UNIVERSITY OF NAPLES FEDERICO II

SCHOOL OF POLYTECHNIC AND BASIC SCIENCES

Department of Chemical, Materials and Production Engineering

PhD in

INDUSTRIAL PRODUCT AND PROCESS ENGINEERING

Innovative methodologies for the production of microbial oils and biodiesel

PhD thesis

SUPERVISOR:

PROF. DOMENICO PIROZZI

PHD STUDENT:

FELICIA RUGARI

XXIX CYCLE

ABSTRACT

Nowadays research has focused on new energy sources to limit the use of traditional fossil fuels and promote the development of biorefineries. In this context, an important goal is the exploitation of lignocellulosic materials obtained from agro-forestry wastes for the synthesis of II-generation biofuels. Among them, one of the most relevant is biodiesel, obtained using as feedstock the lipids extracted from the oleaginous microorganisms such as yeasts, produced by fermentation in hydrolysates of lignocellulosic biomass. These oils can be transformed in biodiesel performing a transesterification reaction.

The production process is still too much expensive and not completely sustainable, due to several energy-intensive steps involved and the use of toxic solvents for the extraction.

The aim of this study is to overcome some of the issues bound to the traditional production process of microbial oils and biodiesel, introducing some innovative techniques, such as microwaves and pulsed electric fields. The latter have been already explored in different branches of research, from the food industry to the medicine and in this thesis have been applied on *Lipomyces starkeyi* and *Metschnikowia pulcherrima* samples.

The main variables investigated during a microwave extraction are the solvent choice, the temperature and the time of extraction, in comparison to the conventional extraction. ^{1}H-NMR analysis were made to characterize the lipid profile of the extracted oils.

Furthermore, some direct transesterification tests were conducted and the fatty acids methyl esters obtained are analyzed by gas-chromatography.

Concerning to the pulsed electric fields, confocal microscopy has been utilized to investigate the possibility of using this treatment on the yeast's cell and to understand the effects of some variables such as the concentration of the cell suspension on the efficiency of the application.

The results obtained pave the way to the opportunity of using those innovative approaches on an industrial scale.

RIASSUNTO

Al giorno d'oggi la ricerca si è concentrata su nuove fonti di energia per limitare l'uso dei combustibili fossili tradizionali e promuovere lo sviluppo di bioraffinerie. In questo contesto, un obiettivo importante è lo sfruttamento di materiali lignocellulosici ottenuti da rifiuti agroforestali per la sintesi di biocarburanti di II generazione. Tra di essi, uno dei più rilevanti è il biodiesel, ottenuto utilizzando come materia prima i lipidi estratti dai microrganismi oleaginosi quali i lieviti, prodotti dalla fermentazione in idrolizzati di biomassa lignocellulosica. Questi oli possono essere trasformati in biodiesel a seguito di una reazione di transesterificazione.

Il processo di produzione è ancora troppo costoso e non completamente sostenibile, a causa di diverse fasi produttive costose dal punto di vista energetico e l'uso di solventi tossici per l'estrazione.

Lo scopo di questo studio è di superare alcuni dei problemi legati al tradizionale processo di produzione di oli microbici e biodiesel, introducendo alcune tecniche innovative, come le microonde e i campi elettrici pulsati. Queste ultime sono state già esplorate in diversi rami della ricerca, dall'industria alimentare alla medicina e in tale lavoro di tesi sono state applicate su campioni di *Lipomyces starkeyi* e *Metschnikowia pulcherrima*.

Le principali variabili indagate durante l'estrazione all'interno di un reattore a microonde sono la scelta del solvente, la temperatura e il tempo di estrazione, rispetto all'estrazione convenzionale. Per caratterizzare il profilo lipidico degli oli estratti sono state condotte analisi ^{1}H-NMR.

Inoltre, sono state effettuate alcune prove di transesterificazione diretta e gli esteri metilici degli acidi grassi ottenuti sono stati analizzati mediante gas-cromatografia.

Per quanto concerne la tecnica dei campi elettrici pulsati, la microscopia confocale è stata utilizzata per esaminare la possibilità di sottoporre le cellule di lievito a questo trattamento e per comprendere gli effetti di alcune variabili come la concentrazione della sospensione cellulare sull'efficienza dell'applicazione.

I risultati ottenuti aprono la strada alla possibilità di utilizzare questi approcci innovativi su scala industriale.

CONTENTS

LIST OF ABBREVIATIONS

^{1}H-NMR	Proton nuclear magnetic resonance
2Me-THF	2-Methyltetrahydrofuran
ASTM	American Society for Testing and Materials International
B100	blend of 100% biodiesel
B20	blend of 20% biodiesel and 80% diesel
B40	blend of 40% biodiesel and 60% diesel
CH	conventional heating
C_{ME}	conversion to biodiesel
EPA U.S.	Environmental Protection Agency
ER	endoplasmic reticulum
EtOH	ethanol
FAME	fatty acid methyl ester
FFA	free fatty acid
GC	Gas chromatography
LCA	Life cycle assessment
LD	lipid droplet
Ls	*Lipomyces starkeyi*
MAE	microwave-assisted extraction
MeOH	methanol
MIBK	methyl isobutyl ketone
MpA	*Metschnikowia pulcherrima* cultivated in condition A
MpB	*Metschnikowia pulcherrima* cultivated in condition B
MW	microwave heating
OD	optical density
Pa	absorbed power
PBS	phosphate-buffer solution
PEF	pulsed electric field
Pi	incident power
PI	propidium iodide
Pr	reflected power
PUFA	polyunsaturated fatty acid

RF	radio frequency
SAR	specific absorption rate
SCO	single cell oil
SE	steryl esters
SMP	standard *Metschnikowia pulcherrima*
TAG/TG	triacylglycerol/triglycerides
TFA	total fatty acid
WG	waveguide
wt%	percentage by weight
ε''	imaginary part of permittivity
ε'	real part of permittivity
ρ	density
σ	electrical conductivity

1. INTRODUCTION

A primary focus of nowadays research is finding alternative energy sources, due to the finite nature of the conventional ones. In particular, the transport sector is one of the most affected areas. In this context, therefore, biofuels represent a sustainable alternative to fossil fuels.

The first biofuels ("first generation") appeared on the market used vegetable oils (soybean, sunflower, rapeseed) as a source of triglycerides (TG). However this method of production is no longer sustainable nor ethically acceptable, due to the use of raw materials in competition with the food industry ones. For this reason, research has turned to the study of so-called "second-generation biofuels", those which use agro-forestry waste and organic fraction of municipal solid wastes as a starting feedstock (Naik *et al.,* 2010).

One of the most important biofuel is biodiesel, defined by ASTM (American Society for Testing and Materials International) as a "fuel comprised of mono-alkyl esters of long chain fatty acids derived from vegetable oils or animal fats, designated B100".

Biodiesel is a non-toxic and highly biodegradable fuel, which can be used for haulage purposes without further treatments, due to its low viscosity not dangerous for the engine (Behçet *et al.*, 2015).

The high cost of biodiesel discourages its use, therefore mixtures (biodiesel blends) are usually preferred, such as B20, formed by conventional diesel and 20% of biodiesel as specified by EPA.

EN 14214 contains requirements and test methods to which biodiesel must be submitted.

Compared to the traditional diesel, biodiesel generates lower emissions of carbon monoxide, unburned hydrocarbons, particulate matters (Van Gerpen, 2005). According to Sarma *et al.,* (2005) SO_2 emission on combustion are also reduced. However it does not offer any advantages regarding the NOx emissions and in some case it could even result in an increase, as underlined by Hoekman & Robbins (2012).

The real challenge of scientists is the development of a biodiesel production system that is actually competitive from the economic point of view compared to fossil fuels and sustainable for the environment.

2. BACKGROUND

2.1 OLEAGINOUS MICROORGANISMS

Oleaginous microorganisms, such as microalgae, bacteria, yeasts and other fungi are capable of accumulating lipids, in the form of triglycerides, above 20% of their biomass, on dry basis (Rossi *et al.*, 2011).

Lipids produced by them, also known as "single cell oils" have an important economic interest because they represent a real alternative to vegetable and animal oils (Li *et al.*, 2008) and can be used for human consumption as nutraceuticals or for biofuels production (Ratledge, 2013).

Oleaginous yeasts represent only a small portion of the large population of existing yeasts: only 5% is capable of accumulating more than 25% of lipids. The oily yeasts include *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon*, and *Lipomyces* (Ageitos *et al.*, 2011).

Compared to the algae, yeasts show several advantages such as a faster growth, a minor susceptibility to viral infections and the possibility of controlling bacterial contamination by providing culture conditions characterized by low pH values (Sitepu *et al.*, 2014). In addition, yeasts have a short production cycle and are not subject to seasonal variations (Rossi *et al.*, 2011).

The biodiesel production process from yeast is a sustainable method, especially if it is based on the use of carbon sources from raw materials, by-products or wastes.

Angerbauer *et al.* (2008) demonstrated that raw sewage sludge, if pretreated by ultrasounds could be a good substrate for *Lipomyces starkeyi.* Matsakas *et al.* (2015) investigated the possibility of using sweet sorghum as a biomass feedstock for samples of *Rhodosporidium toruloides*. Whereas Karatay & Dönmez (2010) studied lipid accumulation of *Candida l.*, *Candida t.* and *Rhodotorula m.* cells grown in molasses medium.

Concerning to *M. pulcherrima*, Chatzifragkou *et al.* (2011) concluded that this strain is not oleaginous. However, when subjected to environmental stress such as nitrogen starvation, this yeast strain showed some evidence of lipid accumulation (Pan *et al.*, 2009). Santomauro *et al.* (2014) demonstrated that *M. pulcherrima* is capable of producing up to 40% lipid by manipulating pH and temperature, under non-sterile conditions.

Bacteria can also accumulate lipids, even if at lower concentrations than other oleaginous microorganisms. However they are characterized by a very high specific growth rate (12-24 h) and can be cultivated in simple conditions (Shi *et al.,* 2011).

At present, biofuels are not competitive with conventional fuels from the economical point of view. It is very difficult to estimate the cost of single cell oils. Algae, for instance, as suggested by Sitepu *et al.* (2014) can be cultivated according to different methodologies (photoautrophic, heterotrophic or mixotrophic metabolism) and in several kind of reactors (raceway ponds, enclosed photobioreactors and dark fermentors). All of these options make the economic calculations very hard to be done.

Concerning the yeasts, Ratledge and Cohen (2008) concluded that the price of SCO would be US$3000/tonn (excluding the costs of extracting the oil and also the cost of the feedstock) which was twice the cost of oils produced by plants.

Huang *et al.* (2013) also studied the production cost of SCO from lignocellulosic biomass and suggested to focus on lipid accumulation process and chemical methods, especially hydrolysis and pretreatment technologies to reduce the price and to make it economically viable.

With the aim of making the production more attractive for industrial applications, Sitepu *et al.* (2014) stated that biodiesel must be considered as "one member of a portfolio of high value coproducts", such as texturized protein, lecithin and sterols.

Developing high lipid content microorganisms or engineered strains could be a promising way to produce biodiesel in the future (Meng *et al.*, 2009).

In Table 2-1 oil contents of the most important oleaginous microorganisms are shown.

Table 2-1 Oil content of some microorganisms (Meng *et al.*, 2009)

Microorganisms	Oil content (% dry wt)	Microorganisms	Oil content (% dry wt)
Microalgae		**Yeast**	
Botryococcus braunii	25–75	*Candida curvata*	58
Cylindrotheca sp.	16–37	*Cryptococcus albidus*	65
Nitzschia sp.	45–47	*Lipomyces starkeyi*	64
Schizochytrium sp.	50–77	*Rhodotorula glutinis*	72
Bacterium		**Fungi**	
Arthrobacter sp.	>40	*Aspergillus oryzae*	57
Acinetobacter calcoaceticus	27–38	*Mortierella isabellina*	86
Rhodococcus opacus	24–25	*Humicola lanuginosa*	75
Bacillus alcalophilus	18–24	*Mortierella vinacea*	66

2.1.1 LIPID ACCUMULATION

Lipid accumulation is a phenomenon that starts during *de novo* synthesis when a primary nutrient exhaust from the culture medium, usually nitrogen. Due to the lack of nitrogen, that is essential for protein and nucleic acid syntheses, cell proliferation stops but the organism continues to assimilate the carbon source. Carbon is involved into lipid synthesis and the results is the storage of triacylglycerols within the cell as discrete lipid bodies. This phenomena happens only for oleaginous microorganisms. The others, in the same nutrient-limiting medium stop cell proliferation or, if they continue to assimilate the available carbohydrate substrate, start producing various polysaccharides, including glycogen and various glucans and mannans. By contrast, lipid accumulation from hydrophobic substrates during primary metabolic growth is independent of the ammonium concentration in the medium (Papanikolaou *et al.*, 2003; Beopoulos *et al.*, 2009; Rossi *et al.,* 2011).

Culture broths are usually characterized by a high carbon-to-nitrogen ratio (C/N). In such conditions the cells' proliferation is prevented, while the accumulation of lipids is favored (Meng *et al.*, 2009; Wild *et al.,* 2010).

The yield of oil strongly depends on the species and the culture conditions, such as pH, salinity, and the sources of nitrogen (Li *et al.*, 2008).

The main fatty acids of the microbial oils are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3), which is similar to that of vegetable oils and is species dependent (Xu *et al.*, 2012). Classification of microorganisms is usually made according to their lipid composition (Lomascolo *et al.,* 1994).

The phenomenon of lipid biogenesis is not well understood yet, as underlined by Athenstaedt & Daum, (2006) who reviewed the life cycle of neutral lipids.

The surface monolayer of lipid droplets (LD) is probably formed starting from the endoplasmic reticulum (ER). The lensing and the bicell formation models, useful to explain the mechanism of lipid biogeneis, have in common the idea of TAG accumulation between the two membrane leaflets of the ER. LD grow until a critical size and then are able to get off from the ER membrane (Fig. 2.1). According to the lensing model, the phospholipid monolayer of LD may develop from the outer leaflet of the ER bilayer membrane, whereas bicell formation from both leaflets (Koch *et al.,* 2014).

Autotrophic microalgae use carbon dioxide as the carbon source and sunlight as the energy source, whereas heterotrophic microalgae can also accumulate oils with organic carbon as the carbon source (Martinez *et al.,* 2015).

In the case of bacteria, biosynthesis of TAG seems to be a common feature of those belonging to the actinomycetes group. These latter can accumulate up to 70% of their biomass as lipids. The accumulation happens during the stationary phase of growth when proteins are not being synthesized (Martinez *et al.,* 2015).

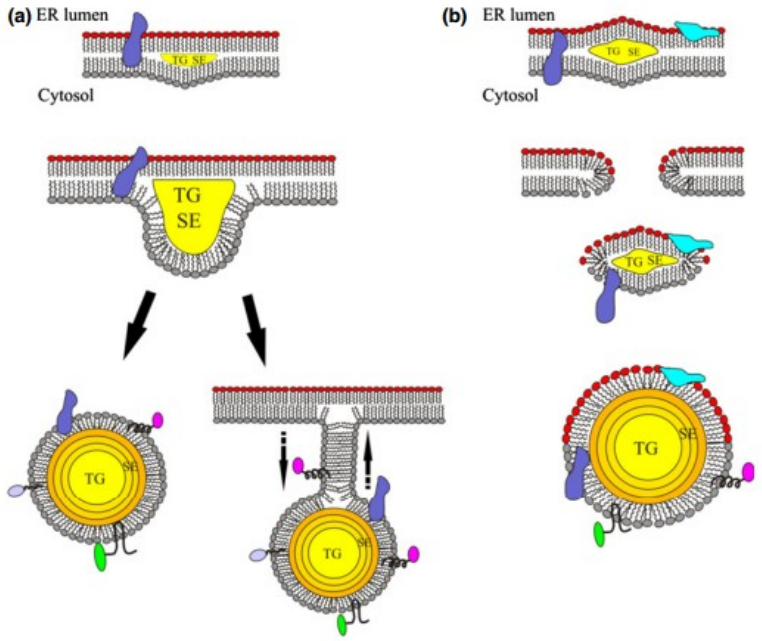


Fig. 2.1 Models of lipid droplet biogenesis: a) Lensing model – b) Bicell formation model (Koch et al., 2014)

2.1.1.1 Lignocellulosic biomass

Mixtures of fermentable sugars, useful to feed the microorganisms, can be obtained from hydrolysis of lignocellulosic materials, such as wheat straw, sugarcane bagasse, rice straw, rice hulls, and corn stover.

Lignocellulosic biomass is constituted for 35-50% of cellulose $(C_6H_{10}O_5)x$, polysaccharide of a linear chain of D-glucose, linked by β-(1,4)-glycosidic bonds to each other, which form the crystalline domains that confer strength and elasticity to the plant fibers.

The 20-25% is represented by hemicellulose, $(C_5H_8O_4)m$, located in secondary cell walls, are heterogeneous branched biopolymers containing pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-glucose, α-D galactose) and/or uronic acids (α-D-glucuronic, α-D-4-O-methylgalacturonic and a-D-galacturonic acids) which constitute the walls of the plant cells and may form hydrogen bonds with cellulose. The remaining 15-20% is formed by lignin, $[C_9H_{10}O_3(OCH_3)0.9–1.7]n$, an aromatic polymer synthesized from phenylpropanoid precursors. The major chemical phenylpropane units of lignin consisting

primarily of syringyl, guaiacyl and p-hydroxy phenol are linked together by a set of linkages to make a complicated matrix (Mood *et al.*, 2013).

An important aspect to be considered is represented by the pretreatment of the lignocellulosic biomass that is useful to improve the yield in the hydrolysis step (Fig.2.2). It could be lead by physical, chemical or biological methods (Kumar *et al.,* 2017).

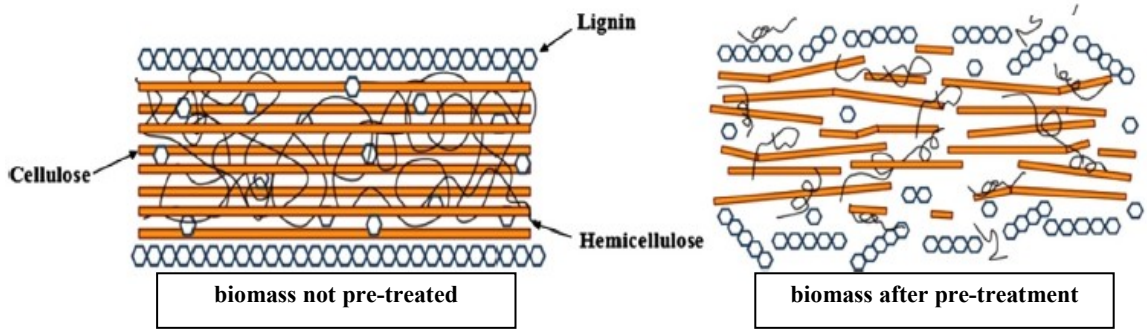


Fig. 2.2 Schematic pretreatment of lignocellulosic material adapted from Mood *et al.* (2013)

2.2 THE TRADITIONAL PRODUCTION PROCESS OF BIODIESEL

The traditional production process of biodiesel from lignocellulosic materials and oleaginous microorganisms is a succession of steps, as illustrated in Fig. 3.1.

The first step involves the hydrolysis of lignocellulosic materials which generates mixtures of sugars that, as already said, could represent the source of carbon for the growth of the oleaginous microorganisms. After a period of fermentation, under the adequate conditions as previously described, oleaginous biomass can accumulate lipids. The structure of yeasts prevents a direct recovery of lipids thus requiring a series of pre-treatments that allow an easier access to organic solvents in the following step of extraction (Lee *et al.*, 2010).

Biodiesel is produced in the final step by means of a transesterification reaction. Glycerol, by-product of the process, can also be recycled and used as a carbon source, as underlined by Xu *et al.* (2012).

The most important steps of the entire process, presented in the following chapters, are pre-treatment, extraction of lipids and transesterification.

2.2.1 PRETREATMENT

An efficient cell disruption method is a pretreatment necessary to maximize the recovery of the lipids from the biomass of microalgae (Günerken *et al.*, 2015) and more generically of all oleaginous microorganisms.

It is of pivotal importance to choose a feasible energy efficient method able to ensure low operating costs and high quality of products.

Pretreatment techniques divide into two main categories: the mechanical methods, such as bead milling, sonication, high pressure and high speed homogenization, ultrasonication, and non-mechanical ones (chemical and enzymatic).

The bead milling is leaded into a jacketed grinding chamber with a rotating shaft through its centre. The shaft is equipped with agitators of different design (concentric or eccentric discs or rings) that transmit kinetic energy to small beads in the chamber, forcing them to collide with each other (Middelberg *et al.,* 1995).

High-pressure homogenization is a method in which cell suspension is pumped to a high pressure and then forced through a narrow orifice of a valve before it is released into a chamber of lower pressure (Halim *et al.,* 2012).

A high-speed homogenizer is a stirring device at high rpm and usually is formed by a stator–rotor assembly, preferably made of stainless steel. The effective cell disruption

mechanisms are hydrodynamic cavitation, generated by stirring at high rpm and shear forces at the solid–liquid interphase (Günerken *et al.,* 2015).

The mechanism of the cell disruption by ultrasounds (sound waves of frequency higher than 15–20 kHz) is connected with the cavitation phenomena, i.e. shear stress developed by viscous dissipative eddies derived by shock waves produced by imploding cavitation bubbles. Shear forces generating eddies larger than the cells are able to move the cells rather than disrupt them. On the contrary, smaller eddies are useful to disrupt the cells. Thus, larger cells are involved in more disruptive eddies than smaller cells (Geciova *et al.,* 2002).

Chemical disruption can be realized by chemical compounds such as antibiotics, chelating agents, chaotropes, detergents, solvents, hypochlorites, acids and alkali (Günerken *et al.*, 2015).

