P. and M.K. Maiti, Molecular characterization of a novel isolate of *Candida tropicalis* for enhanced lipid production. Journal of Applied Microbiology, 2013. 114(5): p. 1357-1368.
- Dias, J.M., M.C.M. Alvim-Ferraz, and M.F. Almeida, Production of biodiesel from acid waste lard. Bioresource Technology, 2009. 100(24): p. 6355-6361.
- Dorado, M.P., et al., An alkali-catalyzed transesterification process for high free fatty acid feedstocks. Transactions of ASAE, 2002. 45(3): p. 525-529.
- Dorado, M.P., et al., An approach to the economics of two vegetable oil-based biofuels in Spain. Renewable Energy, 2006. 31(8): p. 1231-1237.
- Dorado, M.P., et al., Exhaust emissions from a diesel engine fueled with transesterified waste olive oil. Fuel, 2003. 82(11): p. 1311-1315.
- Dorado, M.P., et al., Optimization of alkali-catalyzed transesterification of *Brassica carinata* oil for biodiesel production. Energy & Fuels, 2004. 18(1): p. 77-83.
- Dorado, M.P., et al., Testing waste olive oil methyl ester as a fuel in a diesel engine. Energy & Fuels, 2003. 17(6): p. 1560-1565.
- Dorado, M.P., Raw materials to produce low cost biodiesel. Chapter 4., in Biofuels refining and performance, A. Nag, Editor. 2008, McGraw Hill Professional. p. 107-148.
- Dunn, R.O., Effect of oxidation under accelerated conditions on fuel properties of methyl soyate (biodiesel). Journal of the American Oil Chemists Society, 2002. 79(9): p. 915-920.
- Durrett, T.P., C. Benning, and J. Ohlrogge, Plant triacylglycerols as feedstocks for the production of biofuels The Plant Journal, 2008. 54 (4): p. 593–607.

E

- Eberly, J.O., D.B. Ringelberg, and K.J. Indest, Physiological characterization of lipid accumulation and in vivo ester formation in *Gordonia* sp KTR9. Journal of Industrial Microbiology & Biotechnology, 2013. 40(2): p. 201-208.
- Economou, C.N., et al., Semi-solid state fermentation of sweet sorghum for the biotechnological production of single cell oil. Bioresource Technology, 2010. 101(4): p. 1385-1388.
- Economou, C.N., et al., Single cell oil production from rice hulls hydrolysate. Bioresource Technology, 2011. 102(20): p. 9737-9742.
- Egesel, C.O., M.K. Gul, and F. Kahriman, Changes in yield and seed quality traits in rapeseed genotypes by sulphur fertilization European Food Research and Technology, 2009. 229(5): p. 841-841.
- El Bialy, H., O.M. Gomaa, and K.S. Azab, Conversion of oil waste to valuable fatty acids using Oleaginous yeast. World Journal of Microbiology & Biotechnology, 2011. 27(12): p. 2791-2798.
- El Sabagh, S.M., S.T. Keera, and A.R. Taman, The characterization of biodiesel fuel from waste frying oil. Energy Sources Part a-Recovery Utilization and Environmental Effects, 2010. 33(5): p. 401-409.
- Enweremadu, C.C. and H.L. Rutto, Combustion, emission and engine performance characteristics of used cooking oil biodiesel-A review. Renewable and Sustainable Energy Reviews, 2010. 14(9): p. 2863-2873.
- EU, Regulation (EC) No 715/2007 of the European Parliament and of the Council of 20 June 2007 on type approval of motor vehicles with respect to emissions from light passenger and commercial vehicles (Euro 5 and Euro 6) 2007.

F

- Feddern, V., et al., Physico-chemical composition, fractionated glycerides and fatty acid profile of chicken skin fat. European Journal of Lipid Science and Technology, 2010. 112(11): p. 1277-1284.
- Fei, Q., et al., The effect of volatile fatty acids as a sole carbon source on lipid accumulation by *Cryptococcus albidus* for biodiesel production. Bioresource Technology, 2011. 102(3): p. 2695-2701.
- Fishinger, M., Biofuel from waste oils. 1980, Columbus, Ohio: Ohio State University.
- Fontanille, P., et al., Bioconversion of volatile fatty acids into lipids by the oleaginous yeast *Yarrowia lipolytica.* Bioresource Technology, 2012. 114: p. 443-9.
- Freedman, B. and M. Bagby, Heats of combustion of fatty esters and triglycerides. Journal of the American Oil Chemists Society, 1989. 66(11): p. 1601-1605.
- Freedman, B., E.H. Pryde, and T.L. Mounts, Variables affecting the yields of fatty esters from transesterified vegetable oils. Journal of the American Oil Chemists' Society, 1984. 61: p. 1638–1643.
- Fröhlich, A., B. Rice, and G. Vicente, The conversion of low grade tallow into biodiesel-grade methyl ester. Journal of the American Oil Chemists' Society, 2010. 87: p. 825-833.

G

- Galafassi, S., et al., Lipid production for second generation biodiesel by the oleaginous yeast *Rhodotorula graminis*. Bioresource Technology, 2012. 111(0): p. 398-403.
- Gao, D.F., et al., Microbial lipid production from xylose by *Mortierella isabellina.* Bioresource Technology, 2013. 133: p. 315-321.