Introducing an efficient cell disruption method could also be a method to obtain the same yield of methyl esters, but with a lower volume of solvents used.

2.2.2 LIPID EXTRACTION

The extraction is a process that allow to separate the lipids from the cell, with the aid of organic solvents.

One of the most well-known method for the extraction of lipids, due to its high efficiency is Bligh & Dyer's (1959), which involves the use of a chloroform-methanol mixture.

Other methods require the use of mixtures such as dichloromethane/methanol/water (Ma *et al.*, 2014) or n-hexane/water (Keris-Sen *et al.*, 2014). The use of such solvents, however, is harmful to the environment since they are highly polluting compounds. Furthermore the volatility of these solvents at lower temperatures decrease their contact with molecules to be extracted thus requiring a larger amount of solvents (Iqbal *et al.*, 2013).

Research in recent years has set as its objective the identification of alternative solvents that have yields comparable with those classics, but involve a lesser environmental impact (Zbinden *et al.*, 2013).

The identified environmentally friendly solvents are ethyl acetate (Zbinden *et al.*, 2013), ionic liquids (Young *et al.*, 2010), supercritical fluids, such as carbon dioxide (Cheng *et al.*, 2011).

Ionic liquids are chemical compounds consisting exclusively of ions and combinations of ions, but unlike the salts are liquid at room temperature. They represent a promising alternative to conventional organic solvents due to their non-volatility and thermal stability.

Kim *et al.* (2012) underlined the aforementioned aspects and proposed the use of a mixture ionic liquids/methanol to lead a lipid extraction from algal biomass, obtaining higher yields compared to the Bligh & Dyer's method.

Supercritical fluids are substances that have properties of both liquids and gases when exposed to increased temperatures and pressures. This property makes them good extracting solvents, leaving no residues behind when the system returns to atmospheric pressure and room temperature. Among them, the supercritical carbon dioxide already mentioned presents many advantages: it is very selective (there is no possibility for polar substances to form polymers), allows high yields without requiring additional units for the removal, does not contain heavy metals (Mercer *et al.,* 2011).

Another goal of nowadays research is the possibility of using a wet biomass for the extraction (Chuck *et al.*, 2014; Cui *et al.,* 2014) instead of a dried one in order to reduce the costs of the whole process. Compared to the entire production process of biodiesel, the extraction of lipids is one of the most onerous phases (Wang *et al.*, 2012): dry extraction burden for 90% of total consumption, while wet extraction affects to 70% (Lardon *et al.,* 2009). From these percentages the importance of the development of extraction techniques which operate directly with wet oleaginous biomass is clear. However, the presence of water hampers the efficiency of the extraction step, mostly because it reduces mass transfer and can promote emulsion formation (Dong *et al.*, 2016). Therefore, introducing water can lower the yield of esters, in the following phase of transesterification [§2.2.3].

2.2.3 TRANSESTERIFICATION

The transesterification is a reaction that allows the conversion of triglycerides into alkyl esters and involves the use of an alcohol, typically methanol (Fig. 2.1). This reaction can be catalyzed with acids, bases or enzymes.

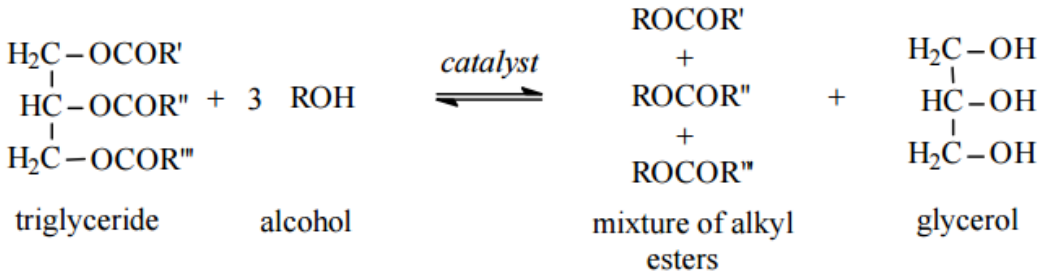


Fig. 2.3 Transesterification reaction (Schuchardt *et al.,* 1998)

The entire process is a sequence of three consecutive reversible reactions, in which diglycerides and monoglycerides are the intermediate, releasing a molecule of ester at each

step. The stoichiometric reaction requires one mole of triglyceride and three moles of alcohol. The excess of alcohol is used to increase the yields of alkyl esters, favoring the equilibrium towards formation of the products and to permit the separation of glycerol, the main by-product of the process (Schuchardt *et al.,* 1998).

The glycerol is an important organic compound that can be used in the production of medicines and cosmetics, if subjected to a purification step for the removal of impurities such as alcohol, salts and water. In the chemical industry it can be used by itself or it can be transformed into other products with a higher added value, such as 1,2-propanediol, useful as anti-freeze agent (Kwak *et al.*, 2012).

The basic transesterification is catalyzed by alkoxides or hydroxides of alkali metals, such as sodium or potassium carbonate. This reaction can be conducted at atmospheric pressure and at a temperature of about 60-70 °C, and requires several hours. The alkali metal hydroxides (KOH and NaOH) are cheaper than metal alkoxides (Helwani *et al.*, 2009), but their final removal is difficult and expensive. When oils contain large amounts of free fatty acids (FFA) as well as water, these latter react with the alkaline catalysts producing soaps which represent the by-products of the process. In this case it is advisable to use acid catalysts. Meher *et al.* (2006) stated that FFA value has to be lower than 3% in order to carry the base catalyzed reaction to completion.

In the case of acid transesterification, sulfuric acid is the most widely used catalyst. Acid catalysts are avoided over basic transesterification since in this case the reaction is characterized by slower kinetics and involves the use of corrosive compounds (Wang *et al.,* 2006).

The enzymatic transesterification provides for the use of lipases, lipolytic enzymes produced by microorganisms such as yeasts, fungi and bacteria by means of fermentative processes. Lipases are soluble in water, even if they act on insoluble substrates in aqueous media, in particular at the interface between the aqueous phase and the organic one (Arpigny & Jaeger, 1999).

The mechanism that explain the behavior of lipase, still not entirely clear, is so called "interfacial activation". In order to explain this mechanism, it has been hypothesized the existence of an additional site inside the lipolytic enzymes, distinct from the active site, capable of reversibly adsorbing the protein at the water-lipid interface (Grochulski *et al.,* 1993).

Lipases that give rise to this mechanism exist in two conformations: a "closed", with a structural element, the “lid”, that covers the active site and an “open” one in which the active

site is accessible to the substrate (Secundo *et al.,* 2006). Conformational transitions in the lid region, that cause the exposition of the active site and allow substrate molecules to bind, happen when lipases interacts with hydrophobic interfaces (Willems *et al.,* 2017).

The enzymatic transesterification has several advantages including easy catalyst recovery and reuse, the possibility to conduct the process at mild operating conditions (Taher *et al.*, 2011), absence of sensitivity to the presence of FFA and water (Lam *et al.*, 2010).

The main limit of the use of lipases, in addition to reduced stability in the conditions generally adopted for biodiesel synthesis, is the high cost. To avoid this problem it is necessary to immobilize the enzymes, to make them reusable. In addition, immobilized enzymes are more stable against changes in temperature and the denaturation phenomena. There are several methods for immobilizing lipases, such as adsorption, entrapment, encapsulation, and the cross-linking, also used in the case of production of biodiesel (Tan *et al.*, 2010).

The great versatility of the lipase, recognized as the most important group of biocatalysts, allows applications in different industries like food, pharmaceutical, textile and cosmetic (Reis *et al.*, 2009).

In recent years research focused on the possibility to lead a transesterification of TAGs in one step (in situ or direct transesterification) with the aim of suppressing the phase of extraction and reducing the volume of solvents involved.

Thliveros *et al.* (2014) investigated the possibility to lead a direct-base catalyzed methanolysis of *R. toruloides*, reaching 97.7 % of FAME yield after 10 h at 50 °C.

Liu & Zhao (2007) studied direct acid-catalyzed transesterification with H_2SO_4 and HCl at 70 °C for 20 h, reaching up to 98% based on total cellular lipid content of *L. starkeyi*, *M. isabellina* and *R. toruloides*.

2.3 INNOVATIVE TECHNOLOGIES FOR SINGLE CELL OILS AND BIODIESEL

2.3.1 PULSED ELECTRIC FIELDS

Pulsed electric field processing is a non-thermal method, which consists in the application of short pulses of high electric field intensity. The effect of this technique is a significant increase in the membrane permeability of the cells subject to the treatment (Aronsson *et al.,* 2005).

The fields of application range from the introduction of small molecules or macromolecules (Kandušer *et al.,* 2009) within the cell to the sterilization of liquid foods (Stoica *et al.*, 2011) or water treatment (Schoenbach *et al.,* 1996) and local control of skin metastasis through electrochemotherapy (Solari *et al.*, 2014). It is possible to vary the electrical parameters to preserve the viability of cells exposed to the pulses or determine the death after the treatment, in the light of the aim of the application.

Electroporation has proven to be effective both in systems with high cell density but with reduced content of liquid, as well as in liquid systems with extremely low cell density (Zbinden *et al.*, 2013).

In order to understand the phenomena of electroporation it is necessary to underline that the cell membrane of most living organisms consists of two phospholipid layers. The fatty acid chains of the phospholipids are hydrophobic facing towards the inside of the bilayer, while the hydrophilic heads are turned towards the cytoplasm or towards the outside of the cell (Kotnik, 2015). In addition to the phospholipids there are other components, such as steroids, proteins.

Zimmermann *et al.* studied the phenomenon of the dielectric breakdown of the cells in 1974. Due to the presence of positively charged ions outside the cell and of predominantly negative charges inside the cell, the application of an external electric field leads to an increase of attraction of charges and then to the electroporation of the cell membrane, which acts as a capacitor. Depending on the strength of the electric field applied, the cells can remain intact, if the so-called Ecrit is not exceeded, can be reversibly permeabilized or irreversibly permeabilized if this value is slightly or considerably exceeded (Stoica *et al.*, 2011).

The lipids in the cell membrane are held together only by weak (noncovalent) interactions, and at sufficiently high temperatures, the bilayer behaves as a two-dimensional liquid, that represents a very impassable barrier. Under specific conditions such as high

temperature and surface tension, this permeation is due to the formation and rapid resealing of small aqueous pores in the lipid bilayer (Kotnik *et al.*, 2015).

Theory of aqueous pore formation can explain the effect of pore formation after the application of an electrical field, that reduces the energy required for spontaneous formation of these holes (Fig. 2.4).

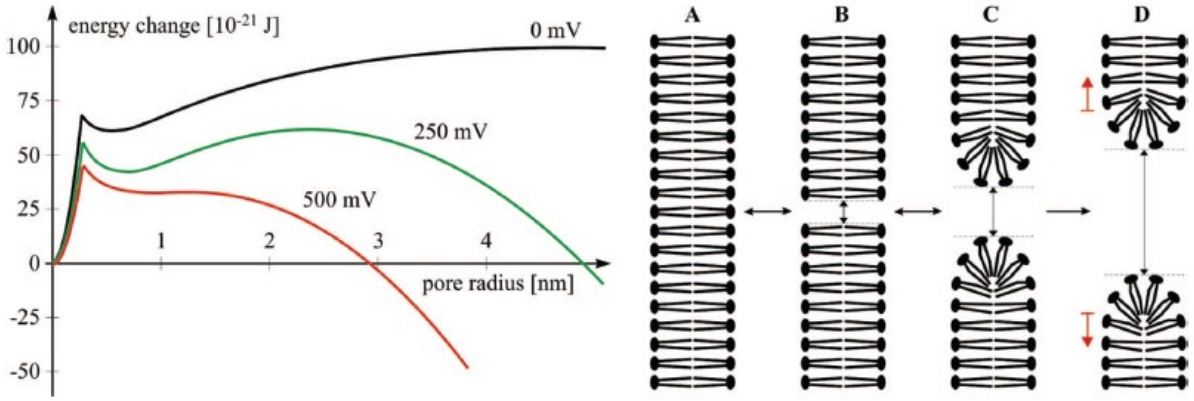


Fig. 2.4 Left: change in lipid bilayer energy caused by formation of an aqueous pore, plotted as a function of the pore radius and voltage across the bilayer. Right: formation of a pore (Kotnik, 2012).

In literature conflicting opinions were diffused about the effect of pulsed electric fields on the cell wall. According to Ganeva *et al.* (2014) and Stirke *et al.* (2014) there are some evidence of this kind of electric influence. The effect of electroporation on cell wall mostly depends on cell configuration (cells in suspension, monolayers or tissues), species of organism and treatment conditions. The increase in permeability and conductivity of electroporation-treated plant tissue is predominantly attributed to the improved permeability of the cell membrane (Mahanič-Kalamiza *et al.*, 2014).

Joannes *et al.* (2015) explained the advantages and limitations of using PEF on microalgae cells as a method to extract lipids.

Zbinden *et al.* (2013) demonstrated that utilizing PEF technology resulted in 90% of the cells being lysed and a significant enhancement in the rate of lipid recovery using ethyl acetate. The increase in lipid recovery was due to the presence of the electric field and not to temperature effects.

The permeabilization of the membrane can be traced using propidium iodide (PI) as a dye. It is an intercalating agent, which emits fluorescence if linked to nucleic acids (Breeuwer & Abee, 2000).

PI is not permeable through the cell walls and usually is excluded from live cells. It is used to identify dead cells in a population and as a counterstain for multicolor fluorescence

techniques (Ganeva *et al.*, 2014). It has also been used to monitor the growth of oleaginous yeasts (Raschke *et al.,* 2009).

2.3.2 MICROWAVES

In recent years microwaves have been applied in several branches of the chemical industry (Jones *et al.*, 2002), for the advantages that their use involves: rapid selective heating, saving energy and time, easy control of the process. The microwave irradiation is in the range of 0.3-300 GHz.

The mechanisms of energy transfer by microwaves are dipolar polarization, ionic conduction and interfacial polarization. They are responsible of a rapid and localized overheating in the reaction materials. If a molecule possesses a dipole moment, when it is exposed to microwave irradiation, the dipole will try to align with the applied electric field (dipolar polarization). At 2.45 GHz, frequency at which conventional microwave reactors generally work, the molecules align with the electric field, but do not follow exactly the oscillatory field. This incessant reorientation of the molecules generates frictions and, therefore, heat. Whereas if a molecule is charged, the electric field component of the microwave irradiation make the ions moving and colliding, producing heat (ionic conduction). The interfacial polarization is a combination of the two mechanisms already explained. (Gude *et al.*, 2013; Lidström *et al.*, 2001).

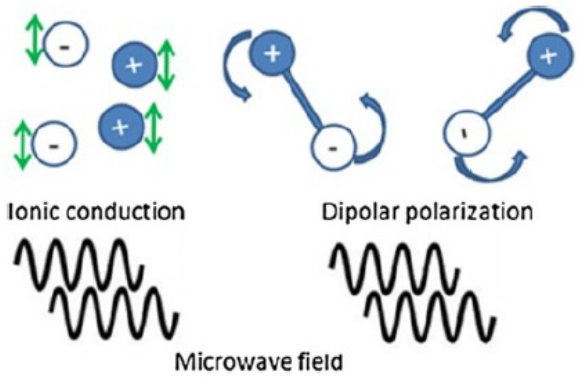


Fig. 2.5 Ionic conduction and dipolar polarization (Gude *et al.*, 2013)

Gude *et al.* (2013) stated that it is possible to use microwaves in biodiesel production to lead a lipid extraction, to enhance the reaction of transesterification or to perform both the steps simultaneously. The ease of extraction with conventional heating depends primarily on the solubility in the solvent, whereas with microwaves a fundamental role is played by the degree of localized overheating. The latter is particularly influenced by the power, frequency and the reactor design used.

Cui *et al.* (2014) pointed out several advantages of microwave extraction (MAE) over a conventional extraction, such as the possibility to use a wet biomass and the easy scale up of the process.

The choice of the solvent is an important decision that has to be taken before starting the experiments: microwaves are only effective with solvents with a dipole moment or a charge (Chuck *et al.*, 2014). In Table 2-2 physical constants and dissipation factors for solvents usually used in MAE were shown.

Methanol is a substance that absorbs microwaves since the presence of the -OH group attached to a large molecule undergoes rotations which generate localized overheating. The latter brings the reaction to completion in a shorter time (Tierney *et al.*, 2009).

The extraction yield of lipids depends on the rate of penetration of the microwaves inside the cell. The disruption of the cells occurs when the temperature of the water molecules inside the cells reaches the boiling point, leading to high pressure gradients and rupture of the cell walls, causing the migration of compounds selected from the matrix of the sample to the extraction solvent. As regards the use of microwaves in the transesterification reaction, its effects can be observed in the improvement of the reaction due to a thermal effect and to a more rapid evaporation of the methanol. The interaction of the reaction compounds (triglycerides and methanol) with the microwave reactor results in a strong reduction of the activation energy due to the increase of the dipolar polarization phenomena (Gude *et al.*, 2013).

Chuck *et al.* (2014) reported an acid-catalyzed direct transesterification method applied on *Rhodotorula glutinis* samples. According to their results, the microwaves did not alter the FAME profile and over 99% of the lipid was esterified when using 25 wt% H_2SO_4 over 20 min at 120 °C. Similar yields were achieved over 30 s when using higher loadings of catalyst, in comparison with 4 h of Soxhlet extractions.

Cui *et al.* (2014) investigated a direct transesterification with wet biomass of *C. curvatus* using microwave heating to disrupt the cell wall. The optimal conditions found were time (2 min), methanol/biomass ratio (50/1, v/m), stirring speed (966 rpm), KOH concentration (5%), and water content (80%). The yield of crude biodiesel (% of total lipids) was 56.1% after the first round reaction. A second round reaction using the residual yeast cells increased the total yield to 92%.

Iqbal *et al.* (2013) used blends of biodiesel B20 and B40 from soybean oil in ethanol to perform a microwave lipid extraction from *Nannochloropsis* cells in order to lead the process

with an eco-friendly solvent and to compare the results with those obtained with MAE using $CHCl_3$/EtOH (1:2) and conventional 8 h Soxhlet extraction.

In Dai *et al.* (2014) some experiments were leaded to realize a microwave lipid extraction from microalgae. The best results in terms of lipid content were obtained using the solvent mixture of n-heptane and iso-propanol (30 wt%).

Table 2-2 Physical constants and dissipation factors for solvents usually used in MAE (Veggi *et al.*, 2012)

Solvent	Dielectric constant,[a] Ɛ'	Dissipator factor tanδ (x10^{-4})	Boiling point,[b] (°C)	Viscosity,[c] (cP)
Acetone	20.7	5.555	56	0.30
Acetonitrile	37.5		82	
Ethanol	24.3	2.500	78	0.69
Hexane	1.89		69	0.30
Methanol	32.6	6.400	65	0.54
2-Propanol	19.9	6.700	82	0.30
Water	78.3	1.570	100	0.80
Ethyl acetate	6.02	5.316	77	0.43
Hexane-acetone (1:1)			52	

[a]Determined at 20 °C

[b]Determined at 101.4 kPa

[c]Determined at 25 °C

3. AIM OF THE WORK

The critical issues of the traditional production process of biodiesel (summarized in Fig. 3.1A) have been identified in the high costs of the process, the difficult scale up and the use of toxic solvents.

Aim of the work is to overcome these problems, with three alternative proposals, intended to make the process more sustainable, faster and cheaper.

The scheme in Fig. 3.1B proposes the use of pulsed electric fields as a pretreatment to explore the possibility to promote the disruption of the yeast cell before the extraction. As said, if a powerful pre-treatment is used before starting the extraction, it is possible to use a greener solvent. In this case, the yield is more bound to the efficiency of the disruption of the cell than to the extraction power of the solvents.

Furthermore, pulsed electric fields could be applied to wet system, reducing the costs associated to the drying of the biomass.

Electrical parameters must be accurately chosen because an irreversible process is advisable. In case of reversible process, it is important to understand how long the cell remain disrupted and bring the extraction to completion before the closing of the pores.

It is believed that this kind of technology works better for an extraction of the oils without the following step of transesterification because the use of a wet biomass could affect the yield of biodiesel.

The scheme in Fig. 3.1C proposes the use of microwaves to enhance the step of extraction.

The role of the microwaves is not yet well understood, however it could have a positive effect in terms of saving time, because it is a quick way to heat samples. It is important to make a general balance of the energy involved in the process to understand if it is more convenient to lead a conventional heating.

The scheme in Fig. 3.1D recommend the possibility to lead a direct transesterification, in particular to use microwaves to improve the production.

This alternative is very attractive from the industrial point of view because using a single reactor instead of two means a saving of space and costs related to energy inputs.