- Gao, J., et al., Effects of manure and fertilizer applications on canola oil content and fatty acid composition. Agronomy Journal, 2010. 102(2): p. 790-797.
- Ghosh, P. and T.K. Ghose, Bioethanol in India: recent past and emerging future. Advances in biochemical engineering/biotechnology, 2003. 85: p. 1-27.
- Gong, Z.W., et al., Co-fermentation of cellobiose and xylose by *Lipomyces starkeyi* for lipid production. Bioresource Technology, 2012. 117: p. 20-24.
- Gonzalez-Delgado, A.-D. and V. Kafarov, Microalgae based biorefinery: issues to consider. Ct&F-Ciencia Tecnologia y Futuro, 2012. 4(4): p. 5-21.
- Gouda, M.K., S.H. Omar, and L.M. Aouad, Single cell oil production by *Gordonia* sp DG using agro-industrial wastes. World Journal of Microbiology & Biotechnology, 2008. 24(9): p. 1703-1711.
- Graboski, M.S., et al., The effect of biodiesel composition on engine emissions from a DDC series60 diesel engine, 2003, National Renewable Energy Laboratory: Golden, Colorado.
- Greenwell, H.C., et al., Placing microalgae on the biofuels priority list: a review of the technological challenges. Journal of the Royal Society Interface, 2010. 7(46): p. 703-726.
- Guan, G., K. Kusakabe, and S. Yamasaki, Tri-potassium phosphate as a solid catalyst for biodiesel production from waste cooking oil. Fuel Processing Technology, 2009. 90(4): p. 520-524.
- Gui, M.M., K.T. Lee, and S. Bhatia, Feasibility of edible oil vs. non-edible oil vs. waste edible oil as biodiesel feedstock. Energy, 2008. 33(11): p. 1646-1653.
- Guru, M., et al., Biodiesel production from waste animal fat and improvement of its characteristics by synthesized nickel and magnesium additive. Energy Conversion and Management, 2009. 50: p. 498-502.
- Guru, M., et al., Biodiesel production from waste chicken fat based sources and evaluation with Mg based additive in a diesel engine. Renewable Energy, 2010. 35: p. 637-643.

- Guzatto, R., et al., Transesterification double step process modification for ethyl ester biodiesel production from vegetable and waste oils. Fuel, 2012. 92(1): p. 197-203.

H

- Haas, M.J., S. Bloomer, and K. Scott, Simple, high-efficiency synthesis of fatty acid methyl esters from soapstock. Journal of the American Oil Chemists' Society, 2000. 77(4): p. 373-379.
- Halim, R., M.K. Danquah, and P.A. Webley, Extraction of oil from microalgae for biodiesel production: A review. Biotechnology Advances, 2012. 30(3): p. 709-732.
- Hall, D.O., et al., The potential applications of Cyanobacterial photosynthesis for clean technologies. Photosynthesis Research, 1995. 46(1): p. 159-167.
- Hall, J., et al., Oil production by a consortium of oleaginous microorganisms grown on primary effluent wastewater. Journal of Chemical Technology and Biotechnology, 2011. 86(1): p. 54-60.
- Harrington, K.J., Chemical and physical properties of vegetable oil esters and their effect on diesel fuel performance. Biomass, 1986. 9: p. 1-17.
- Harvey, A.P., M.R. Mackley, and T. Seliger, Process intensification of biodiesel production using a continuous oscillatory flow reactor. Journal of Chemical Technology & Biotechnology, 2003. 78(2-3): p. 338-341.
- Hingu, S.M., P.R. Gogate, and V.K. Rathod, Synthesis of biodiesel from waste cooking oil using sonochemical reactors. Ultrasonics Sonochemistry, 2010. 17(5): p. 827-832.
- Hu, C.M., et al., Effects of biomass hydrolysis by-products on oleaginous yeast *Rhodosporidium toruloides*. Bioresource Technology, 2009. 100(20): p. 4843-4847.
- Huang, C., et al., Oil production by the yeast *Trichosporon dermatis* cultured in enzymatic hydrolysates of corncobs. Bioresource Technology, 2012. 110: p. 711-4.

- Huang, L.H., et al., Application of fishmeal wastewater as a potential low-cost medium for lipid production by *Lipomyces starkeyi HL*. Environmental Technology, 2011. 32(16): p. 1975-1981.

I

- Ito, T., et al., Biodiesel production from waste animal fats using pyrolysis method. Fuel Processing Technology, 2012. 94(1): p. 47-52.

J

- Jacobson, K., et al., Solid acid catalyzed biodiesel production from waste cooking oil. Applied Catalysis B: Environmental, 2008. 85(1,2): p. 86-91.
- Jain, S., M.P. Sharma, and S. Rajvanshi, Acid base catalyzed transesterification kinetics of waste cooking oil. Fuel Processing Technology, 2010. 92(1): p. 32-38.
- Jayasinghe, P. and K. Hawboldt, A review of bio-oils from waste biomass: Focus on fish processing waste. Renewable & Sustainable Energy Reviews, 2012. 16(1): p. 798-821.

K

- Kalscheuer, R., T. Stolting, and A. Steinbuchel, Microdiesel: *Escherichia coli* engineered for fuel production. Microbiology-Sgm, 2006. 152: p. 2529-2536.
- Karatay, S.E. and G. Donmez, Improving the lipid accumulation properties of the yeast cells for biodiesel production using molasses. Bioresource Technology, 2010. 101(20): p. 7988-7990.