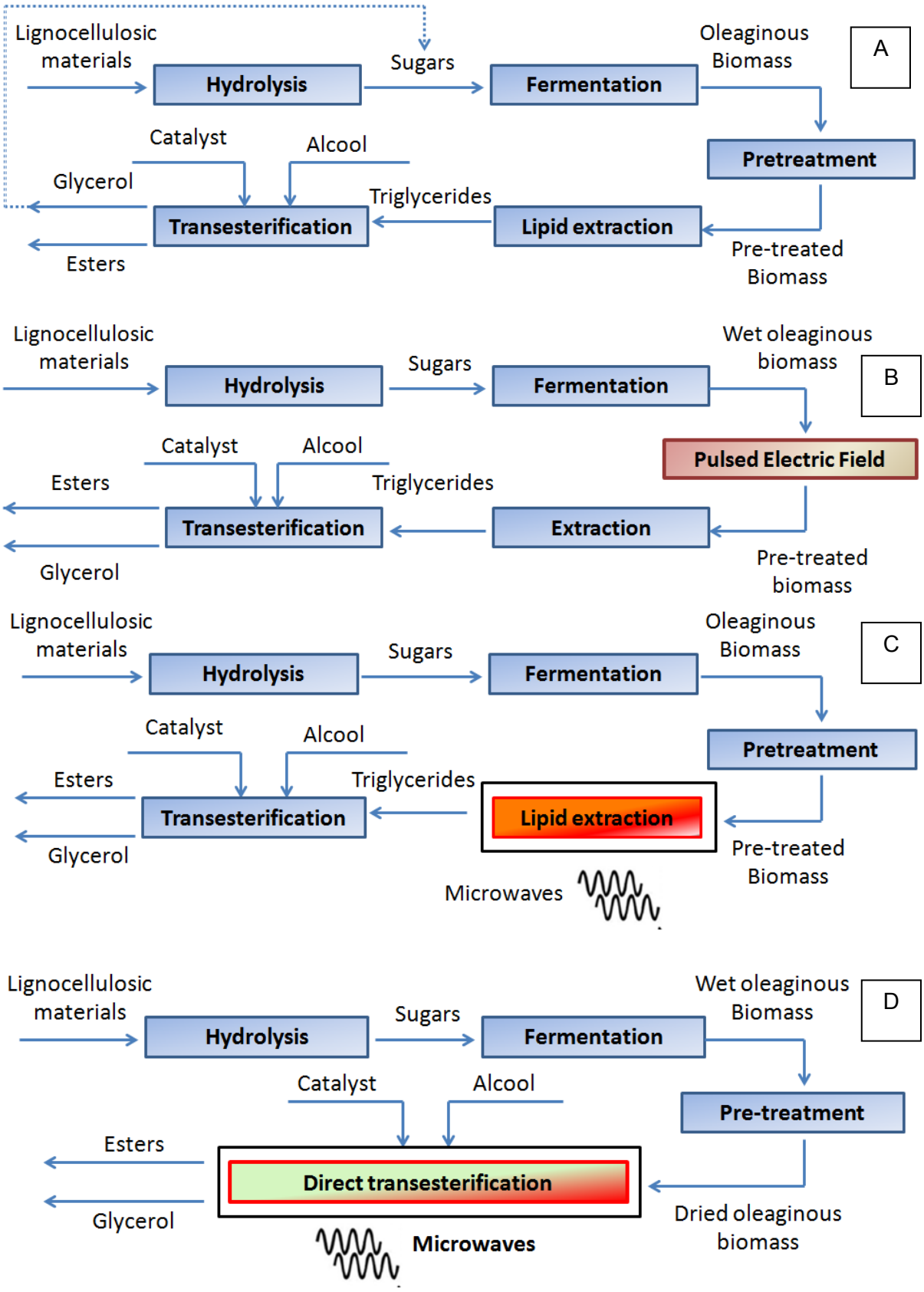


Fig. 3.1 Traditional production process [A] – Pulsed electric fields [B] – Lipid extraction with microwaves [C] – Direct transesterification with microwaves [D]

4. MATERIALS AND METHODS

4.1 MICROBIAL CULTIVATION

Lipomyces starkeyi (DBVPG 6193) was grown on solid YPD agar medium (Sigma-Aldrich), previously sterilized in an autoclave (121 °C - 20 minutes) and incubated at 25 °C until the appearance of an appropriate amount of colonies.

Tests were carried out in batch mode, by varying the fermentation time, in 250 mL Erlenmeyer flasks containing 2% by volume of inoculum and liquid synthetic medium with the following composition: yeast extract 10 g/L, peptone 10 g/L and glucose 20 g/L. The flasks were then put on the rotating plate in an incubator (Infors HT Minitron) and kept at a constant temperature of 30 °C with rotation of 160 rpm.

The biomass was monitored daily by measuring the turbidity of the culture medium, with analysis in a UV-Vis 1700-spectrophotometer (Shimadzu), with a wavelength of 600 nm.

After the fermentation, the biomass was centrifuged with a centrifuge Rotanta 460R for 20 minutes at 4500 rpm.

For the tests requiring the use of a dried biomass, it was necessary to use the oven at 106 °C.

Metschnikowia pulcherrima (wild type strain) in condition A (MpA) was cultured aerobically in six 2.0 L jacketed, airlift fermentors at 20 °C, with a pH of 4, 300 rpm (Department of Chemical Engineering at the University of Bath). The culture medium was a minimum media containing glucose 66 g/L and xylose 33 g/L (KH_2PO_4 1 g/L; $ZnSO_4 \cdot 7H_2O$ 0.02 g/L; $CaCl_2 \cdot 2H_2O$ 0.15 g/L; $(NH_4)_2SO_4$, 0.0625 g/L; NH_4Cl 0.354 g/L; $MgCl_2 \cdot 6H_2O$ 1.08 g/L; yeast extract 1.0 g/L and $MgSO_4 \cdot 7H_2O$ 0.188 g/L).

The dissolved oxygen for three bioreactors was 50% and for the remaining three 10%. Prior to the harvest of the biomass, the medium of all of the six bioreactors were mixed.

After 7 days of fermentation, the biomass was centrifuged for 20 minutes at 3000 rpm, the supernatant removed and the resulting biomass was freeze dried. The resulting powder was stored at room temperature prior to use.

Metschnikowia pulcherrima (directly evolved strain) in condition B (MpB) was provided by the Department of Biology and Biochemistry of the University of Bath.

80% oxygenation was maintained at the determined concentration through agitation (650 rpm max) and sparging (max 3:1 v/v), pH was maintained at 4 through the addition of

nitric acid (1 M) or sodium hydroxide (1 M) and temperature was maintained at 20 °C. These parameters were controlled by the Fermac 320 bioreactor control unit.

The bioreactor was inoculated using 25 mL of an SMP grown overnight, with an OD between the region of 30-50. The culture was performed to replicate likely industrial conditions, and as such, were under non-sterile conditions. Culturing volume used was 1 L to allow for sufficient headspace in the event of foaming. 10 mL of polypropylene glycol P2000 antifoam was added upon inoculation of each culture. Media composition was KH_2PO_4 1 g/L; $MgSO_4 \cdot 7H_2O$ 0.3725 g/L; $MgCl_2 \cdot 6H_2O$ 0.9278 g/L; $ZnSO_4 \cdot 7H_2O$ 0.02 g/L; $(NH_4)_2SO_4$ 0.295 g/L; NH_4Cl 1.671 g/L; yeast extract 1 g/L; $CaCl_2 \cdot 2H_2O$ 0.15 g/L; glucose 66.6 g/L and xylose 33.3 g/L.

The two strains were tested in the same conditions at the University of Bath. According to the results MpA have a lipid content of almost 20% and MpB of 38%.

4.2 Application of pulsed electric fields

Tests were conducted to understand the effects of the exposure to pulsed electric fields on sample of Lipomyces starkeyi (Ls), using a modified protocol by Ganeva *et al.* (2014). The latter suggested to collect the biomass in late exponential or stationary phase and diluted it to a concentration corresponding to 25 or 50 mg dry weight/mL. According to this approach, in all the tests a wet biomass of 125 mg/mL has been used to start, which corresponds to 25 mg dry weight/mL, considering that moisture content inside a yeast sample is almost 80 %.

Dilution were made by deionized water.

Yeast samples were subjected to electric pulses in standard electroporation cuvettes by using a commercial devise (Cliniporator, IGEA, SpA, Italy).

Cells images were collected using a Leica TCS SP5 confocal microscope.

Propidium iodide (Sigma Aldrich) used as a dye is diluted with PBS from a starting concentration of 1.5 mM. It has been stored in the dark.

Yeast suspension and PI were introduced before the starting of the exposure.

4.3 Lipid extraction

4.3.1 Conventional extraction

The freeze-dried biomass of MpA (0.1 g or 0.5 g in the experiments containing a higher amount of biomass) was suspended in 10 mL of a solvent such as $CHCl_3$:MeOH (1:1) ((2:1) for the experiments aiming the exploitation of a different molar ratio between the solvents), ethyl acetate, hexane, acetone in a tube with a stir bar.

In the tests with the hydrolysis step prior to the extraction, 10 mL of 6 M HCl were added in the tubes and the reaction has been conducted for 1 h at 80 °C. The same procedure has been applied to the water instead of the hydrochloric acid for the experiments with water.

For the runs at room temperature the tubes were put in the hotplate with stirring, but without heating.

For the runs at higher temperatures (80-100-120 °C), it is necessary to wait for the hotplate becoming warm before starting the reaction. After the reaction, tubes were put in a water bath at room temperature to cool them down.

After the extraction step the biomass has been separated by centrifugation (10 minutes, 4500 rpm). The oil content was determined by gravimetric method, after the removal of the solvents under reduced pressure.

4.3.2 Microwave extraction

Microwave extractions were undertaken using an Anton Paar monowave 300 microwave reactor equipped with a MAS 24 autosampler capable of loading 10 mL sealable reaction vessels.

The biomass of MpA (0.1 g) was suspended in a 10 mL of a solvent such as $CHCl_3$:MeOH (1:1), hexane or ethyl acetate.

The microwave reaction consists of three phases:

- heating to the set temperature (80-100-120 °C)
- reaction (0-10-20 min)
- cooling of the samples to 55 °C

For each step the stirring is 1000 rpm.

4.4 Direct transesterification

4.4.1 Experiments with Metschnikowia pulcherrima

The biomass of MpA (0.1 g) was suspended in a $CHCl_3$/MeOH (1:1) mixture (10 ml) with NaOH, 1 wt% (0.001 g), 10 wt% (0.01 g) and a stir bar. The reaction was performed in the microwave reactor previously described.

The organic layer was washed three times with water to remove the basic catalyst and glycerol and then the chloroform was removed under reduced pressure prior to ^1H NMR analysis.

4.4.2 EXPERIMENTS WITH LIPOMYCES STARKEYI

First tests on Ls biomass were conducted using a modified protocol by Thliveros *et al.* (2014), to investigate the possibility to lead a direct transesterification (extraction of lipids from the yeast's cell and transesterification reaction both in one single step).

0.2 g of Ls dried biomass were put in a falcon F1 with 4 mL of a solution of 4 g/L of NaOH inside an incubator (Minitron HT Infors) at the reaction temperature (50 °C or 60 °C) with an agitation of 160 rpm. After the reaction time (1-3-8-15 h), the supernatant and the FAMEs obtained were put in a new falcon F2 and brought to dryness.

For the following tests, direct transesterification was conducted with and without microwaves, at the same conditions, to compare the results.

For the tests with microwaves, 0.2 g of dried biomass of Ls were put in a glass vial V1 with 4 mL of methanol with a concentration of 4 g/L of NaOH. The vial V1 was placed inside a standardized exposure device (Romeo *et al.,* 2013): a short-circuit waveguide (WG, WR 430; 54.6-mm wide, 109.2-mm high, and 350-mm long) with a coaxial adapter (Maury Microwave R213A2, VSWR:1.05, Mont Clair, CA, USA) at the feeding end. The E4432B ESG-D series generator (Agilent Technologies, Santa Clara, CA, USA) provided the RF signal, which was then amplified (Microwave Amplifiers, LtdAM38A-092S-40-43, North Somerset, Bristol, U.K.) and fed into the exposure chamber through a bidirectional NRT-Z43 power sensor (Rohde and Schwarz, Munich, Germany), used to measure the incident (Pi) and reflected (Pr) powers. Power measurements were used to calculate the average Pa = Pi-Pr (where Pa is the absorbed power, assuming negligible the power lost on the walls of the applicator). The WG was specifically provided with customized tubes allowing the circulation of thermostated water, provided by a RC20 LAUDA (Lauda, Könishofen, Germany) bath with a water flow of 20 L/min, in order to control the environmental temperature during the exposures at 30 °C. The position of the sample (inserted on a polystyrene support and at a distance of 18.3 cm from the shortcircuit), was set on the basis of dosimetric evaluations as described in the following paragraphs. The temperature was continuously monitored by means of an optical fiber thermometer LUXTRON inserted in a cut-off cylindrical WG placed on the short side of the WR430 guide. Before starting the microwave exposure the vial was left inside the waveguide for ten minutes to make sure that the test started when the sample and the waveguide reached 30 °C. At this point the exposure of the sample to microwaves started, by opportunely setting the incident power in order to ensure that the Pa was sufficient to reach the established temperature that didn't exceed 50 °C (reaction temperature) or

decreased. Once finished the reaction, before recovering the supernatant phase, V1 was left for ten minutes in order to pellet all the dry biomass. The liquid phase has been put in a glass test tube PV1 and the solvents were evaporated.

For the tests with conventional heating, 0.2 g of dry biomass of Ls were put into a vial V1 with 4 mL of methanol with 4 g/L of NaOH. To reproduce the same conditions of the test with the microwaves, the vial V1 was placed in a water bath at 30 °C for ten minutes and in another one at 50 °C for the whole reaction time. The test continued as the microwaves'one.

FAME remained in falcon F2 or in vial PV1 tube were dissolved in a octadecane (as an internal standard) solution in heptane and analyzed by GC.

4.5 LIPID ANALYSIS

The lipid profile was shown by ^{1}H NMR by means of 400 MHz Bruker. The oil has been dissolved in $CDCl_3$ before the analysis.

Olive and rapeseed oils are of commercial use.

4.6 FAME ANALYSIS

The lipid content and FAME profile were calculated by gaschromatography calibrated to known standards. The analysis were carried out using a GC-2010 Shimadzu Gas Chromatograph equipped with a capillary column Supelco Omegawax 250 (30 m x 0.25mm ID, 0:25 μM) 24136. The injector and FID detector temperatures are respectively 250 °C and 260 °C. The initial temperature of 50 °C is maintained for 2 minutes and then brought to 220 °C at a rate of 4 °C/min and then held for 15 minutes. Helium was used as carrier gas (flow rate: 30 cm/s) at 205 °C. The injection is of 1 μL, 100:1 split.

5. RESULTS AND DISCUSSION

5.1 PULSED ELECTRIC FIELDS

Several tests were performed to investigate the effects of the application of pulsed electric fields on Ls samples fermented for 96 h.

The first aim was finding the ideal concentration of propidium iodide and cell suspension.

Some parameters were established before starting an electrical exposure. Concerning to physical parameters, the concentration of cell suspension, the dilution mean, the concentration of the dye (PI) and the volume of the treatment were chosen. Electrical parameters, such as amplitude [V], number of pulses, length of the pulse [ms] and the frequency [Hz] were varied. Concerning to the design of the chamber treatment, type of electrocuvette were selected.

First applications were made using a cell concentration of 125 mg/mL and a concentration of PI equal to 0.5 mM, following the indications in Ganeva *et al.* (2014). Electrocuvettes with a gap between the electrodes of 0.2 cm were chosen, able to contain a maximum volume of 300 µL.

The volume of the whole sample was 270 µL (180 µL of cell suspension and 90 µL of PI) to not exceed the height of the electrodes.

The amplitude used was 800 V, the number of the pulses was 40, the duration of the exposure 2 ms and the frequency 2 Hz.

The first image collected (Fig. 5.1A) is related to a "sham" which is a sample not subjected to electroporation, but prepared in the same conditions (same container and environmental conditions).

As shown, the concentration of PI is too high and is not possible to understand if the red color is due to a real penetration of the dye inside the cell.

The same results are shown in Fig. 5.1B and B', related to the electroporated sample.

Fig. 5.1A' and B' are the magnification (x3) of the samples illustrated in Fig. 5.1A and B.

Some tests were performed adding glycerol (as written in Ganeva *et al.*, 2013) but this insertion seems only to give more sharpness to the images. Those images were realized in the same electrical conditions, but with a concentration of PI of 0.05 mM (9 µL of PI for a total volume of 270 µL).

Following tests were conducted changing the dilution medium (saline instead of water) and the physical parameters of the sample (after the fermentation and not), to understand the role of the medium and the differences between different aged cells.

The presence of salts in the dilution medium hampers the appropriate propagation of the pulses. It was not possible to use the same electrical condition with saline, because the Cliniporator devise stopped.

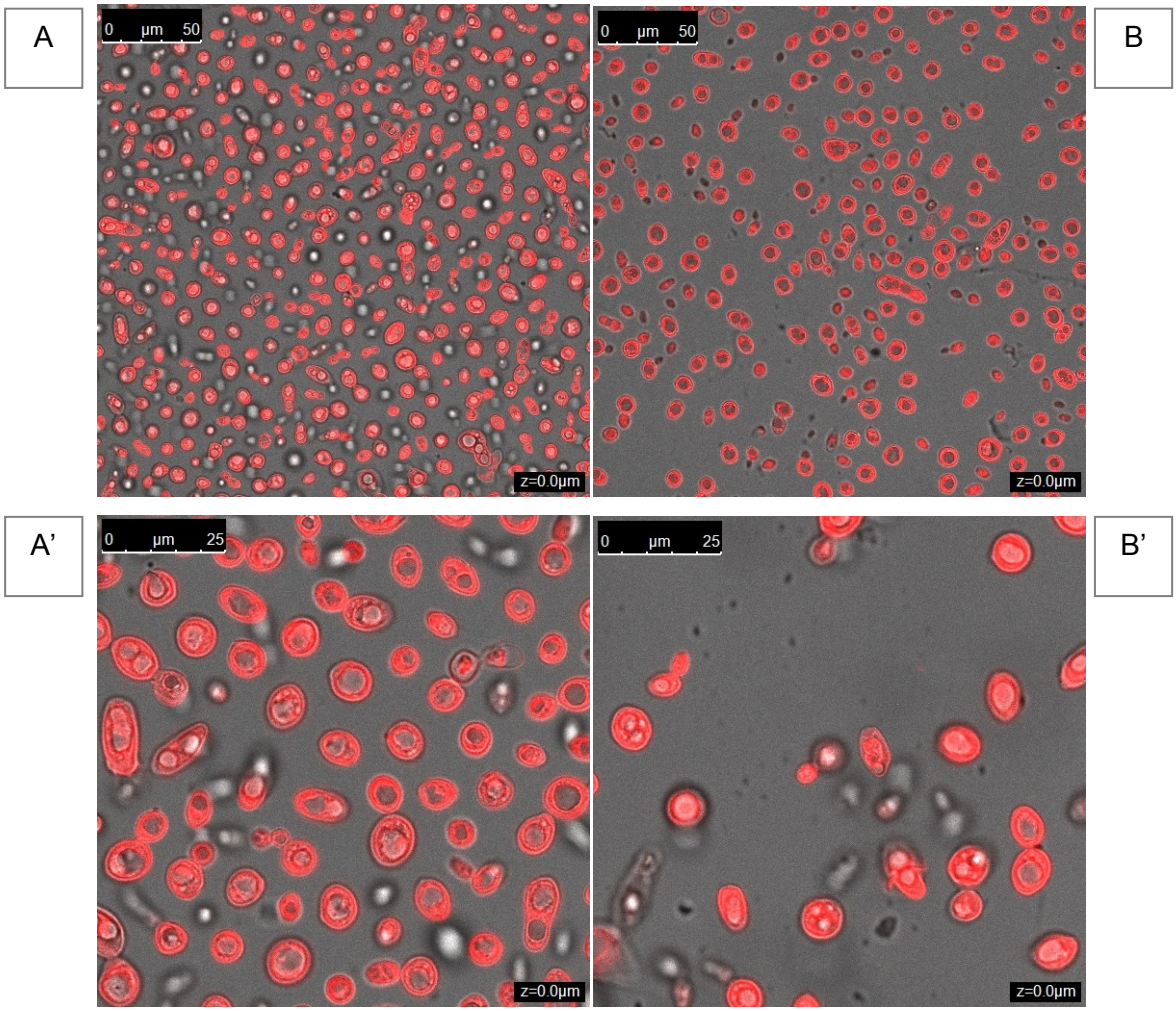


Fig. 5.1 Sham sample [A] - Electroporated sample [B]-
Sham sample (x3) [A'] – Electroporated sample (x3) [B']

After these experimental runs, it was possible to conclude that the ideal concentration of PI is 0.005 mM and the optimal concentration of cell suspension is 12.5 mg/mL.

The following test was conducted to understand if it is possible to electroporate a yeast cell, with the ideal concentration and the same exposure condition of the previous treatment (summarized in Table 5-1).

Table 5-1 Exposure conditions

Fig.	Cuvette	gap [cm]	Amplitude [V]	N pulse	L pulse [ms]	F [Hz]
AA'A''	Blue	0.2	-	-	-	-
BB'B''	Blue	0.2	800	40	2	2

Fig. 5.2A (sham) shows a very weak fluorescence in comparison with Fig. 5.2B. It is interesting to see the magnification (x3) and (x5) of the samples (5.2 A'A''B'B'').