- Karimi, K. and A. Zamani, Mucor indicus: Biology and industrial application perspectives: A review. Biotechnology Advances, 2013. 31(4): p. 466-481.
- Keselman, H.J. and J.C. Rogan, A comparison of the modified-Tukey and Scheffe methods of multiple comparisons for pairwise contrasts. Journal of the American Statistical Association, 1978. 73(361): p. 47-52.
- Keskin, A., et al., Using of cotton oil soapstock biodiesel-diesel fuel blends as an alternative diesel fuel. Renewable & Sustainable Energy Reviews, 2008. 33: p. 553-557.
- Khanna, S., A. Goyal, and V.S. Moholkar, Microbial conversion of glycerol: present status and future prospects. Critical Reviews in Biotechnology, 2012. 32(3): p. 235-262.
- Khot, M., et al., Single cell oil of oleaginous fungi from the tropical mangrove wetlands as a potential feedstock for biodiesel. Microbial Cell Factories, 2012. 11.
- Kitcha, S. and B. Cheirsilp, Screening of oleaginous yeasts and optimization for lipid production using crude glycerol as a carbon source, in 9th Eco-Energy and Materials Science and Engineering Symposium, P.P. Yupapin, S. PivsaArt, and H. Ohgaki, Editors. 2011, Elsevier Science Bv: Amsterdam.
- Knothe, G. and K.R. Steidley, Kinematic viscosity of biodiesel fuel components and related compounds. Influence of compound structure and comparison to petrodiesel fuel components. Fuel, 2005. 84(9): p. 1059-1065.
- Knothe, G. and R.O. Dunn, Dependence of oil stability index of fatty compounds on their structure and concentration and presence of metals Journal of the American Oil Chemists Society, 2003. 72: p. 1155-1160.
- Knothe, G., "Designer" biodiesel: Optimizing fatty ester composition to improve fuel properties. Energy & Fuels, 2008. 22(2): p. 1358-1364.
- Knothe, G., A.C. Matheaus, and T.W. Ryan, Cetane numbers of branched and straight-chain fatty esters determined in an ignition quality tester. Fuel, 2003. 82(8): p. 971-975.

- Knothe, G., C.A. Sharp, and T.W. Ryan, Exhaust emissions of biodiesel, petrodiesel, neat methyl esters, and alkanes in a new technology engine. Energy & Fuels, 2006. 20(1): p. 403-408.
- Knothe, G., Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Processing Technology, 2005. 86(10): p. 1059-1070.
- Knothe, G., S.C. Cermak, and R.L. Evangelista, Cuphea oil as source of biodiesel with improved fuel properties caused by high content of methyl decanoate Energy & Fuels, 2009. 23(3): p. 1743-1747.
- Knothe, G., Some aspects of biodiesel oxidative stability. Fuel Processing Technology, 2007. 88(7): p. 669-677.
- Kondamudi, N., et al., A green process for producing biodiesel from feather meal. Journal of Agricultural and Food Chemistry, 2009. 57(14): p. 6163-6166.
- Kosa, M. and A.J. Ragauskas, Bioconversion of lignin model compounds with oleaginous *Rhodococci.* Applied Microbiology and Biotechnology, 2012. 93(2): p. 891-900.
- Kosa, M. and A.J. Ragauskas, Lipids from heterotrophic microbes: advances in metabolism research. Trends in Biotechnology, 2011. 29(2): p. 53-61.
- Koutinas, A., et al., Production of Fermentation Feedstock from Jerusalem Artichoke Tubers and its Potential for Polyhydroxybutyrate Synthesis. Waste and Biomass Valorization, 2013. 4(2): p. 359-370.
- Koutinas, A.A., et al., Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. Fuel, 2014. 116(0): p. 566-577.
- Krawczyk, T., Biodiesel, in International News on Fats, Oils and Related Materials. 1996, American Oil Chemists Society Press: Champaign, Illinois. p. 801.
- Krisnangkura, K., T. Yimsuwan, and R. Pairintra, An empirical approach in predicting biodiesel viscosity at various temperatures. Fuel, 2006. 85(1): p. 107-113.
- Kumar Tiwari, A., A. Kumar, and H. Raheman, Biodiesel production from *jatropha* oil (*Jatropha curcas*) with high free

fatty acids: An optimized process. Biomass and Bioenergy, 2007. 31(8): p. 569-575.
- Kumar, A., et al., Enhanced CO(2) fixation and biofuel production via microalgae: recent developments and future directions. Trends in Biotechnology, 2012. 28(7): p. 371-380.
- Kumar, A.K., P. Vatsyayan, and P. Goswami, Production of Lipid and Fatty Acids during Growth of *Aspergillus terreus* on Hydrocarbon Substrates. Applied Biochemistry and Biotechnology, 2010. 160(5): p. 1293-1300.

L

- Lam, M.K. and K.T. Lee, Microalgae biofuels: A critical review of issues, problems and the way forward. Biotechnology Advances, 2012. 30(3): p. 673-690.
- Lam, M.K. and K.T. Lee, Renewable and sustainable bioenergies production from palm oil mill effluent (POME): Win-win strategies toward better environmental protection. Biotechnology Advances, 2012. 29(1): p. 124-141.
- Lam, M.K., M.T. Lee, and A.R. Mohamed, Homogeneous, heterogeneous and enzymatic catalysis for transesterification of high free fatty acid oil (waste cooking oil) to biodiesel: A review. Biotechnology Advances, 2011. 28(4): p. 500-518.
- Lapuerta, M., J. Rodríguez-Fernández, and E.F. de Mora, Correlation for the estimation of the cetane number of biodiesel fuels and implications on the iodine number. Energy Policy, 2009. 37(11): p. 4337-4344.
- Lapuerta, M., J. Rodríguez-Fernández, and F. Oliva, Determination of enthalpy of formation of methyl and ethyl esters of fatty acids. Chemistry and Physics of Lipids, 2010. 163(2): p. 172-181.
- Lapuerta, M., J. Rodriguez-Fernandez, and J.R. Agudelo, Diesel particulate emissions from used cooking oil biodiesel. Bioresource Technology, 2008. 99(4): p. 731-740.