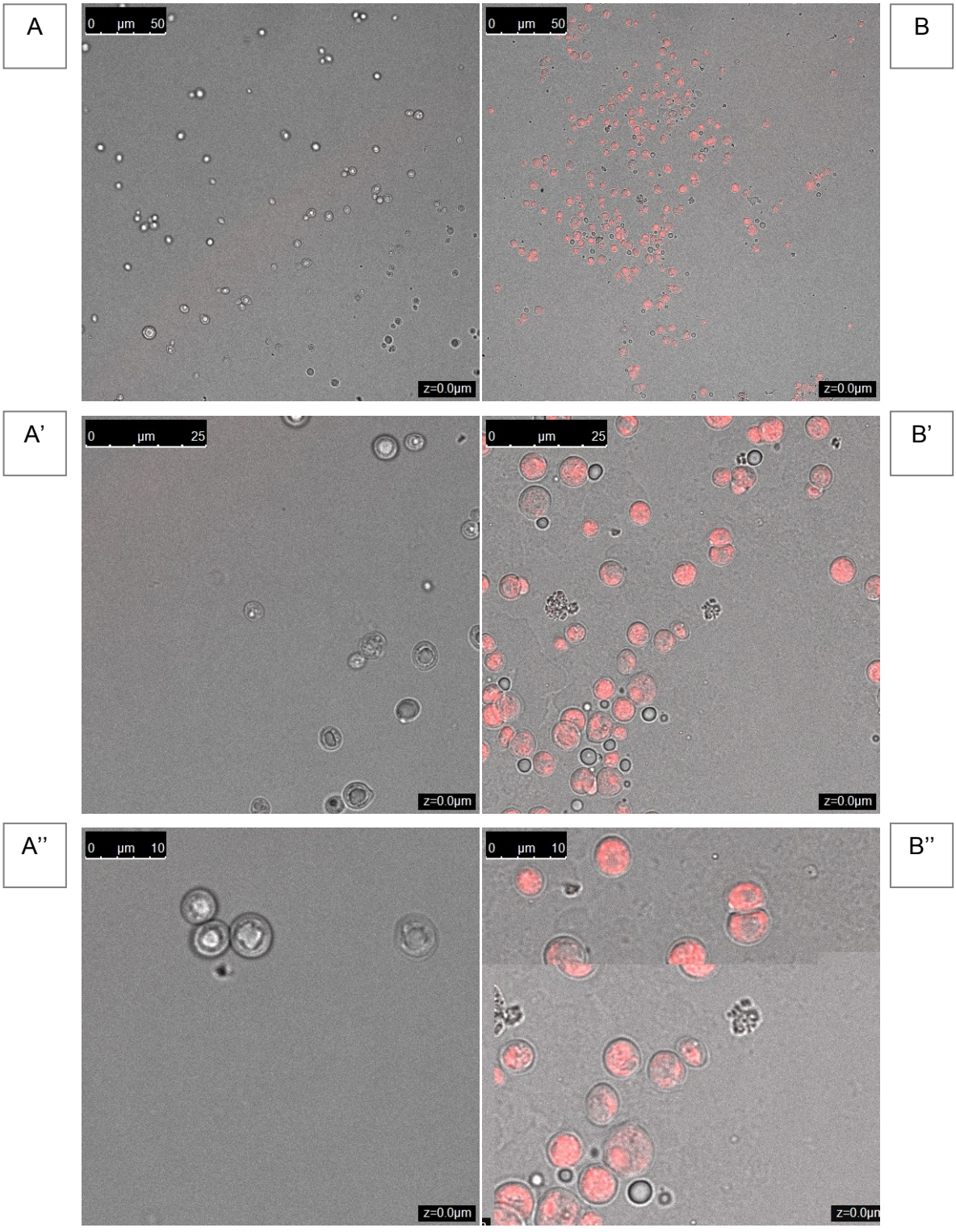


Fig. 5.2 Sham sample [A] – Electroporated sample [B]
Sham sample (x3) [A'] – Electroporated sample (x3) [B']
Sham sample (x5) [A''] – Electroporated sample (x5) [B'']

Electroporated sample showed a higher fluorescence in comparison to the sham sample and this is probably due to an easier access of the dye after the application of pulsed electric fields. Other applications are necessary to confirm these results and to make sure that the electroporation is irreversible.

5.2 CONVENTIONAL EXTRACTION

5.2.1 EFFECT OF SOLVENT

Several tests of conventional lipid extractions were performed with MpA biomass in order to select the ideal solvent to which the best results in terms of lipid extracted are related.

The solvents chosen are acetone that is cheap and very easy to find in a laboratory, ethyl acetate due to its green behavior, hexane that is widely used for lipid extractions and $CHCl_3$:MeOH (1:1). The latter has been performed with and without the previous step of hydrolysis with HCl or water. The reason why some runs were leaded with water instead of HCl is to understand the chemical role of the acid in improving the access of the solvents.

In Fig. 5.3 results are shown. It is important to underline that values in the graphs are points and do not represent a trend line and that they are the average of three experiments (unless otherwise specified). Lipid extracted Y_L [%] are calculated as following:

$$Y_L = \frac{m_L}{m_B} \times 100 \quad (5.1)$$

Where m_L is the mass of lipid extracted and m_B the mass of dry biomass.

According to the results, for an extraction longer than 18 h, the mixture $CHCl_3$/MeOH (1:1) with the previous step of hydrolysis at 80 °C with HCl seems to be more effective (31.1% after 24 h) than process without this step. This is an avoidable condition due to the costs related to the energy for the stirring, the time and the environmental concerns about using an acid.

Apart from the latter point, the most working solvent (20.6% in 4 h) is $CHCl_3$/MeOH (1:1) mixture.

Results also show that is not useful to lead an extraction for more than 4 h, because each curve (except for the HCl-$CHCl_3$:MeOH (1:1)) in the graph reaches a plateau after that time.

Lipid extracted using water instead of hydrochloric acid are low (7.1% after 4 h of extraction) and it is reasonable to say that the unique effect of temperature is not enough to disrupt yeast cells for an easy release of lipids.

Due to the results found it has been decided to use the $CHCl_3$:MeOH (1:1) as a solvent to explore high temperatures extractions and microwave heating.

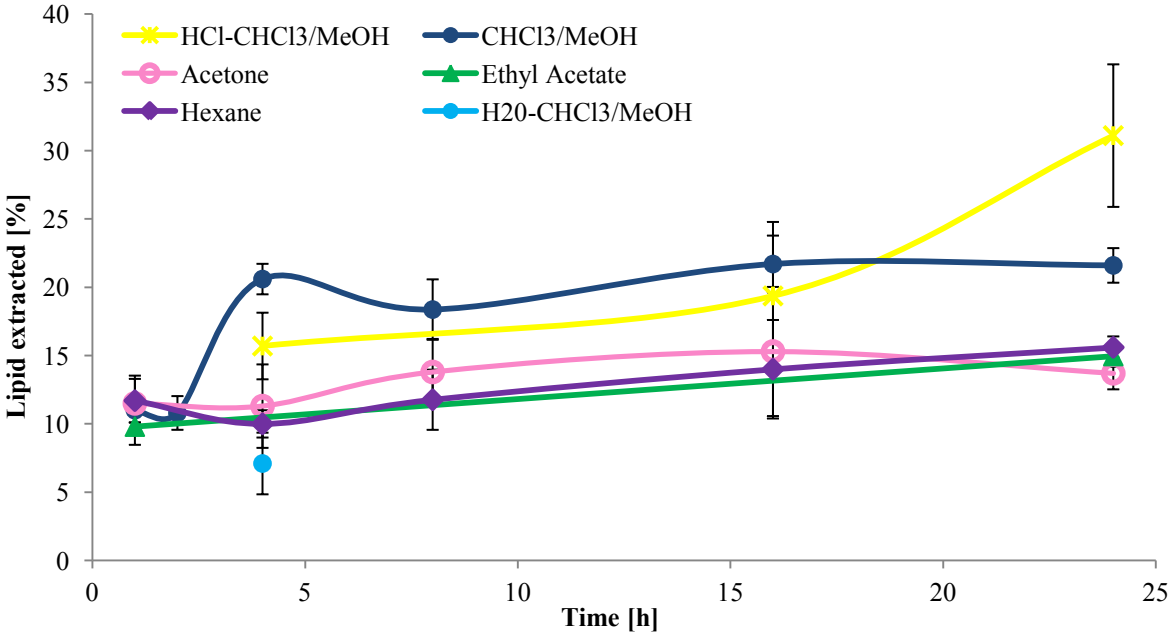


Fig. 5.3 Lipid extracted with different solvents at T=room

5.2.2 EFFECT OF TEMPERATURE

Two temperatures (100-120 °C) were selected to lead conventional extractions with $CHCl_3$:MeOH (1:1). Increasing the temperature can accelerate the step of extraction thus reducing the time of the whole process. Hold time is the time of reaction after the reaching of the set temperature.

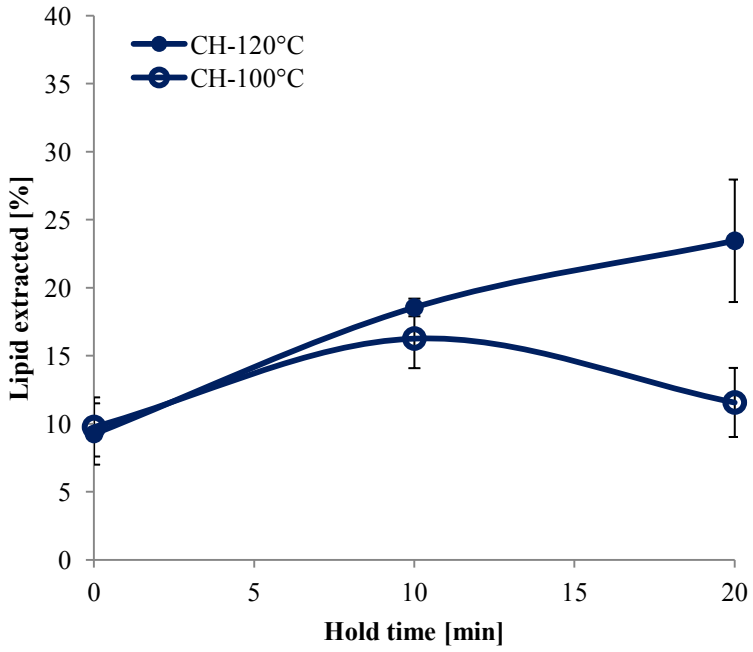


Fig. 5.4 Lipid extracted at 100 °C and 120 °C with $CHCl_3$/MeOH (1:1)

Temperature can have a positive effect on the lipid yield. Otherwise heating up a hotplate means energy costs and also the risk of losing solvent by evaporation.

In Fig. 5.4 results from lipid extraction at 100 °C and 120 °C are shown.

Extraction at 100 °C lead to a lower yield in terms of lipid extracted for each time in comparison with the curve related to 120 °C. It is interesting to observe that in the curve related to 100 °C there is a maximum at 10 minutes (16.3%) and then a decrease. Whereas at 120 °C the lipid extracted linearly increased as time passed.

By comparing the graphs in Fig. 5.3 and Fig. 5.4, it is possible to notice that results at 120 °C and 20 minutes (23.4%) are similar to those at room temperature after 4 h (20.6%).

Following tests were made to understand the increase of lipid extracted, raising the temperature during the extraction time. Unlike the previous experiments, where the chosen temperature was constant, tubes were put when the hotplate was at room temperature monitoring the probable increase of yield as the time passed.

The results are illustrated in Fig.5.5.

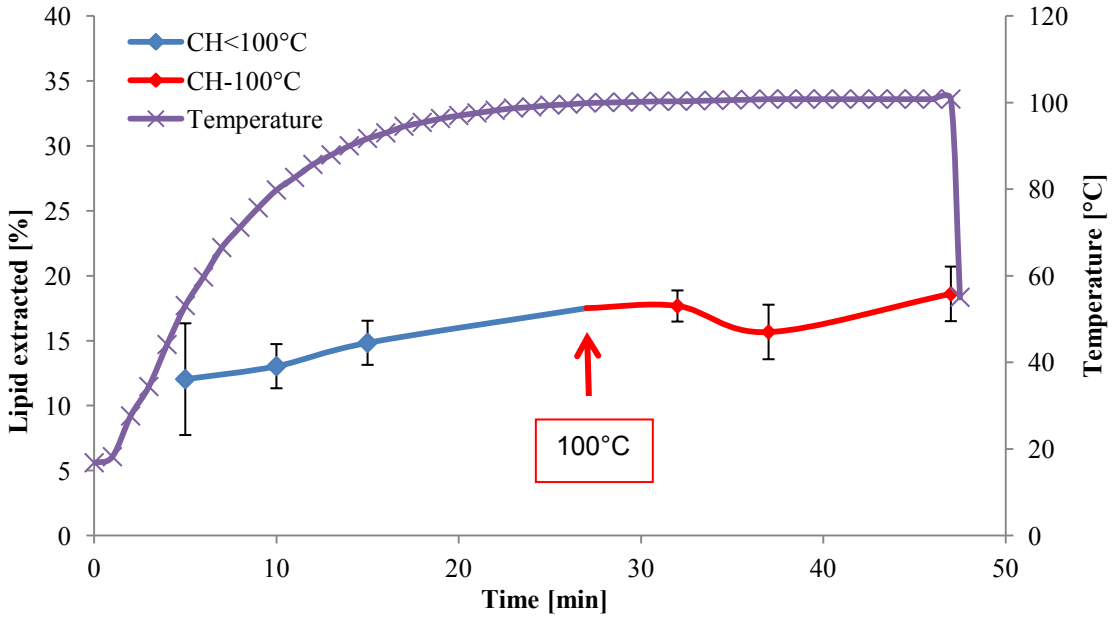


Fig. 5.5 Lipid extracted increasing temperature from 16 °C to 120 °C

A change of lipid extracted from 12% to 14.8% related to an increase of temperature from 53.1 °C to 91.7 °C was observed in the blue curve, achieved after respectively 5 minutes and 15 minutes. After other 5 minutes of extraction at 100 °C it has been possible to reach 17.6% (red curve). Leading the extraction for other 5 minutes allow to extract 15.6% and the last value of the red curve is 18.6 %, 47 minutes later since the starting of the experiment.

According to the results, it is possible to state that the lipid increase in the red curve is not so pronounced, thus it might be useful to stop the reaction already after 5 minutes at 100° C.

The results obtained could be compared to those shown in Fig. 5.4. Both the curves presented a decrease in the lipid extracted after some minutes.

5.2.3 EFFECT OF A DIFFERENT MOLAR RATIO

Following experiments were conducted to investigate the results obtained using a different molar ratio of (2:1) instead of (1:1) between chloroform and methanol used for the extraction.

According to the results shown in Fig. 5.6, until almost six minutes of extraction, the ratio (2:1) seems to be more efficient than (1:1). After that, there is an increase of lipid content with a molar ratio of (1:1).

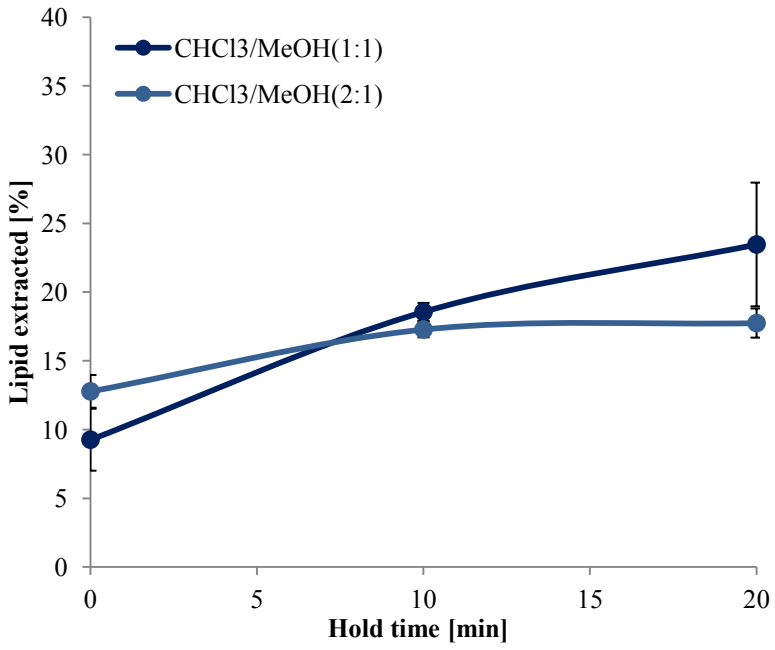


Fig. 5.6 Lipid extracted with different molar ratio of $CHCl_3$/MeOH ((1:1) and (2:1)) at T=120 °C

5.3 MICROWAVE EXTRACTION

5.3.1 POLARITY OF SOLVENTS

An Anton Paar 300 monowave was used to extract the lipid from Mp (A and B) biomass.

Before starting microwave reactions, some considerations have to be done about the solvents. It is important to consider their polarity, that is a characteristic useful to understand the ability of a compound to absorb microwaves [§ 2.3.2].

In the first part of the chapter the same solvents used for the conventional extraction were analyzed, to understand their behavior if subject to microwave heating with MpA yeasts.

In the second part, other solvents or mixtures (limonene:ethanol (1:1), 2-methyl tetrahydrofuran and 2-methyl isobutyl ketone) were analyzed, as green alternatives to conventional solvents, with MpB yeasts. Ethanol has been added to limonene, that is an extraction solvent, only to improve its microwave absorption.

Fig. 5.7A shows the temperature profile related to chloroform and methanol (1:1). It took almost 67 seconds to heat up, whereas adding an amount of biomass decreased the time to 62 seconds. This reduction of time is probably due to a residual moisture content inside the freeze dried biomass that enhance the polarity of the content.

In Fig. 5.7B temperature profiles of hexane alone, with 0.1 g of biomass and in mixture with other polar solvents such as acetone and water in different molar ratios are shown.

Due to its non-polarity, hexane took much time to heat up at 120 °C (153 seconds). In this case, its heating is improved by the presence of the stir bar and an amount of polar substances contained in this solvent. After adding 0.1 g of yeast biomass, hexane takes 144 seconds to heat up.

Furthermore adding 1 mL of a polar solvent, such as acetone or water can drastically reduce the time of heating (109 seconds and 101 respectively). With acetone:hexane ratio of (1:1) the time useful to heat up was 80 seconds.

In Fig. 5.7C temperature profiles of ethyl acetate alone and with the adding of biomass are displayed. Water:ethyl acetate (1:9) had also been plotted. Both the two curves without water took 65 seconds to become warm, whereas the other one spent 76 seconds. It is interesting to see that ethyl acetate alone, unlike the other solvents took less time than with the adding of 1 mL of water. The mixture water:ethyl acetate is less polar than ethyl acetate alone.

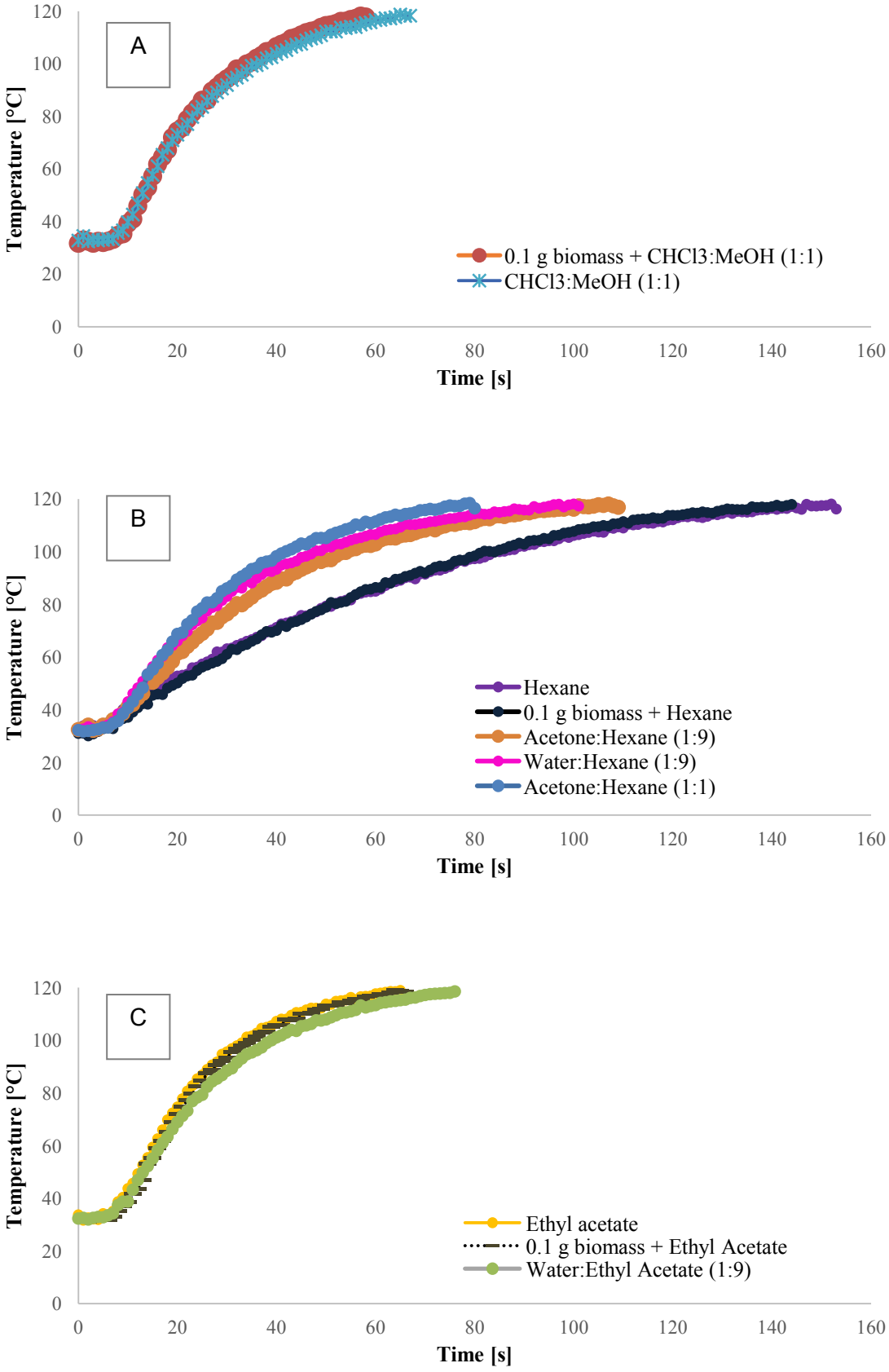


Fig. 5.7 Temperature profiles of chloroform:methanol [A], hexane [B], ethyl acetate [C] alone, in different mixture with other solvents and with the adding of 0.1 g of biomass