- Lapuerta, M., O. Armas, and J. Rodriguez-Fernandez, Effect of biodiesel fuels on diesel engine emissions Progress in Energy and Combustion Science, 2009. 35(4): p. 383-383.
- Latif Ahmad, A., S. Ismail, and S. Bhatia, Water recycling from palm oil mill effluent (POME) using membrane technology. Desalination, 2003. 157(1,3): p. 87-95.
- Lee, S., D. Posarac, and N. Ellis, An experimental investigation of biodiesel synthesis from waste canola oil using supercritical methanol. Fuel, 2012. 91(1): p. 229-237.
- Leiva-Candia, D.E., et al., Influence of nitrogen fertilization on physical and chemical properties of fatty acid methyl esters from *Brassica napus* oil. Fuel, 2013. 111(0): p. 865-871.
- Lertsathapornsuk, V., et al., Microwave assisted in continuous biodiesel production from waste frying palm oil and its performance in a 100-kW diesel generator. Fuel Processing Technology, 2008. 89(12): p. 1330-1336.
- Leung, D., et al., Biodiesel from *Zophobas morio* Larva Oil: Process Optimization and FAME Characterization. Industrial & Engineering Chemistry Research, 2012. 51(2): p. 1036-1040.
- Li, Q., et al., Bioconversion of dairy manure by black soldier fly (Diptera: *Stratiomyidae*) for biodiesel and sugar production. Waste Management, 2011. 31(6): p. 1316-1320.
- Li, Q., et al., From organic waste to biodiesel: Black soldier fly, *Hermetia illucens*, makes it feasible. Fuel, 2011. 90(4): p. 1545-1548.
- Li, Q., W. Du, and D. Liu, Perspectives of microbial oils for biodiesel production. Applied Microbiology and Biotechnology, 2008. 80(5): p. 749-756.
- Li, Y., et al., Using rice straw hydrolysate for microbial oil production by *Trichosporon fermentans HWZ004*. Chinese journal of biotechnology, 2011. 27(9): p. 1309-16.
- Li, Z., et al., *Chrysomya megacephala* (Fabricius) larvae: A new biodiesel resource. Applied Energy, 2012. 94(0): p. 349-354.
- Liang, Y.N., et al., Converting crude glycerol derived from yellow grease to lipids through yeast fermentation. Bioresource Technology, 2010. 101(19): p. 7581-7586.

- Liang, Y.N., et al., Lipid production from sweet sorghum bagasse through yeast fermentation. Renewable Energy, 2012. 40(1): p. 130-136.
- Lie, S., The EBC-ninhydrin method for determination of free alpha amino nitrogen J. Inst. Brewery, 1973. 79: p. 37-41.
- Lim, S.J., et al., Biological nutrient removal with volatile fatty acids from food wastes in sequencing batch reactor. Korean Journal of Chemical Engineering, 2008. 25(1): p. 129-133.
- Lin, L., et al., Opportunities and challenges for biodiesel fuel. Applied Energy, 2011. 88(4): p. 1020-1031.
- Liu, B. and Z. Zhao, Biodiesel production by direct methanolysis of oleaginous microbial biomass. Journal of Chemical Technology and Biotechnology, 2007. 82(8): p. 775-780.
- Liu, S.Y., et al., Fast biodiesel production from beef tallow with radio frequency heating. Renewable Energy, 2010. 36(3): p. 1003-1007.
- Liu, X.J., Y. Jiang, and F. Chen, Fatty acid profile of the edible filamentous Cyanobacterium nostoc flagelliforme at different temperatures and developmental stages in liquid suspension culture. Process Biochemistry, 2005. 40(1): p. 371-377.
- Lou, W.-Y., M.-H. Zong, and Z.-Q. Duan, Efficient production of biodiesel from high free fatty acid-containing waste oils using various carbohydrate-derived solid acid catalysts. Bioresource Technology, 2008. 99(18): p. 8752-8758.
- Lu, J., et al., Enzymatic synthesis of fatty acid methyl esters from lard with immobilized Candida sp. Process Biochemistry, 2007. 42: p. 1367-1370.
- Lu, X., H. Vora, and C. Khosla, Overproduction of free fatty acids in *E. coli*: Implications for biodiesel production. Metabolic Engineering, 2008. 10(6): p. 333-339.
- Lunin, V.V., et al., Biodiesel fuel production from lipids of filamentous fungi. Applied Biochemistry and Microbiology, 2013. 49(1): p. 46-52.
- Lv, X., et al., Variations in lipid yields and compositions of marine microalgae during cell growth and respiration, and

within intracellular structures. Journal of Experimental Marine Biology and Ecology, 2010. 391(1-2): p. 73-83.