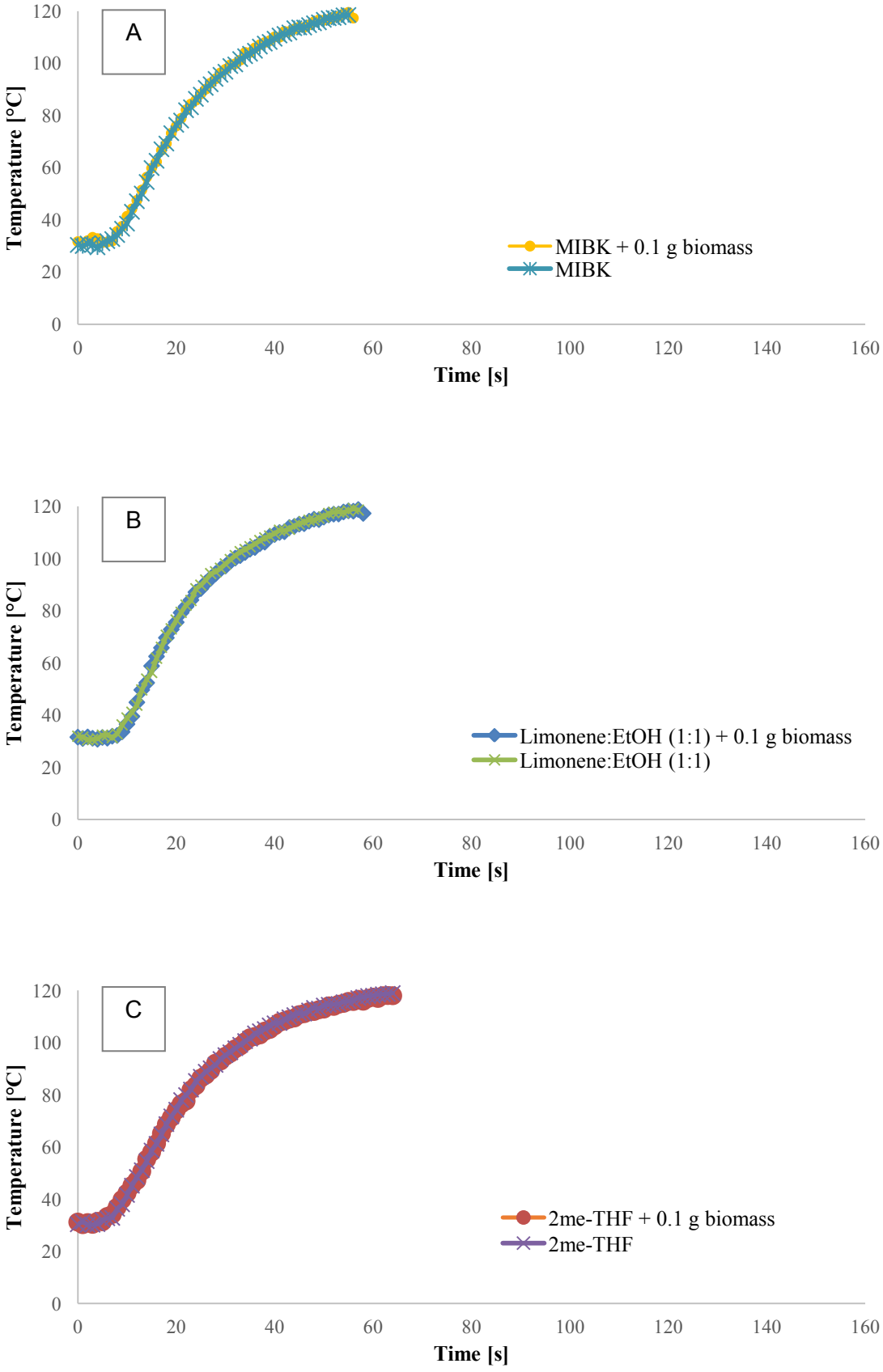


Fig. 5.8 Temperature profiles of MIBK [A], Limonene:EtOH (1:1) [B], 2Me-THF [C] alone and with the adding of 0.1 g of biomass

In Fig. 5.8A, B and C temperature profiles of MIBK, limonene:EtOH (1:1) and 2Me-THF alone and also the curves with the adding of biomass are shown.

There is no significant time variation in the case of adding the biomass.

The results are very encouraging, because all three solvents analyzed took almost one minute or less to heat up.

Their behaviors are very similar to the $CHCl_3$:MeOH (1:1) profiles in terms of warming up time, but they are not toxic such as the latter.

5.3.2 *Effect of temperature*

Three reaction times (0-10-20 minutes) and three temperatures (80-100-120 °C) were selected to lead microwave lipid extractions with MpA biomass with $CHCl_3$:MeOH (1:1).

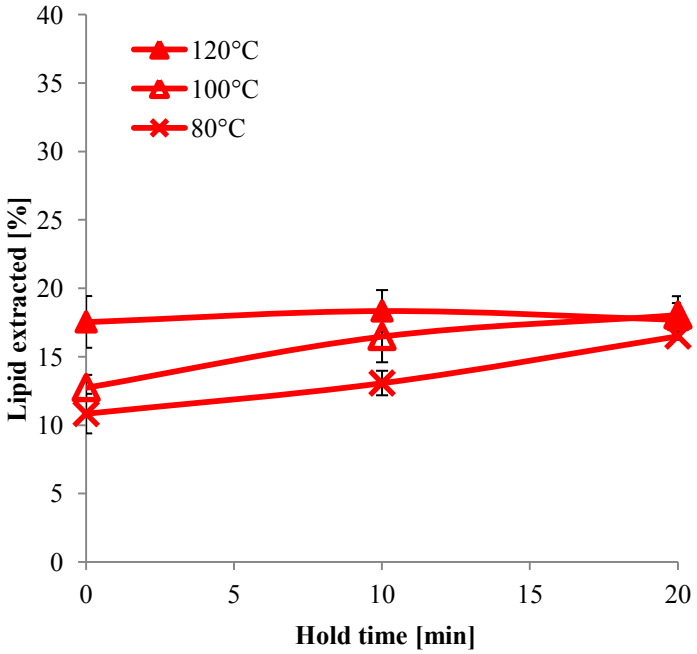


Fig. 5.9 Lipid extracted with microwaves with $CHCl_3$/MeOH (1:1) at T=80-100-120 °C

Fig. 5.9 shows lipid extracted varying the hold time and the set temperatures. Curve related to 80 °C exhibited the lowest yield for each hold time investigated (10.8% at 0 minutes; 13.1% at 10 minutes; 16.5% at 20 minutes).

Concerning the 100 °C curve, values are higher than the previous results (12.7% at 0 minutes; 16.5% at 10 minutes and 18% at 20 minutes). It is interesting to observe that increasing the temperature of 20 °C allows to extract the same amount of lipids by reducing the time of 10 minutes (16.5%).

The highest amount of lipid extracted are related to 120 °C curve for each extraction time (17.5% at 0 minutes; 18.3% at 10 minutes; 17.7% at 20 minutes). The latter curve is

quite flat, thus meaning that 0 minutes of hold time is a sufficient period to extract the largest quantity of lipids.

It should be noted that energy calculations are needed to decide the most convenient choice concerning temperature and extraction time [§5.6 Fig. 5.30].

5.3.3 EFFECT OF SOLVENT

Following runs in the microwave reactor were conducted with ethyl acetate, because it is rapid to heat as previously shown and it is a green solvent. As shown in Fig. 5.10 the lipid extracted are less than 10% for all the times of extraction The maximum is 9.9% after 10 minutes. The curve seems to be flat such as the graphs related to $CHCl_3$:MeOH (1:1).

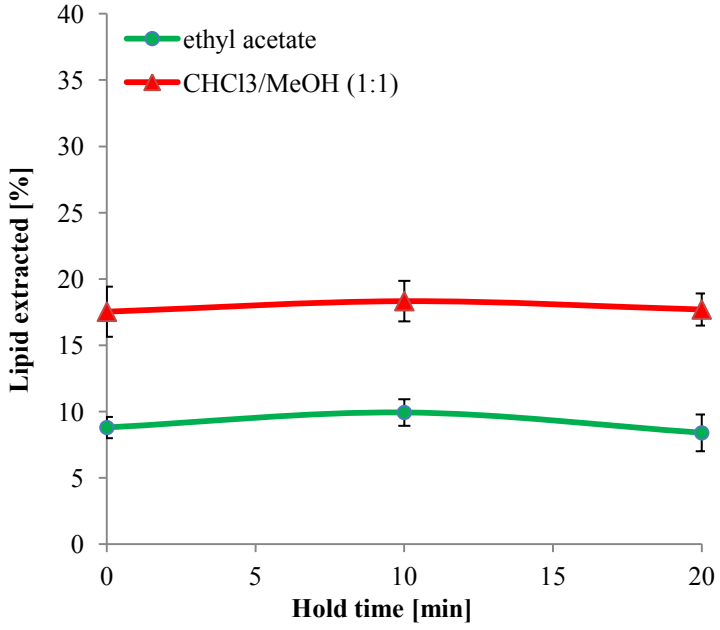


Fig. 5.10 Lipid extracted from MpA biomass with microwave extraction with ethyl acetate and $CHCl_3$/MeOH (1:1) at T=120 °C

5.3.4 EFFECT OF YEAST LOADING

Some experiments were made to understand the best amount of yeast loading between 0.1 g and 0.5 g with MpA biomass.

According to the results (Fig. 5.11), it is clear that raising biomass amount does not entail an increase in percentage of lipid extracted. This is probably due to a worst solvent/biomass ratio or an inadequate mass transfer during the stirring.

The best value obtained with 0.5 g of biomass is 13.9% for an hold time of 10 minutes. At 0 minutes it is possible to extract 9.5% of lipids whereas after 20 minutes 13.7%.

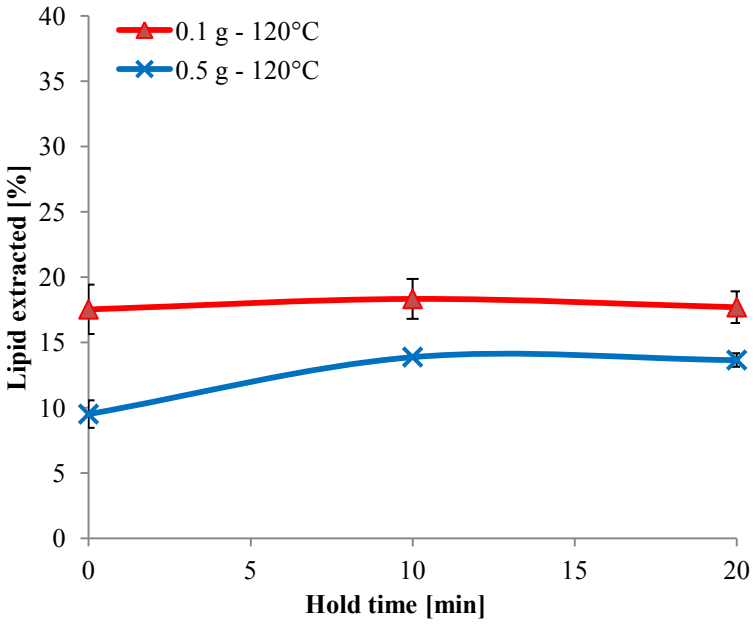


Fig. 5.11 Lipid extracted with conventional heating with a different amount of MpA yeast biomass (0.1 g and 0.5 g)

5.3.5 EXTRACTION WITH MPB BIOMASS

In Fig 5.12 results of extraction with $CHCl_3$:MeOH (1:1) and hexane from MpB biomass are shown (data are the average of two experiments).

With $CHCl_3$:MeOH (1:1) it is possible to achieve 32.2% of lipid at 0 minutes, 29.9% at 10 minutes and 31.6% at 20 minutes. With hexane results obtained are 13.2% 14.5% and 14.5% respectively at 0, 10 and 20 minutes.

Hexane demonstrated to be an inefficient solvent compared to $CHCl_3$:MeOH (1:1).

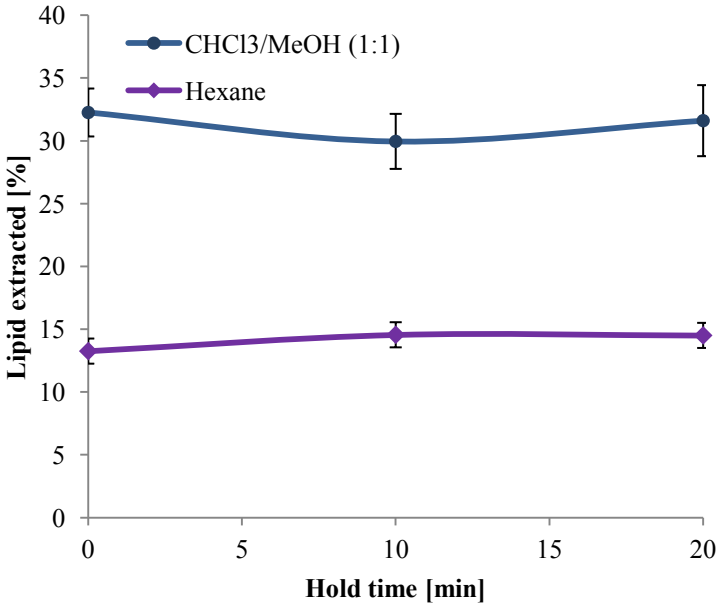


Fig. 5.12 Lipid extracted from MpB with microwave extraction with $CHCl_3$:MeOH (1:1) and hexane at T=120 °C

In Fig. 5.13 first results from an extraction with 2Me-THF are shown.

It is interesting to notice that the yield at 80 °C is higher than that at 120 °C, differently to all the graphs previously presented in this chapter, where the increase of temperature was useful to reach an higher amount of lipids. The best result obtained is 20.5% after 20 minutes at 80 °C (in comparison to 15.9% at 120 °C). At 0 minutes, with a temperature of 80 °C it has been possible to obtain 19.2% of lipid extracted whereas 17.8% at 120 °C.

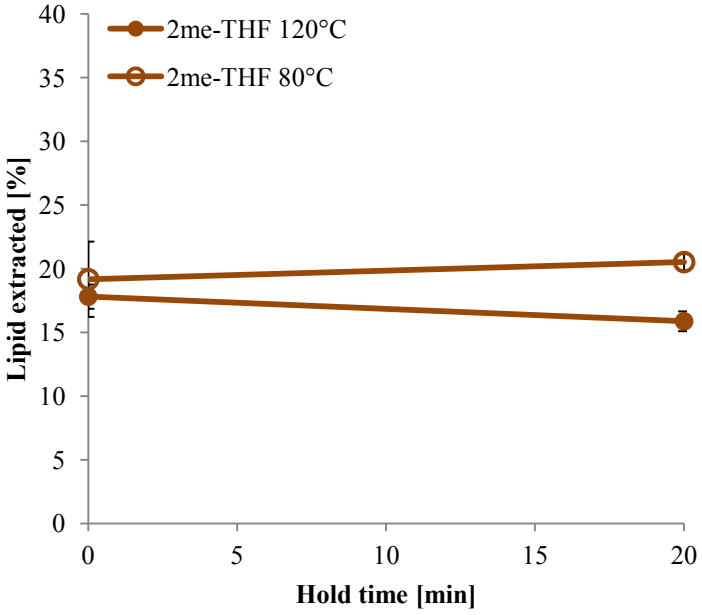


Fig. 5.13 Lipid extracted with microwave heating with 2Me-THF

Some tests were started with limonene:ethanol (1:1). Several difficulties are bound to the use of limonene, due to its high boiling point (176 °C) that make the removal of the solvents very difficult with conventional methods.

As already shown by Breil *et al.,* (2016) it is necessary to create an azeotrope adding 50% (v/v) of water in the extraction solvents.

5.4 COMPARISON BETWEEN CONVENTIONAL AND MICROWAVE EXTRACTION

In order to compare conventional and microwave extractions in terms of lipid content, Fig. 5.14A and B show contemporarily the curves related to both different experiments, at 120 °C and 100 °C with MpA biomass.

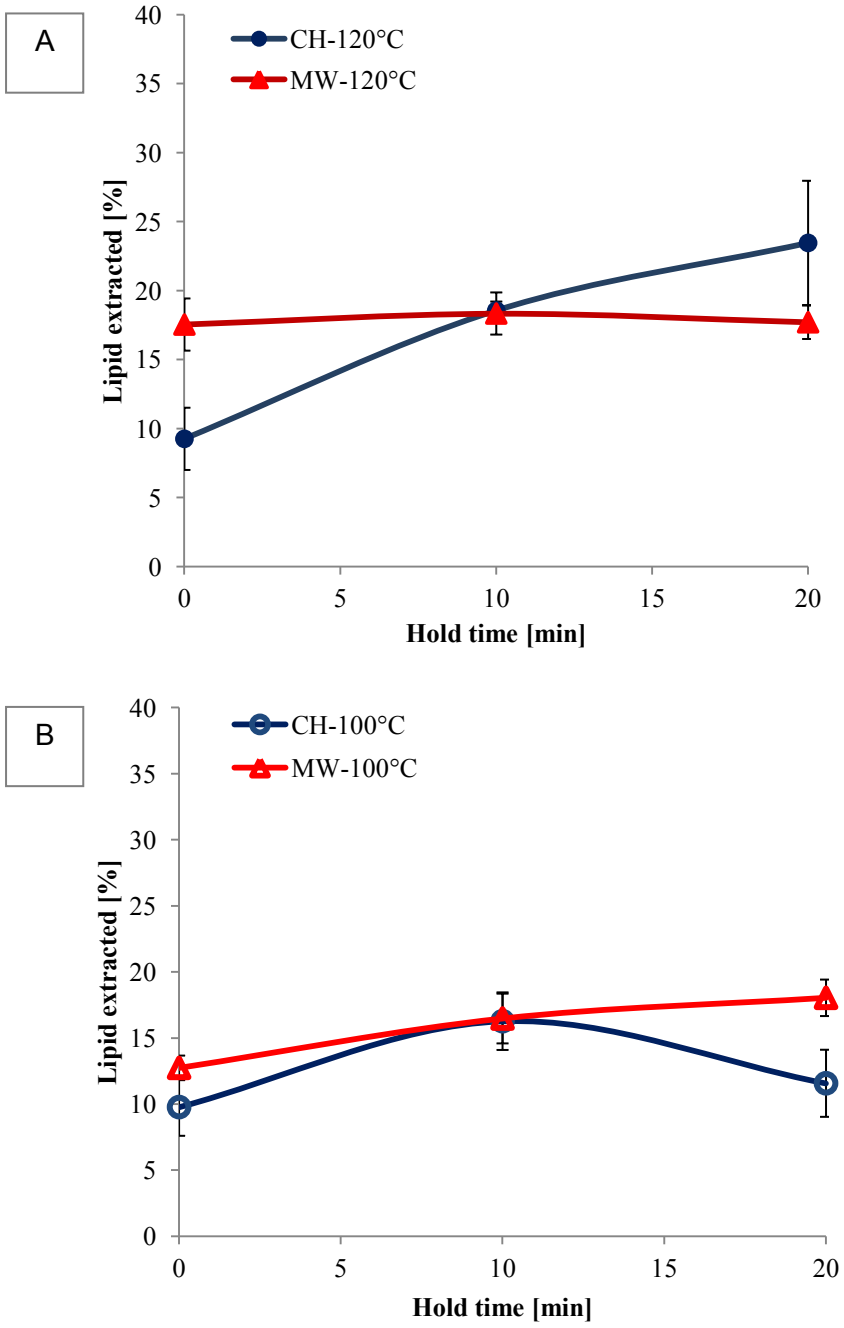


Fig. 5.14 Lipid extracted with conventional and microwave heating at T=120 °C [A] and at T=100 °C [B]

According to Fig. 5.14A at 120 °C it is clear that until 10 minutes of extraction is better to lead the reaction by microwave heating, whereas after 10 minutes conventional heating allow to obtain a higher amount of lipids. At minute 10 there is a point of intersection

between the two curves (18.3% for microwave extraction and 18.6% for conventional heating). The results show that is convenient to use microwave heating for 0 minutes (17.5%) or conventional heating for longer reaction time (at hold time of 20 minutes, 23.4%).

Fig. 5.14B shows lipid extracted related to conventional and microwave heating at 100 °C. At all the times (0, 10 and 20 minutes) the best results were obtained using a microwave reactor rather than conventional heating. Equally to the curve related to microwave extraction at 120 °C, also at 100 °C the curve seems to be quite flat. At minute 10 the two curves reach almost the same lipid content (16.5% with microwaves and 16.3% with conventional heating).

At 100 °C, the optimum (18%) is at hold time equal to 20 minutes with microwaves, whereas with conventional heating the maximum percentage of lipid extracted is 16.3% after 10 minutes.

A comparison between conventional and microwave heating cannot be conceived without the consideration of the time to heat up.

As shown in Fig. 5.15, to heat up at temperature equal to 100 °C microwave reactor took less than a minute is as opposed to at least 20 minutes by conventional heating. This is an important issue due to the will of scaling up the process.

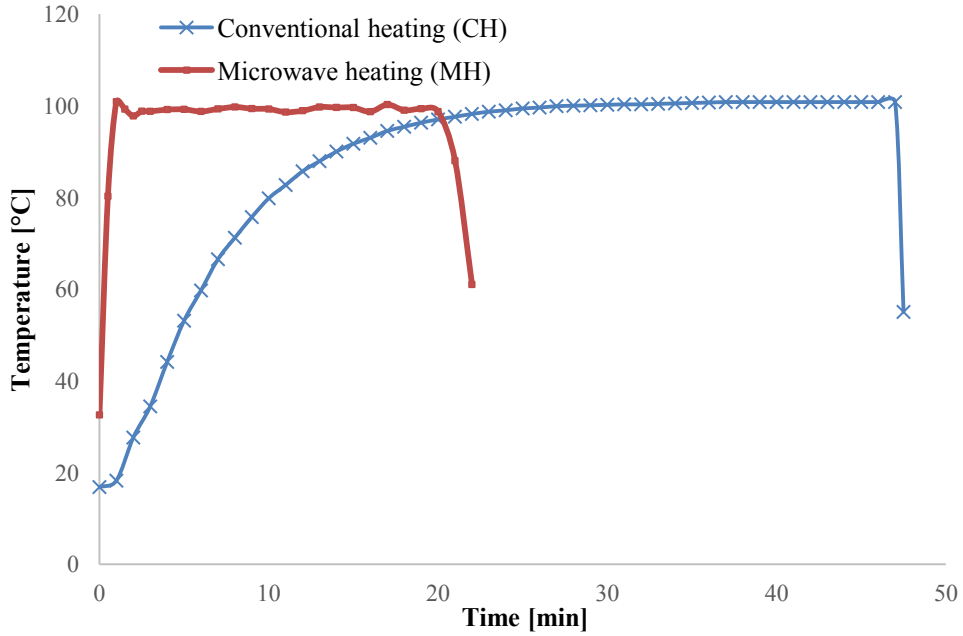


Fig. 5.15 Temperature profiles of conventional and microwave heating (T=100 °C, 20 min)

5.5 ANALYSIS OF ^{1}H- NMR SPECTRA

The ^{1}H NMR spectral features of yeast biomass are similar to those of vegetable oils.