M

- Ma, F., L.D. Clements, and M.A. Hanna, The effects of catalyst, free fatty acids and water on transesterification of beef tallow. Transaction of ASAE, 1998. 41: p. 1261–1264.
- Ma, F.R. and M.A. Hanna, Biodiesel production: a review. Bioresource Technology, 1999. 70(1): p. 1-15.
- Maceiras, R., et al., Effect of methanol content on enzymatic production of biodiesel from waste frying oil. Fuel, 2009. 88(11): p. 2130-2134.
- Manzano-Agugliaro, F., et al., Insects for biodiesel production. Renewable and Sustainable Energy Reviews, 2012. 16: p. 3744– 3753.
- Mata, T.M., A.A. Martins, and N.S. Caetano, Microalgae for biodiesel production and other applications: A review. Renewable and Sustainable Energy Reviews, 2012. 14(1): p. 217-232.
- Mattingly, B.G., Production of biodiesel from chicken fat containing free fatty acids. 2006, University of Arkansas.
- Mbaraka, I.K., K.J. McGuire, and B.H. Shanks, Acidic mesoporous silica for the catalytic conversion of fatty acids in beef tallow. Industrial & Engineering Chemistry Research, 2006. 45(9): p. 3022–3028.
- Mehta, P.S. and K. Anand, Estimation of a lower heating value of vegetable oil and biodiesel fuel. Energy & Fuels, 2009. 23(8): p. 3893-3898.
- Melero, J.A., et al., Zr-SBA-15 acid catalyst: Optimization of the synthesis and reaction conditions for biodiesel production from low-grade oils and fats. Catalysis Today, 2012. 195(1): p. 44-53.
- Meng, X., et al., Biodiesel production from oleaginous microorganisms. Renewable Energy, 2009. 34(1): p. 1-5.

- Mitra, D., et al., Value-added oil and animal feed production from corn-ethanol stillage using the oleaginous fungus *Mucor circinelloides*. Bioresource Technology, 2012. 107(0): p. 368-375.
- Mittelbach, M. and H. Enzelsberger, Transesterification of heated rapeseed oil for extending diesel fuel. Journal of the American Oil Chemists' Society, 1999. 76(5): p. 545–550.
- Mittelbach, M., B. Pokits, and A. Silberholz. Diesel fuels derived from vegetable oils, IV: Production and fuel properties of fatty acid methyl esters from used frying oil. in Liquid Fuels from Renewable Resources. Proceedings of an Alternative Energy Conference. 1992. Nashville, Tennesse (USA).
- Mondal, P., M. Basu, and N. Balasubramanian, Direct use of vegetable oil and animal fat as alternative fuel in internal combustion engine. Biofuels Bioproducts & Biorefining-Biofpr, 2008. 2(2): p. 155-174.
- Moser, B., Biodiesel production, properties, and feedstocks. In Vitro Cellular & Developmental Biology - Plant, 2009. 45(3): p. 229-266.
- Mosier, N., et al., Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology, 2005. 96(6): p. 673-686.
- Mulbry, W., et al., Recycling of manure nutrients: use of algal biomass from dairy manure treatment as a slow release fertilizer. Bioresource Technology, 2005. 96(4): p. 451-458.
- Muniraj, I.K., et al., Microbial lipid production from potato processing wastewater using oleaginous filamentous fungi *Aspergillus oryzae*. Water Research, 2013. 47(10): p. 3477-3483.

N

- Nebel, B.A. and M. Mittelbach, Biodiesel from extracted fat out of meat and bone meal. European Journal of Lipid Science Technology, 2006. 108: p. 398–403.

- Neste oil, the only way is forward, annual report 2012, N. oil, Editor. 2012. p. 131.
- Nye, M.J., et al., Conversion of used frying oil to diesel fuel by transesterification: preliminary tests. Journal of the American Oil Chemists' Society, 1983. 60(8): p. 1598–1601.

O

- Oner, C. and S. Altun, Biodiesel production from inedible animal tallow and an experimental investigation of its use as alternative fuel in a direct injection diesel engine. Applied Energy, 2009. 86: p. 2114-2120.

P

- Papanikolaou, S., et al., Kinetic profile of the cellular lipid composition in an oleaginous *Yarrowia lipolytica* capable of producing a cocoa-butter substitute from industrial fats. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 2001. 80(3-4): p. 215-224.
- Parmar, A., et al., Cyanobacteria and microalgae: A positive prospect for biofuels. Bioresource Technology, 2012. 102(22): p. 10163-10172.
- Patnayak, S. and A. Sree, Screening of bacterial associates of marine sponges for single cell oil and PUFA. Letters in Applied Microbiology, 2005. 40(5): p. 358-363.
- Pattamaprom, C., W. Pakdee, and S. Ngamjaroen, Storage degradation of palm-derived biodiesels: Its effects on chemical properties and engine performance. Renewable Energy, 2012. 37(1): p. 412-418.
- Pinzi, S. and M.P. Dorado, Feedstocks for advanced biodiesel production, in Advances in biodiesel production: Processes and technologies, R. Luque and J.A. Melero, Editors. 2012, Woodhead Cambridge, UK.

- Pinzi, S. and M.P. Dorado, Vegetable-based feedstocks for biofuels production, in Handbook of biofuels production: Processes and technologies, R. Luque, J.M. Campelo, and J.H. Clark, Editors. 2010, Woodhead Publishing Limited: Cambridge, UK.
- Pinzi, S., et al., Influence of vegetable oils fatty acid composition on reaction temperature and glycerides conversion to biodiesel during transesterification. Bioresource Technology, 2011. 102(2): p. 1044-1050.
- Pinzi, S., et al., Influence of vegetable oils fatty acid composition on reaction temperature and glycerides conversion to biodiesel during transesterification. Bioresource Technology, 2011. 102: p. 1044-1050.
- Pinzi, S., et al., Multiple response optimization of vegetable oils fatty acid composition to improve biodiesel physical properties. Bioresource Technology, 2011. 102(15): p. 7280-7288.
- Pinzi, S., et al., Response surface modeling to predict biodiesel yield in a multi-feedstock biodiesel production plant. Bioresource Technology, 2010. 101(24): p. 9587-9593.
- Pinzi, S., et al., The effect of biodiesel fatty acid composition on combustion and diesel engine exhaust emissions. Fuel, 2013. 104(0): p. 170-182.
- Pinzi, S., et al., The ideal vegetable oil-based biodiesel composition: a review of social, economical and technical implications. Energy & Fuels, 2009. 23: p. 2325–2341.
- Priego-Capote, F., J. Ruiz-Jimenez, and M.D. Luque de Castro, Identification and quantification of trans fatty acids in bakery products by gas chromatography-mass spectrometry after focused microwave Soxhlet extraction. Food Chemistry, 2007. 100(2): p. 859-867.

Q

- Quintana, N., et al., Renewable energy from cyanobacteria: energy production optimization by metabolic pathway

engineering. Applied Microbiology and Biotechnology, 2011. 91(3): p. 471-490.

R

- Raksakantong, P., et al., Fatty acids and proximate composition of eight Thai edible terricolous insects. Food Research International, 2010. 43(1): p. 350-355.
- Ramadhas, A.S., S. Jayaraj, and C. Muraleedharan, Biodiesel production from high FFA rubber seed oil. Fuel, 2005. 84(4): p. 335-340.
- Ramos-Elorduy, J., Energy supplied by edible insects from Mexico and their nutritional and ecological importance. Ecology of Food and Nutrition, 2008. 47(3): p. 280-297.
- Ramos, M.J., et al., Influence of fatty acid composition of raw materials on biodiesel properties. Bioresource Technology, 2009. 100(1): p. 261-268.
- Ravindran, B. and G. Sekaran, Bacterial composting of animal fleshing generated from tannery industries. Waste Management, 2012. 30(12): p. 2622-2630.
- Refaat, A.A. and S.T. El-Sheltawy. Comparing three options for biodiesel production from waste vegetable oil. in Waste management and the environment IV. International Conference on Waste Management and the Environment. 2008. Granada, Spain.
- Rittmann, B.E., Opportunities for renewable bioenergy using microorganisms. Biotechnology and Bioengineering, 2008. 100(2): p. 203-212.
- Rosenberg, J.N., et al., A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. Current Opinion in Biotechnology, 2008. 19(5): p. 430-436.
- Rottig, A., et al., Fatty acid alkyl esters: perspectives for production of alternative biofuels. Applied Microbiology and Biotechnology, 2011. 85(6): p. 1713-1733.

- Ruan, Z., et al., Evaluation of lipid accumulation from lignocellulosic sugars by Mortierella isabellina for biodiesel production. Bioresource Technology, 2012. 110(0): p. 198-205.
- Rucker, J., et al., Engineering *E. coli* for triglyceride accumulation through native and heterologous metabolic reactions. Applied Microbiology and Biotechnology, 2013. 97(6): p. 2753-2759.
- Ruenwai, R., S. Cheevadhanarak, and K. Laoteng, Overexpression of Acetyl-CoA Carboxylase Gene of *Mucor rouxii* Enhanced Fatty Acid Content in Hansenula polymorpha. Molecular Biotechnology, 2009. 42(3): p. 327-332.
- Rumpold, B.A. and O.K. Schluter, Nutritional composition and safety aspects of edible insects. Molecular Nutrition & Food Research, 2013. 57(5): p. 802-823.
- Ryu, B.-G., et al., High-cell-density cultivation of oleaginous yeast *Cryptococcus curvatus* for biodiesel production using organic waste from the brewery industry. Bioresource Technology, 2013. 135(0): p. 357-364.

S

- Sabudak, T. and M. Yildiz, Biodiesel production from waste frying oils and its quality control. Waste Management, 2011. 30(5): p. 799-803.
- Saenge, C., et al., Efficient concomitant production of lipids and carotenoids by oleaginous red yeast *Rhodotorula glutinis* cultured in palm oil mill effluent and application of lipids for biodiesel production. Biotechnology and Bioprocess Engineering, 2011. 16(1): p. 23-33.
- Saenge, C., et al., Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. Process Biochemistry, 2011. 46(1): p. 210-218.
- Sahoo, P.K., et al., Biodiesel development from high acid value polanga seed oil and performance evaluation in a CI engine. Fuel, 2006. 86 p. 448-454.