For this reason, in this thesis comparisons to olive and rapeseed oil spectra were made, to underline differences and similarities of the profiles.

Most interesting spectra were reported in this chapter and analyzed according to the indications about the peaks illustrated by Sarpal *et al.,* (2014).

Gelbard *et al*. (1995) proposed a method to calculate the conversion C_{ME} (%) of vegetable oil to biodiesel, considering the peak of the methyl ester (at approximately 3.7 ppm) and the peak of $\alpha - CH_2$ (about 2.3 ppm).

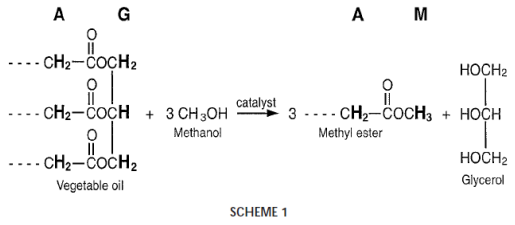
Knothe (2000) presented an alternative method to rapidly calculate the conversion of vegetable oil to biodiesel, based on glyceridic peaks in the triacylglycerides and rewrote the Gelbard's approach as in the following equation:

$$C_{ME} = 100 \cdot \frac{2 \cdot I_{ME}}{3 \cdot I_{\alpha - CH_2}} \qquad (5.2)$$

Where I_{ME} is the intensity of the methyl ester peak (M) and $I_{\alpha-CH_2}$ is the intensity of the peaks of the $\alpha - CH_2$ protons (A) (Fig. 5.16).

In this chapter calculations of C_{ME} were done according to (5.2), in spectra which show the peak of OCH_3 at 3.66 ppm.

The software TopSpin 3.5 was used to integrate the peaks and to plot the data.



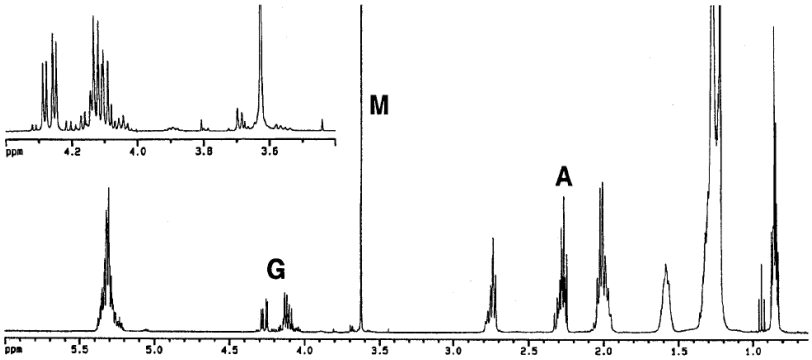


Fig.5.16 ^{1}H-NMR spectrum of a progressing transesterification reaction.The letters A, G and M are the $\alpha - CH_2$, glyceridic and methyl esters respectively (Knothe, 2000)

5.5.1 CONVENTIONAL HEATING

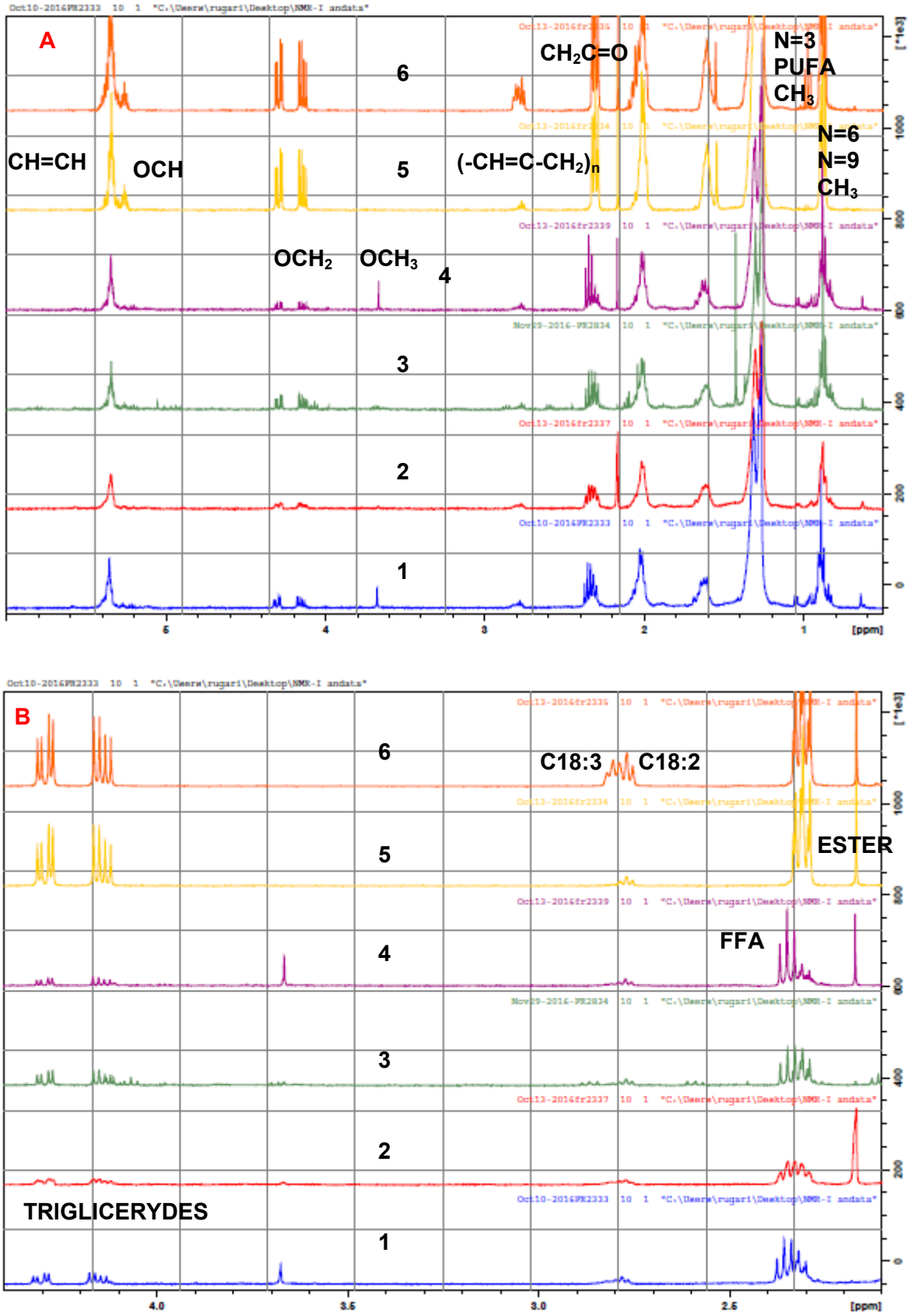


Fig. 5.17 400 MHz ^{1}H NMR spectra of chloroform:methanol (1:1) (1), acetone (2), ethyl acetate (3), hexane (4) extracts at T=room for t=24h, olive oil (5) and rapeseed oil (6). These latter are shown for comparison [A]. Expanded spectra [B].

In Fig. 5.17A spectra of oils derived from the conventional extraction with different solvents at T=room after 24 h are shown. The pattern seems to be analogue to the one relative to olive oil. The spectra of part expanded are shown in Fig. 5.17B.

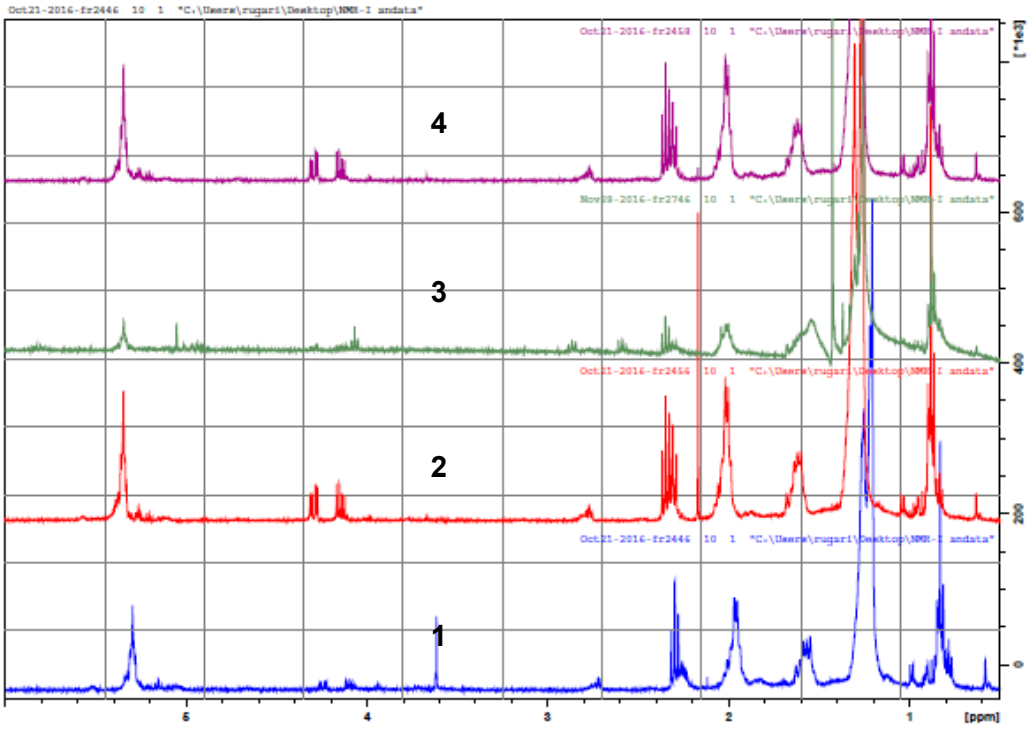


Fig. 5.18 400 MHz ^{1}H NMR spectra of chloroform:methanol (1:1) (1), acetone (2), ethyl acetate (3), hexane (4) extracts at T=room for t=1h

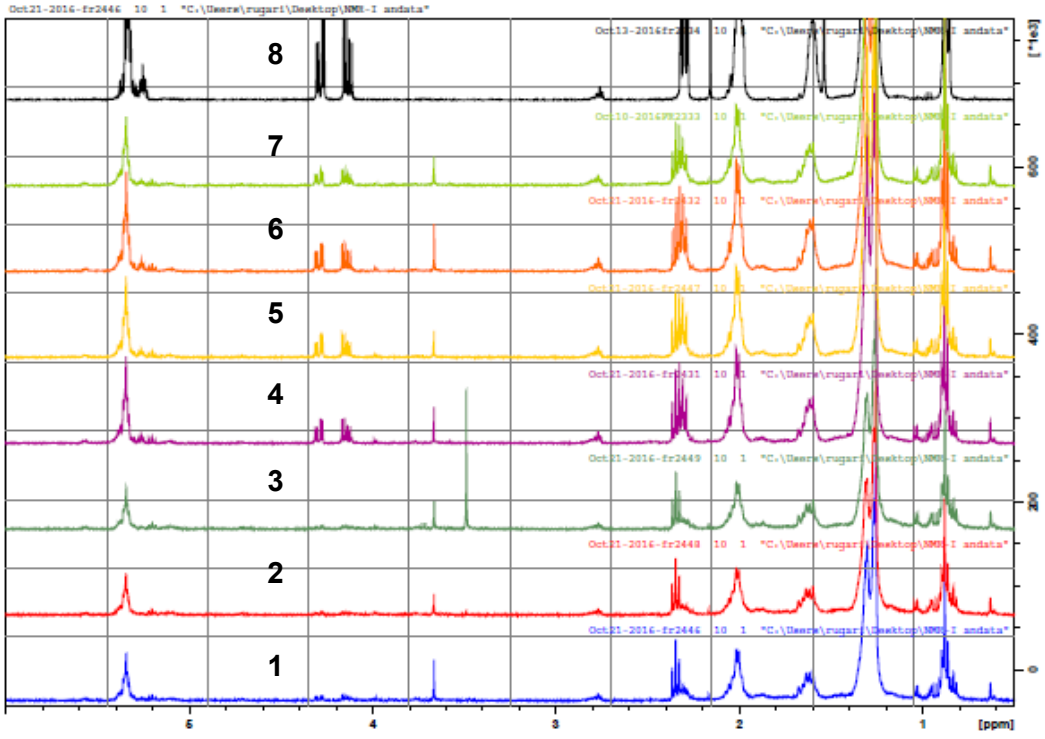


Fig. 5.19 400 MHz ^{1}H NMR spectra of chloroform:methanol varying the time of extraction 1h (1), 2h (2), 3h (3), 4h (4), 8h (5), 16h (6), 24h (7), olive oil (8)

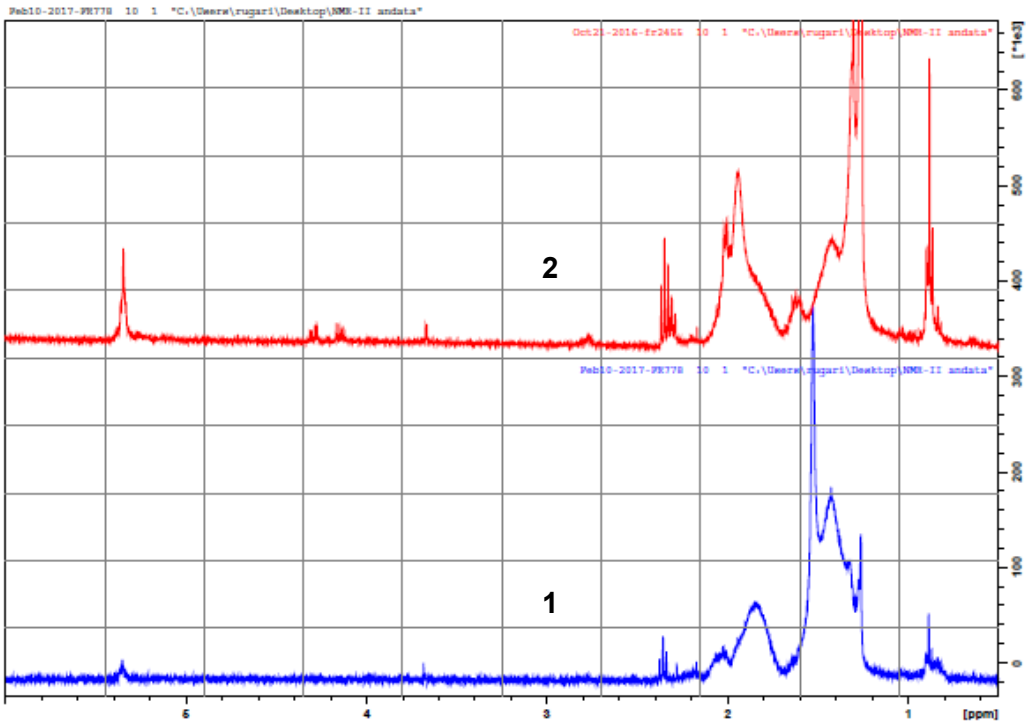


Fig. 5.20 400 MHz ^{1}H NMR spectra of chloroform:methanol (1:1) with a previous step of 1h of hydrolysis with water (1) and with HCl (2) after 4h of extraction

In Fig. 5.18 spectra of oils derived from the conventional extraction with different solvents at T=room after 1 h are shown. Fig. 5.19 illustrated the differences between spectra obtained after $CHCl_3$/MeOH extraction at different times.

In Fig. 5.20 the comparison between a sample subject to a HCl step of hydrolysis and another one subject to an hydrolysis of water both at 80 °C is shown. The latter doesn't show peaks due to triglycerides. The explanation could be the inefficiency of hot water as a pretreatment.

Table 5-2 Conversion of yeast oil to biodiesel according to Knothe (2000)

Fig.	Spectrum	C_{ME} [%]
5.17 A and B	1	4.8
5.17 A and B	4	8.3
5.18 - 5.19	1	17.4
5.19	2	11.3
5.19	3	15.0
5.19	4	5.4
5.19	5	5.4
5.19	6	5.1
5.19	7	4.8

5.5.2 MICROWAVE HEATING

In Fig. 5.21 a comparison between a spectrum from a sample extracted with microwaves and a sample heated in a conventional way is shown.

Results from microwave extraction at 120 °C, 100 °C and 80 °C were represented in Fig. 5.22, 5.23 and 5.24A.

Part expanded spectra in Fig. 5.24B are reported to underline the presence of the triplet related to $\alpha - CH_2$, probably due to the presence of FFA as explained by Satyarthi *et al.,* (2009).

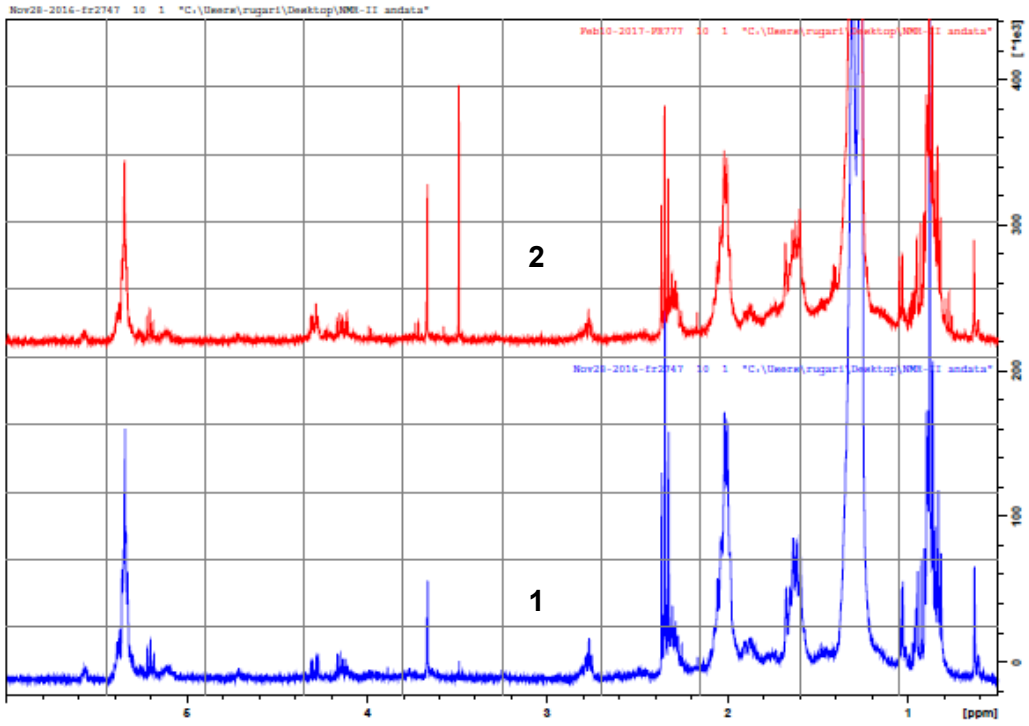


Fig. 5.21 400 MHz ^{1}H NMR spectra of chloroform:methanol after 20 minutes (1:1) with conventional heating (1), with microwaves (2) at 120°C

Results of microwave extraction with ethyl acetate at different hold time were presented in Fig. 5.25.