- Sanford, S.D., et al., Feedstock and Biodiesel Characteristics Report. 2009, Renewable Energy Group.
- Sankh, S., et al., Biodiesel production from a newly isolated Pichia kudriavzevii strain. Fuel, 2013. 106(0): p. 690-696.
- Sarin, A., et al., Blends of biodiesels synthesized from non-edible and edible oils: Effects on the cold filter plugging point. Energy & Fuels, 2010. 24: p. 1996–2001.
- Schönborn, A., et al., The influence of molecular structure of fatty acid monoalkyl esters on diesel combustion. Combustion and Flame, 2009. 156(7): p. 1396-1412.
- Schulte, W.B., Biodiesel production from tall oil and chicken fat via supercritical methanol treatment. 2007, University of Arkansas.
- Schütz, K., et al., Cyanobacterial H2 production — a comparative analysis. Planta, 2004. 218(3): p. 350-359.
- Sheehan, J., et al., A look back at the US Department of Energy's aquatic species program—biodiesel from algae. 1998, National Renewable Energy Laboratory: Golden, CO.
- Shi, S.B., et al., Prospects for microbial biodiesel production. Biotechnology Journal, 2011. 6(3): p. 277-285.
- Shin, H.Y., et al., Biodiesel production from waste lard using supercritical methanol. Journal of Supercritical Fluids, 2012. 61: p. 134-138.
- Singh, J. and S. Cu, Commercialization potential of microalgae for biofuels production. Renewable & Sustainable Energy Reviews, 2010. 14(9): p. 2596-2610.
- Sivakumar, P., K. Anbarasu, and S. Renganathan, Bio-diesel production by alkali catalyzed transesterification of dairy waste scum. Fuel, 2010. 90(1): p. 147-151.
- Sorrell, S., et al. An assessment of the evidence for a near-term peak in global oil production. 2009
- St-Hilaire, S., et al., Fish Offal Recycling by the Black Soldier Fly Produces a Foodstuff High in Omega-3 Fatty Acids. Journal of the World Aquaculture Society, 2007. 38(2): p. 309-313.

- Subramaniam, R., et al., Microbial lipids from renewable resources: production and characterization. Journal of Industrial Microbiology & Biotechnology, 2010. 37(12): p. 1271-1287.

T

- Thanh, L., et al., A two-step continuous ultrasound assisted production of biodiesel fuel from waste cooking oils: A practical and economical approach to produce high quality biodiesel fuel. Bioresource Technology, 2010. 101(14): p. 5394-5401.
- Tsukahara, K. and S. Sawayama, Liquid fuel production using microalgae. Journal of the Japan Petroleum Institute, 2005. 48: p. 251–259.
- Taha, E.M., et al., Lipid biosynthesis in *Cunninghamella bainieri* 2A1 in N-limited and N-excess media. Annals of Microbiology, 2010. 60(4): p. 615-622.
- Tamagnini, P., et al., Cyanobacterial hydrogenases: diversity, regulation and applications. FEMS Microbiology Reviews, 2007. 31(6): p. 692-720.
- Tutunea, D., Thermal investigation of biodiesel blends derived from rapeseed oil. Journal of Thermal Analysis and Calorimetry, 2013. 111(1): p. 869-875.
- Takakuwa, N. and K. Saito, Conversion of beet molasses and cheese whey into fatty acid methyl esters by the yeast *Cryptococcus curvatus*. Journal of Oleo Science, 2010. **59**(5): p. 255-260.
- Thiru, M., S. Sankh, and V. Rangaswamy, Process for biodiesel production from *Cryptococcus curvatus*. Bioresource Technology, 2011. 102(22): p. 10436-10440.
- Tsigie, Y.A., et al., Lipid production from *Yarrowia lipolytica* Po1g grown in sugarcane bagasse hydrolysate. Bioresource Technology, 2011. 102(19): p. 9216-9222.

U

- US imports threaten EU biodiesel. Biofuels Bioproducts & Biorefining, 2008. 2(1): p. 4-4.
- Usta, N., et al., Combustion of biodiesel fuel produced from hazelnut soapstock/waste sunflower oil mixture in a diesel engine. Energy Conversion and Management, 2005. 46: p. 741-755.
- Uckun Kiran, E., A. Trzcinski, and C. Webb, Microbial oil produced from biodiesel by-products could enhance overall production. Bioresource Technology, 2013. 129(0): p. 650-654.
- Uckun Kiran, E., et al., Enhancing the value of nitrogen from rapeseed meal for microbial oil production. Enzyme and Microbial Technology, 2012. 50(6-7): p. 337-42.

V

- Vicente, G., et al., Biodiesel production from biomass of an oleaginous fungus. Biochemical Engineering Journal, 2009. 48(1): p. 22-27.
- Venkata Subhash, G. and S. Venkata Mohan, Biodiesel production from isolated oleaginous fungi *Aspergillus* sp. using corncob waste liquor as a substrate. Bioresource Technology, 2011. 102(19): p. 9286-9290.
- Vongsangnak, W., et al., Genome-scale analysis of the metabolic networks of oleaginous *Zygomycete* fungi. Gene, 2013. 521(1): p. 180-190.
- Vyas, A.P., J.L. Verma, and N. Subrahmanyam, A review on FAME production processes. Fuel, 2010. 89(1): p. 1-9.