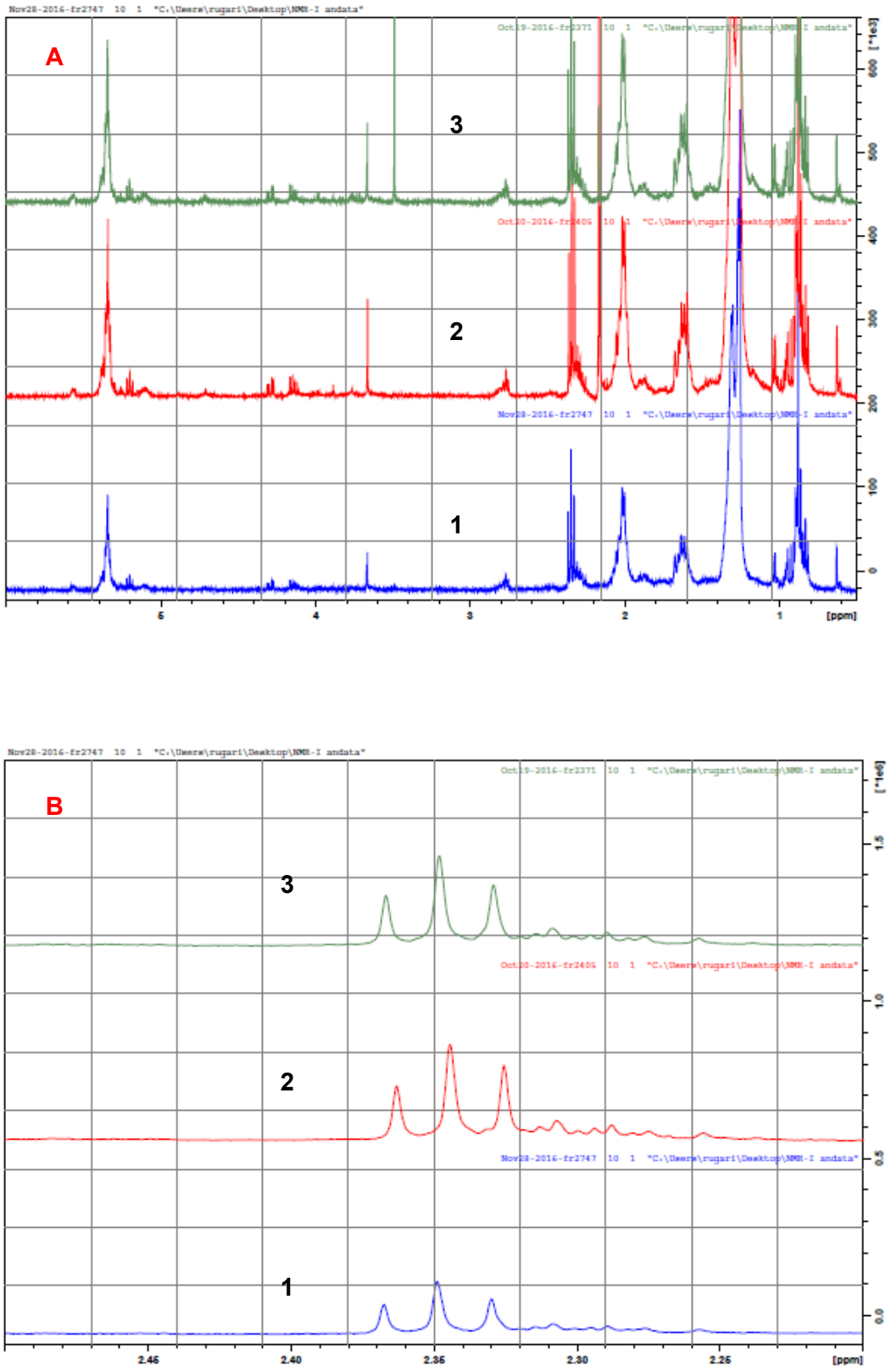


Fig. 5.22 400 MHz ^{1}H NMR spectra of chloroform:methanol (1:1) after 20 minutes (1), 10 minutes (2), 0 minutes (3) with microwave extraction at 120 °C [A]. Part expanded spectra [B]

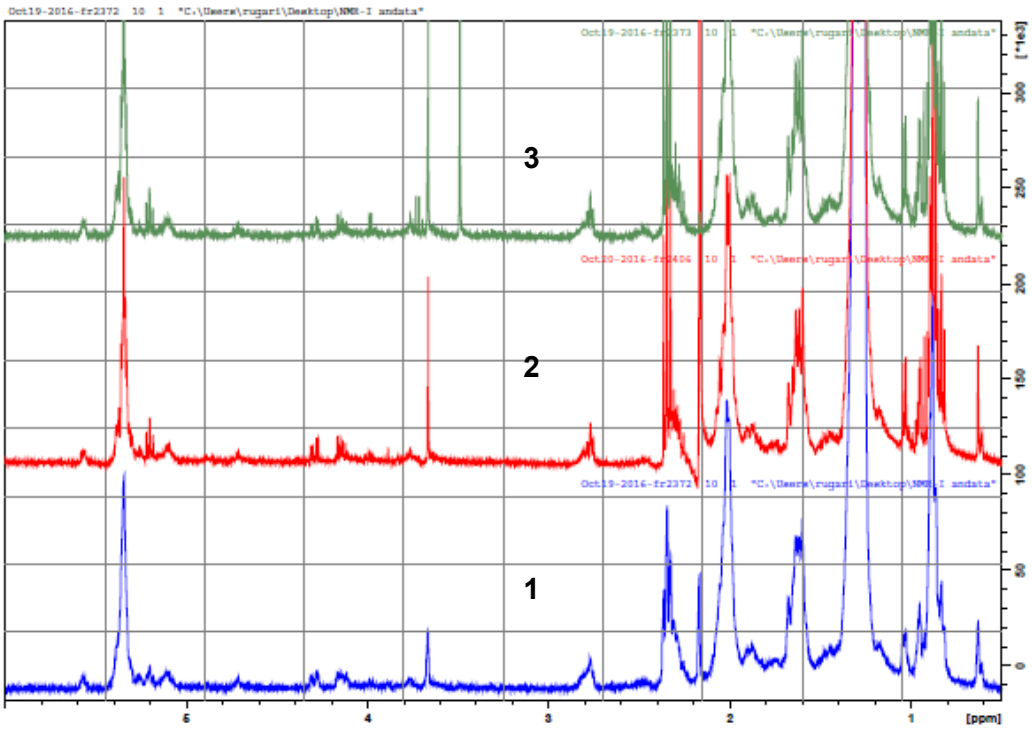


Fig. 5.23 400 MHz ^{1}H NMR spectra of chloroform:methanol (1:1) after 20 minutes (1), 10 minutes (2), 0 minutes (3) with microwave extraction at 100°C

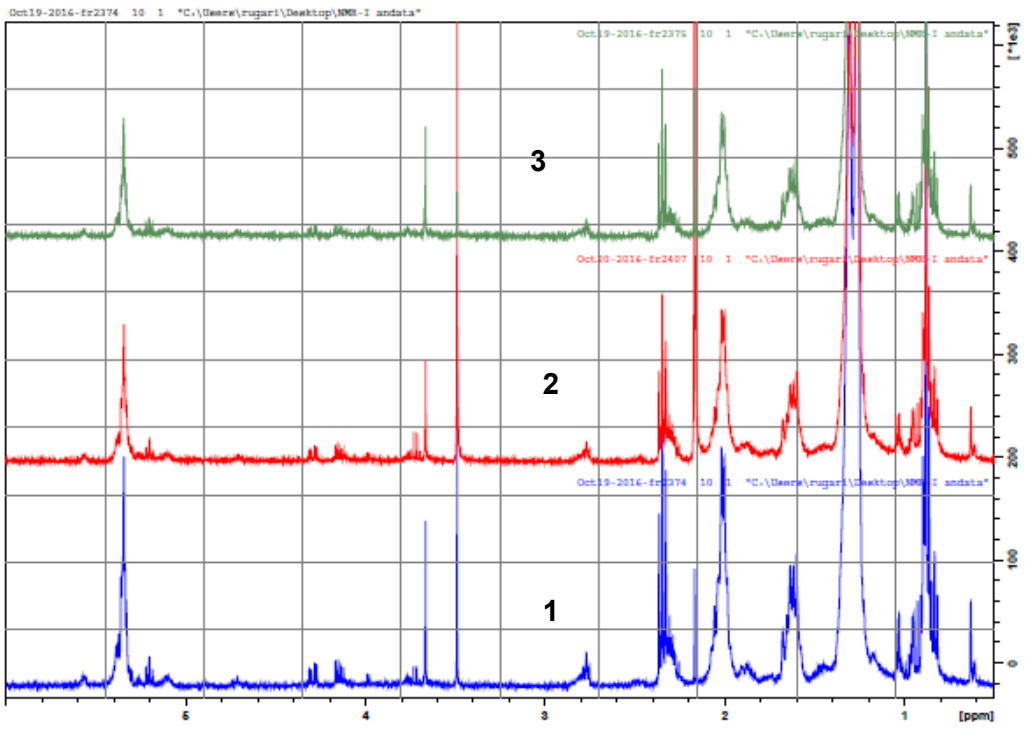


Fig. 5.24 400 MHz ^{1}H NMR spectra of chloroform:methanol (1:1) after 20 minutes (1), 10 minutes (2), 0 minutes (3) with microwave extraction at 80°C

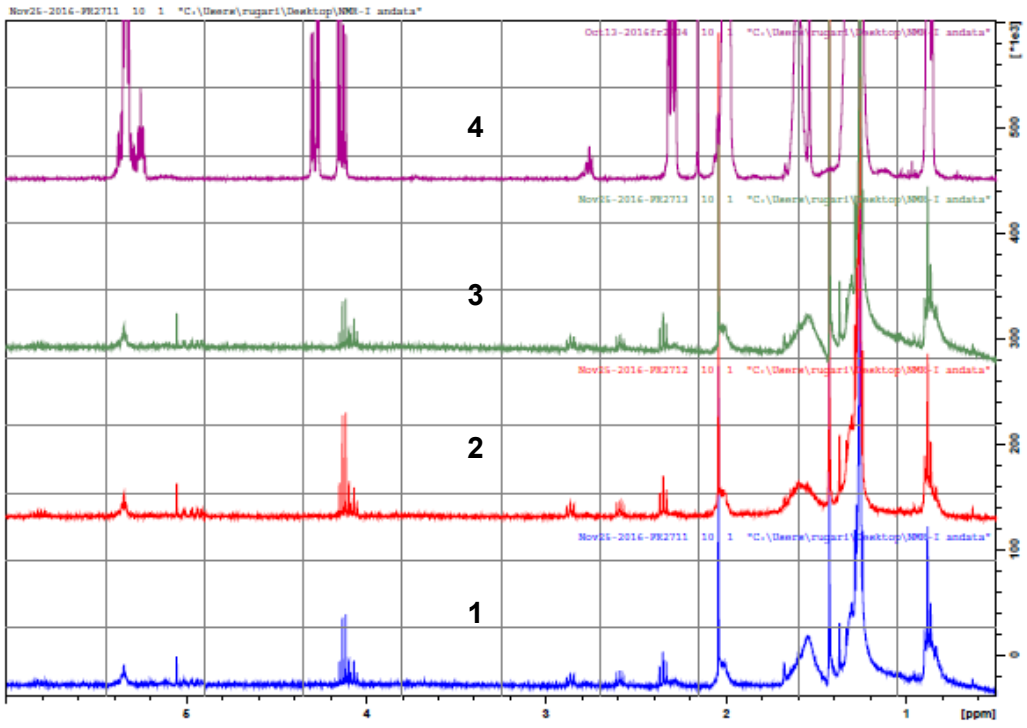


Fig. 5.25 400 MHz ^{1}H NMR spectra of ethyl acetate after 20 minutes (1), 10 minutes (2), 0 minutes (3) with microwave extraction at 120°C and olive oil (4)

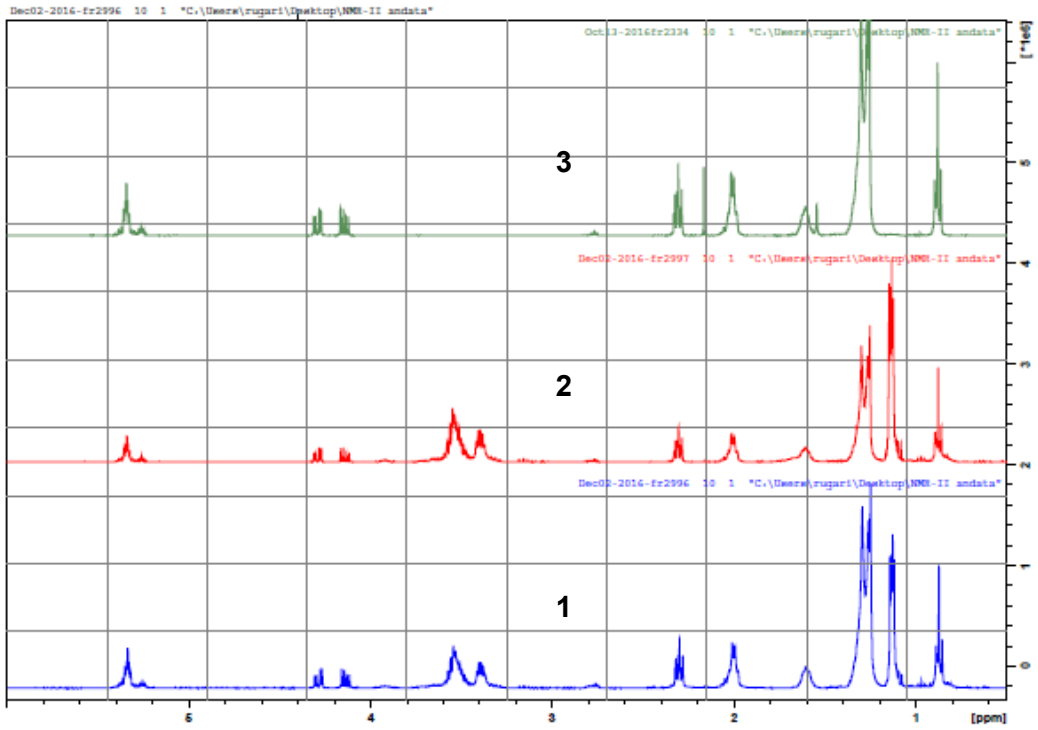


Fig. 5.26 400 MHz ^{1}H NMR spectra of chloroform:methanol (1:1) (1), hexane (2) extract of lipid from MpB biomass after 20 minutes with microwave heating and olive oil (3)

According to Sarpal *et al.* (2014) the peaks in the region 3-4 ppm in the Fig. 5.26 (microwave extraction of lipids from MpB biomass with hexane and $CHCl_3$/MeOH) could belong to phospho/glycolipids.

In Table 5-3 the results obtained after the integration of the interesting peaks are shown.

Table 5-3 Conversion of yeast oil to biodiesel according to Knothe (2000)

Fig.	Spectrum	CME [%]
5.21 and 5.22	1	36.0
5.21	2	9.1
5.22	2	66.7
5.22	3	10.7
5.23	1	13.2
5.23	2	11.6
5.23	3	41.5
5.24	1	16.0
5.24	2	50.6
5.24	3	10.8

5.5.3 DIRECT TRANSESTERIFICATION WITH METSCHNIKOWIA PULCHERRIMA

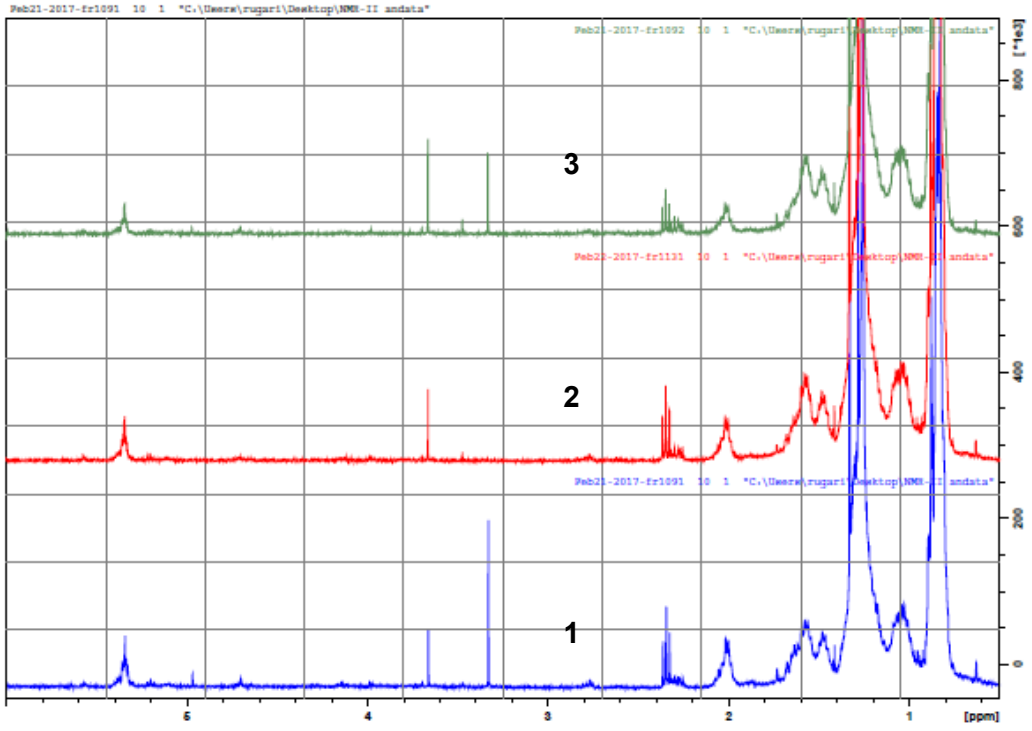


Fig. 5.27 400 MHz ^{1}H NMR spectra of biodiesel samples after a direct transesterification with NaOH (1%wt) after 20 minutes (1) 10 minutes (2) and 0 minutes (3)

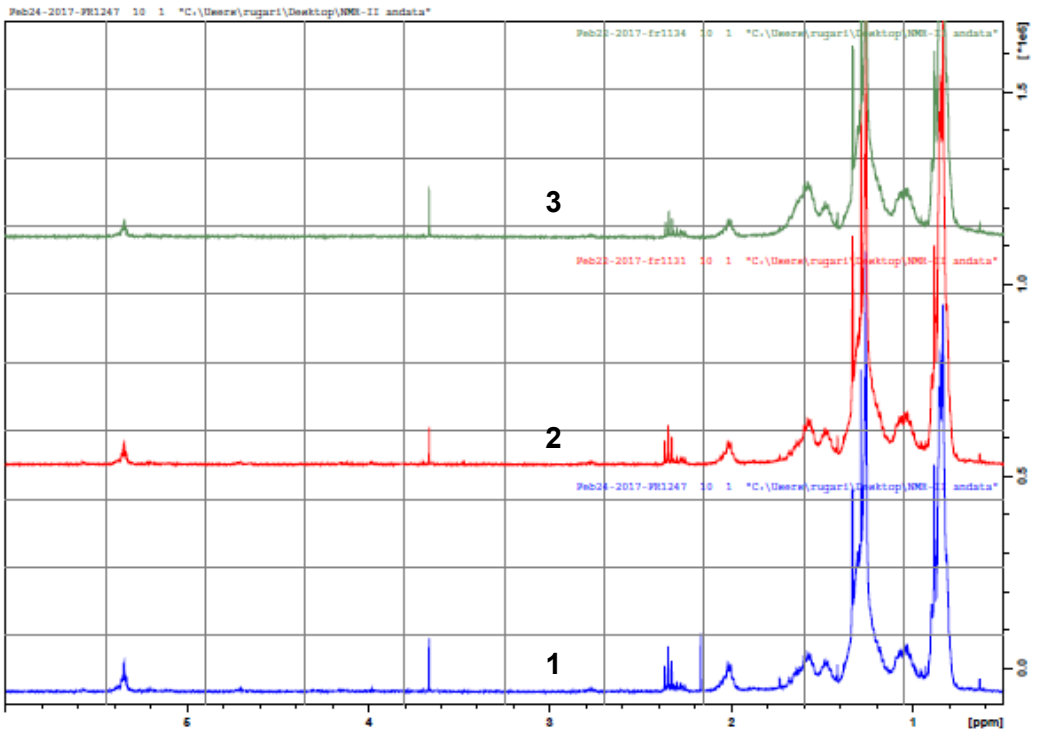


Fig. 5.28 400 MHz ^{1}H NMR spectra of biodiesel samples after a direct transesterification with NaOH (10%wt) after 20 minutes (1) 10 minutes (2) and 0 minutes (3)

In Fig. 5.27 e 5.28 (direct transesterification with 1% wt and 10% wt of NaOH respectively) it is possible to see the lack of the triglycerides. They could be converted in methyl esters.

Table 5-4 Conversion of yeast oil to biodiesel according to Knothe (2000)

Fig.	Spectrum	CME [%]
5.27	1	35.0
5.27	2	15.2
5.27	3	36.5
5.28	1	22.0
5.28	2	6.0
5.28	3	30.4

The spectra presented in this chapter demonstrated to have a lipid profile that is very similar to olive oil.

Biodiesel samples did not show triglycerides peaks and that could be a good indication of the transesterification reaction.

5.6 ENERGY CALCULATIONS

After the investigation of the best solvent in terms of lipid extracted, it is important to search the most convenient one in terms of energy consumptions, using a microwave reactor.

In this chapter energy calculations were made to compare the different experiments conducted in order to understand the most energy efficient method, able to ensure a good yield in terms of lipid extracted.

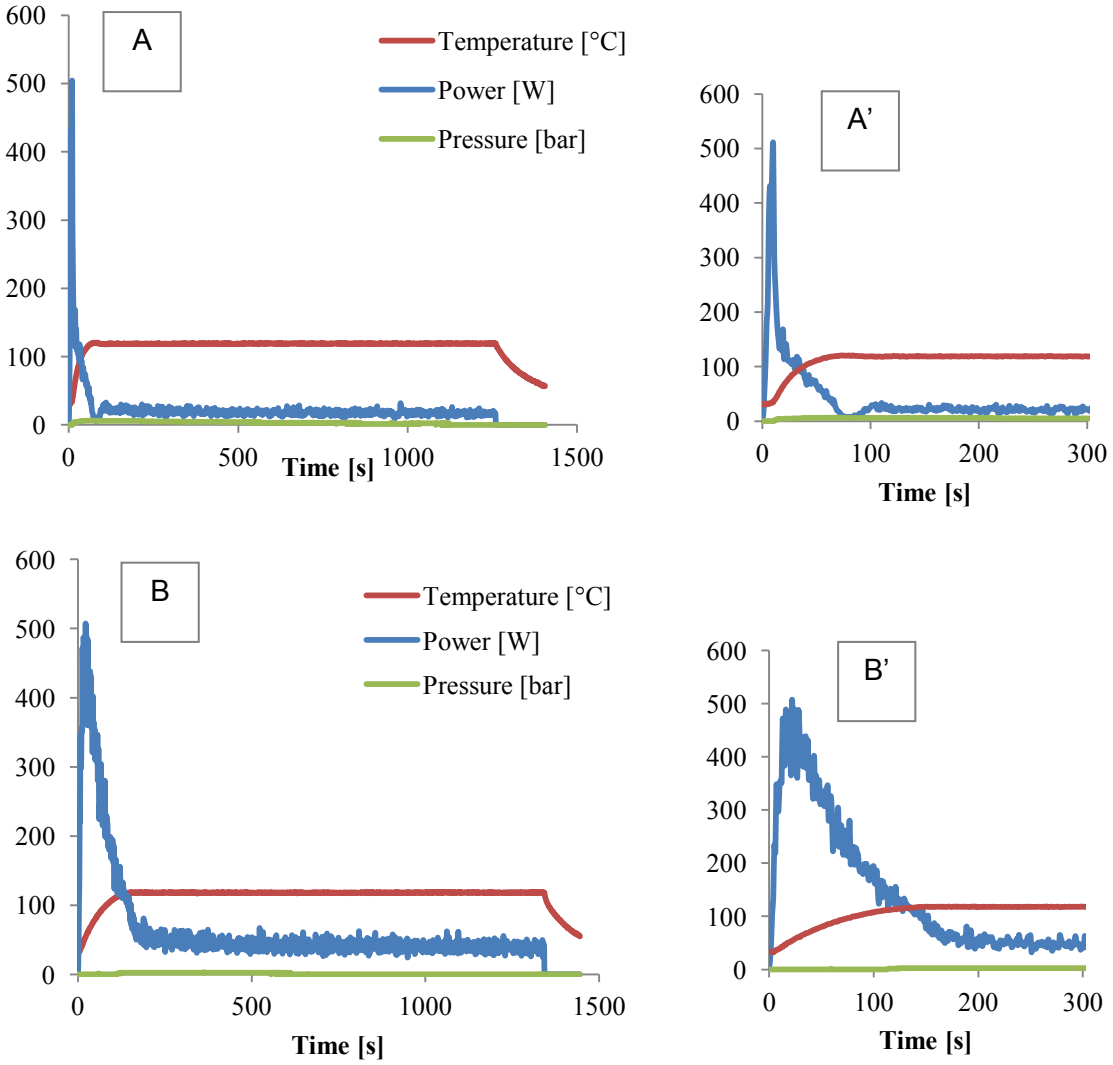


Fig. 5.29 Reaction conditions for Anton Paar monowave 300, showing the pressure, temperature and power of the microwave extraction with $CHCl_3$:MeOH (1:1) at 120°C over 20 min with MpA [A], zoom of the first 5 minutes [A'], with hexane at 120°C over 20 min with MpB [B], zoom of the first 5 minutes [B']

In Fig. 5.29A and A' pressure, temperature and power profiles of a typical experiment in a microwave reactor with $CHCl_3$:MeOH (1:1) are shown. During the initial period of the reaction the pressure and temperature increased gradually as the microwave

heated the samples to the set temperature. In the first minute the power input rapidly increased until the temperature has been reached. Then the power drastically reduced during the reaction. The sample temperature was then held for the length of the reaction (hold time), while the pressure remained almost constant. After the hold time, the system was cooled to 55° C and the pressure returned to the same value of the beginning.