W

- Wahlen, B.D., et al., Biodiesel from Microalgae, Yeast, and Bacteria: Engine Performance and Exhaust Emissions. Energy & Fuels, 2012. 27(1): p. 220-228.
- Walsh, K., G.J. Jones, and R. Hugh Dunstan, Effect of irradiance on fatty acid, carotenoid, total protein composition and growth of *Microcystis aeruginosa.* Phytochemistry, 1997. 44(5): p. 817-824.
- Wan Omar, W.N.N. and N.A.S. Amin, Biodiesel production from waste cooking oil over alkaline modified zirconia catalyst. Fuel Processing Technology, 2012. 92(12): p. 2397-2405.
- Wang, J.-X., et al., Lipase-catalyzed production of biodiesel from high acid value waste oil using ultrasonic assistant. Chinese Journal of Biotechnology, 2007. 23(6): p. 1121-1128.
- Wang, Q., et al., Lipid production from hydrolysate of cassava starch by *Rhodosporidium toruloides* 21167 for biodiesel making. Renewable Energy, 2012. **46**: p. 164-168.
- Wang, R.H., et al., Bioconversion of rapeseed meal for the production of a generic microbial feedstock. Enzyme and Microbial Technology, 2010. **47**(3): p. 77-83.
- Wang, W., et al., Effect of structural changes on enzymatic hydrolysis of eucalyptus, sweet sorghum bagasse, and sugarcane bagasse after liquid hot water pretreatment. Bioresources, 2012. **7**(2): p. 2469-2482.
- Wang, Y., et al., Comparison of two different processes to synthesize biodiesel by waste cooking oil. Journal of Molecular Catalysis A: Chemical, 2006. 252: p. 107–112.
- Weselake, R.J., et al., Increasing the flow of carbon into seed oil. Biotechnology Advances, 2009. 27(6): p. 866-878.
- Whipple, T. Peak oil review. in International peak oil conference 2009. Denver, Colorado: Asociation for the Study of Peak Oil and Gas (ASPO).
- Wijffels, R.H., M.J. Barbosa, and M.H.M. Eppink, Microalgae for the production of bulk chemicals and biofuels. Biofuels Bioproducts & Biorefining, 2010. 4(3): p. 287-295.

- Wu, S.G., et al., Phosphate-limitation mediated lipid production by *Rhodosporidium toruloides*. Bioresource Technology, 2010. 101(15): p. 6124-6129.
- Wynn, J.P., A.B.A. Hamid, and C. Ratledge, The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi. Microbiology-Uk, 1999. 145: p. 1911-1917.

X

- Xia, C.J., et al., A new cultivation method for microbial oil production: cell pelletization and lipid accumulation by *Mucor circinelloides*. Biotechnology for Biofuels, 2011. 4.
- Xu, H., X. Miao, and Q. Wu, High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. Journal of Biotechnology, 2006. 126(4): p. 499-507.
- Xu, J.Y., et al., Microbial conversion of biodiesel byproduct glycerol to triacylglycerols by oleaginous yeast *Rhodosporidium toruloides* and the individual effect of some impurities on lipid production. Biochemical Engineering Journal, 2012. 65: p. 30-36.
- Xu, J.Y., et al., Microbial oil production from various carbon sources and its use for biodiesel preparation. Biofuels Bioproducts & Biorefining-Biofpr, 2013. 7(1): p. 65-77.

Y

- Yen, H.-W. and D.E. Brune, Anaerobic co-digestion of algal sludge and waste paper to produce methane. Bioresource Technology, 2007. 98(1): p. 130-134.
- Yen, H.-W., Y.-C. Yang, and Y.-H. Yu, Using crude glycerol and thin stillage for the production of microbial lipids through the cultivation of *Rhodotorula glutinis*. Journal of Bioscience and Bioengineering, 2012. 114(4): p. 453-6.

- Yousuf, A., Biodiesel from lignocellulosic biomass-prospects and challenges. Waste Management, 2012. 32(11): p. 2061-2067.
- Yousuf, A., et al., Microbial conversion of olive oil mill wastewaters into lipids suitable for biodiesel production. Journal of Agricultural and Food Chemistry, 2010. 58(15): p. 8630-8635.
- Yu, X.C., et al., Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. Bioresource Technology, 2011. 102(10): p. 6134-6140.

Z

- Zeng, J., et al., Lignocellulosic biomass as a carbohydrate source for lipid production by *Mortierella isabellina*. Bioresource Technology, 2013. 128(0): p. 385-391.
- Zhang, G.C., et al., Microbial lipid production as biodiesel feedstock from N-acetylglucosamine by oleaginous microorganisms. Journal of Chemical Technology and Biotechnology, 2011. 86(5): p. 642-650.
- Zhang, Y., et al., Biodiesel production from waste cooking oil: 1. Process design and technological assessment. Bioresource Technology, 2003. 89(1): p. 1-16.
- Zhao, X., et al., Lipid production by *Rhodosporidium toruloides* Y4 using different substrate feeding strategies. Journal of Industrial Microbiology & Biotechnology, 2011. 38(5): p. 627-632.
- Zhao, X., et al., Lipid production from Jerusalem artichoke by *Rhodosporidium toruloides Y4*. Journal of Industrial Microbiology & Biotechnology, 2010. 37(6): p. 581-585.
- Zhao, X.B., et al., Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast *Rhodosporidium toruloides* and preparation of biodiesel by enzymatic transesterification of the lipid. Bioprocess and Biosystems Engineering, 2012. 35(6): p. 993-1004.

- Zheng, L., et al., Biodiesel production from rice straw and restaurant waste employing black soldier fly assisted by microbes. Energy, 2012. 47(1): p. 225-229.
- Zheng, L., et al., Exploring the potential of grease from yellow mealworm beetle (*Tenebrio molitor*) as a novel biodiesel feedstock. Applied Energy, 2013. 101(0): p. 618-621
- Zheng, Y.B., et al., Feasibility of filamentous fungi for biofuel production using hydrolysate from dilute sulfuric acid pretreatment of wheat straw. Biotechnology for Biofuels, 2012. 5.
- Zikou, E., et al., Evaluating glucose and xylose as cosubstrates for lipid accumulation and γ-linolenic acid biosynthesis of *Thamnidium elegans.* Journal of Applied Microbiology, 2013. 114(4): p. 1020-1032.


Universidad de Córdoba
UNIVERSIDAD
D
CORDOBA
SAHE