Otherwise in Fig. 5.29B and B' pressure, temperature and power profiles of a typical experiment in a microwave reactor with hexane are shown. They are similar to those related to chloform:methanol (1:1), with the difference that the decrease of the power after the reaching of the set temperature was milder and the constant value of the power during the hold time was higher.

The energy used for the microwave extraction was calculated by integrating the power output using the trapezium rule. It is clear that energy involved in an extraction with hexane is higher than that related to $CHCl_3$/MeOH (1:1).

In order to understand if the higher energy consumption is useful in order to obtain a larger amount of lipids, it is necessary to plot the Energy/Lipid extracted [MJ/g].

The calculation were done considering the lipid average values of three experiments unless otherwise specified.

Values in Fig. 5.30A are related to the energy consumption during test with $CHCl_3$/MeOH (1:1). The highest is 0.011 MJ (20 minutes at 120 °C).

Results in Fig. 5.30B show that with $CHCl_3$/MeOH (1:1), at 0 minutes of hold time it is better to use a temperature of 120 °C, at 10 minutes it is very similar to use a T of 80 °C or 100 °C and at 20 minutes it is better to use a 80 °C. Passing time is better to use a lower temperature.

Values in Fig. 5.31A are related to experiments changing the amount of biomass.

There is no significant difference between the two curves, thus meaning that no much energy consumption are associated to an higher amount of biomass (~ 0.030 MJ after 20 minutes of extraction). Results in Fig. 5.31B demonstrate that from the energetic point of view is better to use a biomass of 0.1 g for an hold time of 0 minutes (0.076 MJ) whereas it is better to use a biomass of 0.5 g for hold time of 10 and 20 minutes (respectively 0.216 MJ/g and 0.423 MJ/g). But from the chemical point of view the situation is the opposite because the volume of solvents is not sufficient, as already shown in Fig 5.12.

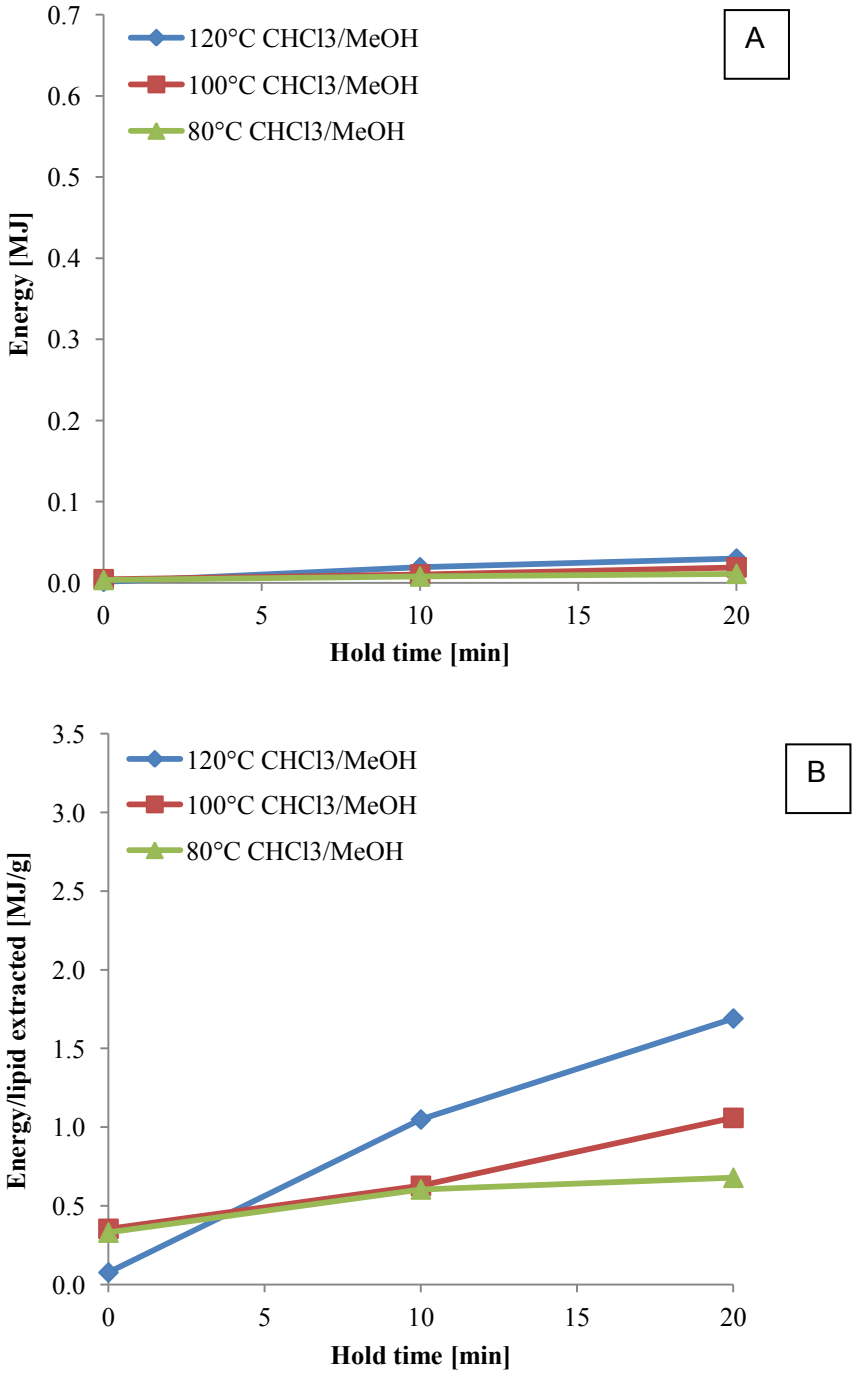


Fig. 5.30 Effect of temperature. Energy [A] - Energy/lipid extracted [B]

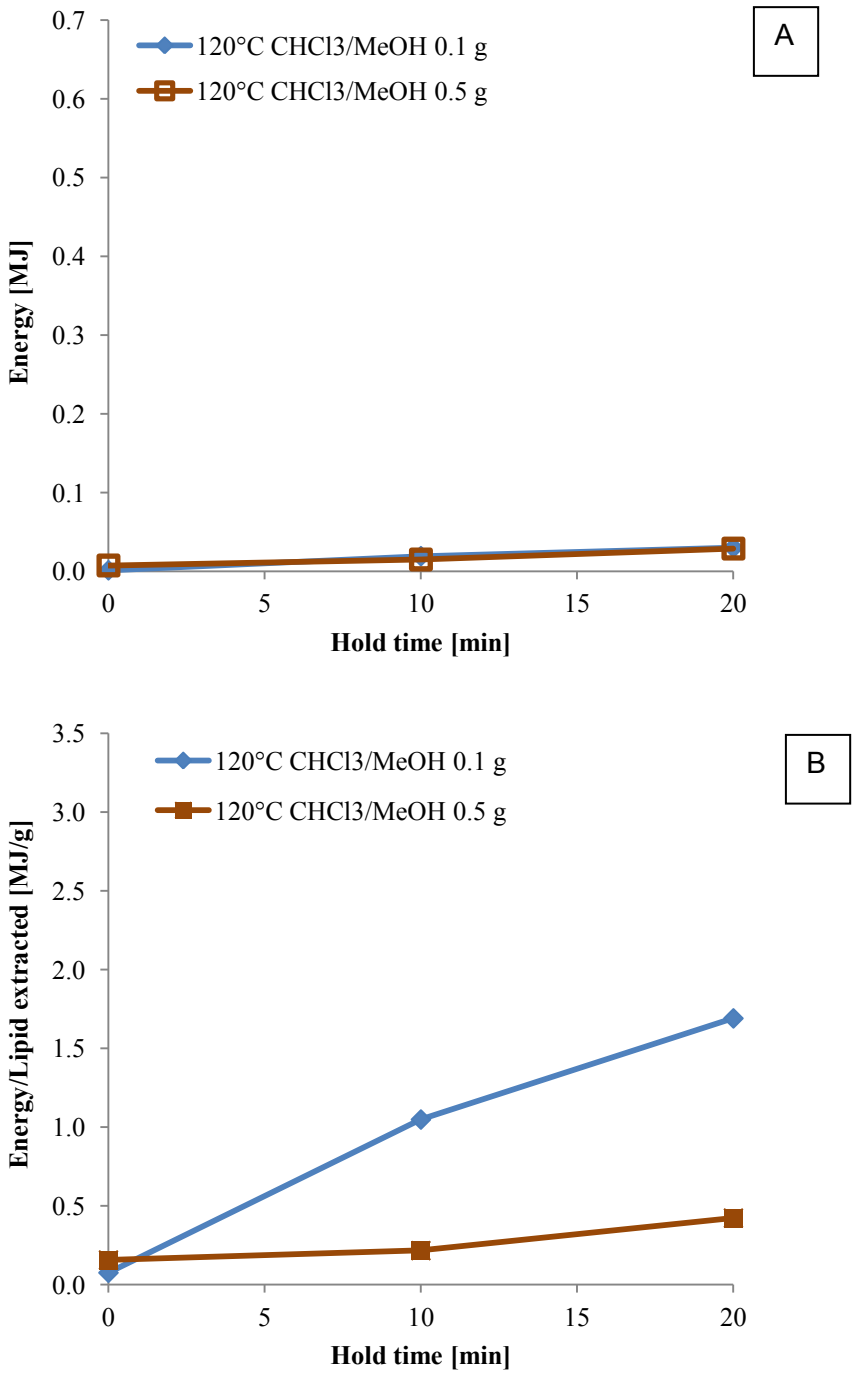


Fig. 5.31 Effect of yeast loading. Energy [A]- Energy/lipid extracted [B]

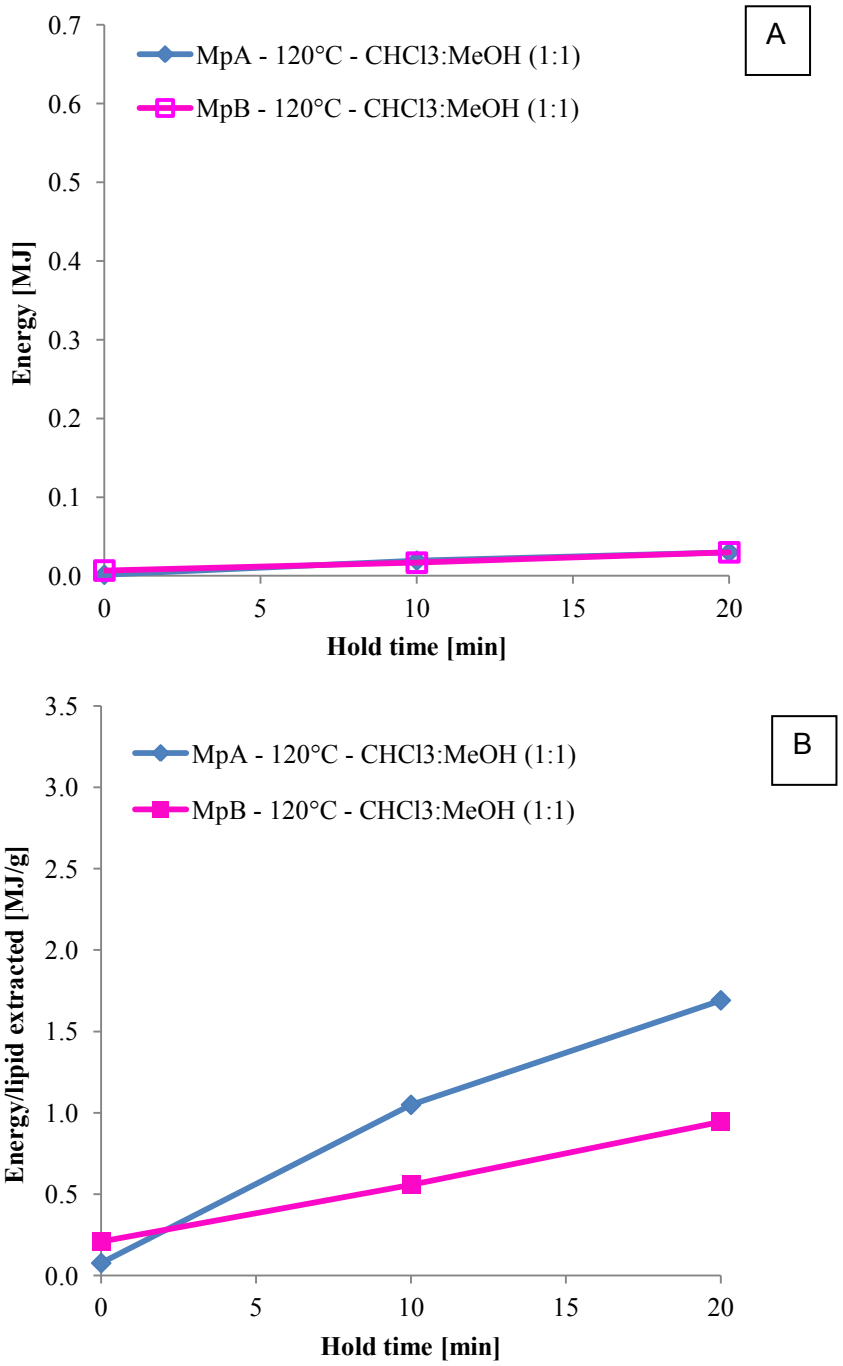


Fig. 5.32 Effect of a different biomass. Energy [A]- Energy/lipid extracted [B] (MpB values are the average of two experiments)

In Fig. 5.32A a comparison between MpA and MpB was shown. There is no significant difference between the two curves. The highest value for both the curve is at 20 minutes and is equal to 0.030 MJ.

Results in Fig. 5.32B demonstrate that from the energetic point of view is better to use a biomass with a higher lipid content for the first minute (0.076 MJ/g). After that, it is much useful to use a biomass with a less content of lipids (0.557 MJ/g and 0.945 MJ/g respectively at 10 and 20 minutes).

In Fig 5.33A it is shown that energy used to extract lipid with $CHCl_3$:MeOH and ethyl acetate are very similar (at 20 minutes, 0.030 MJ and 0.025 MJ respectively). Due to the different yield in terms of lipid extracted, in Fig. 5.33B it is clear that for all the hold times investigated it is better to use a mixture of $CHCl_3$:MeOH (1:1) instead of ethyl acetate (e.g. 1.691 MJ/g instead of 3.025 MJ/g for an hold time of 20 minutes).

The evaluation of the total energy to lead an extraction depends also to the energy demand for the production of the solvents used.

In order to consider this aspect, in Table 5.5 data for the cumulative energy demand of the solvents are shown. The LCA software used is Simapro 7 and the database set for data is Ecoinvent 3. The aim is to have a global average of production for 1 kg of each solvent.

Cumulative Energy Demand v1.09 was used to calculate the embodied energy of each solvent.

Table 5-5 Embodied energy of solvents used

	Embodied energy [MJ/Kg]	Density [g/mL]	Volume [mL]	Energy [MJ]
Chloroform	0.0371	1.470	5	0.2727
Methanol	0.0331	0.792	5	0.1311
Hexane	0.0210	0.655	10	0.1375
Ethyl acetate	0.0713	0.902	10	0.6431

Through the value of density it has been possible to calculate the energy used for the production of the amount of solvent used for each experiment (10 mL).

The cheapest solvent in terms of energy costs is the hexane (0.1375 MJ), whereas the most expensive is ethyl acetate (0.6431 MJ).

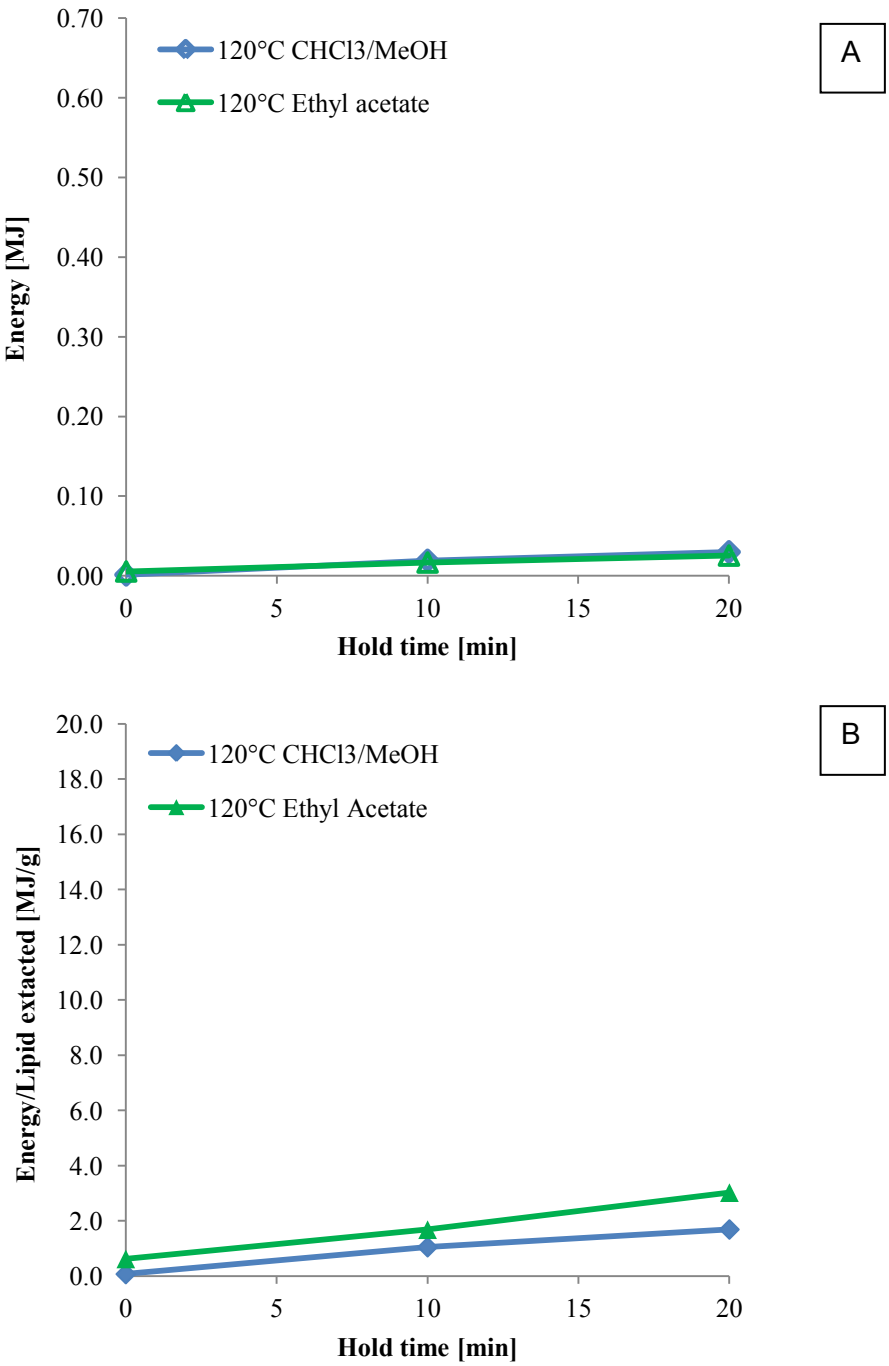


Fig. 5.33 Effect of the solvent. Energy [A]- Energy/lipid extracted [B]

In Fig. 5.34A it is possible to see the plot of the total energy involved in a microwave extraction considering also the energy to produce the solvent, according to the embodied energy of each solvent.

In Fig. 5.34B the graph related to the energy/lipid extracted considering also the energy associated to the production of the solvent are shown. The latter confirm the situation already shown in Fig. 5.33B.

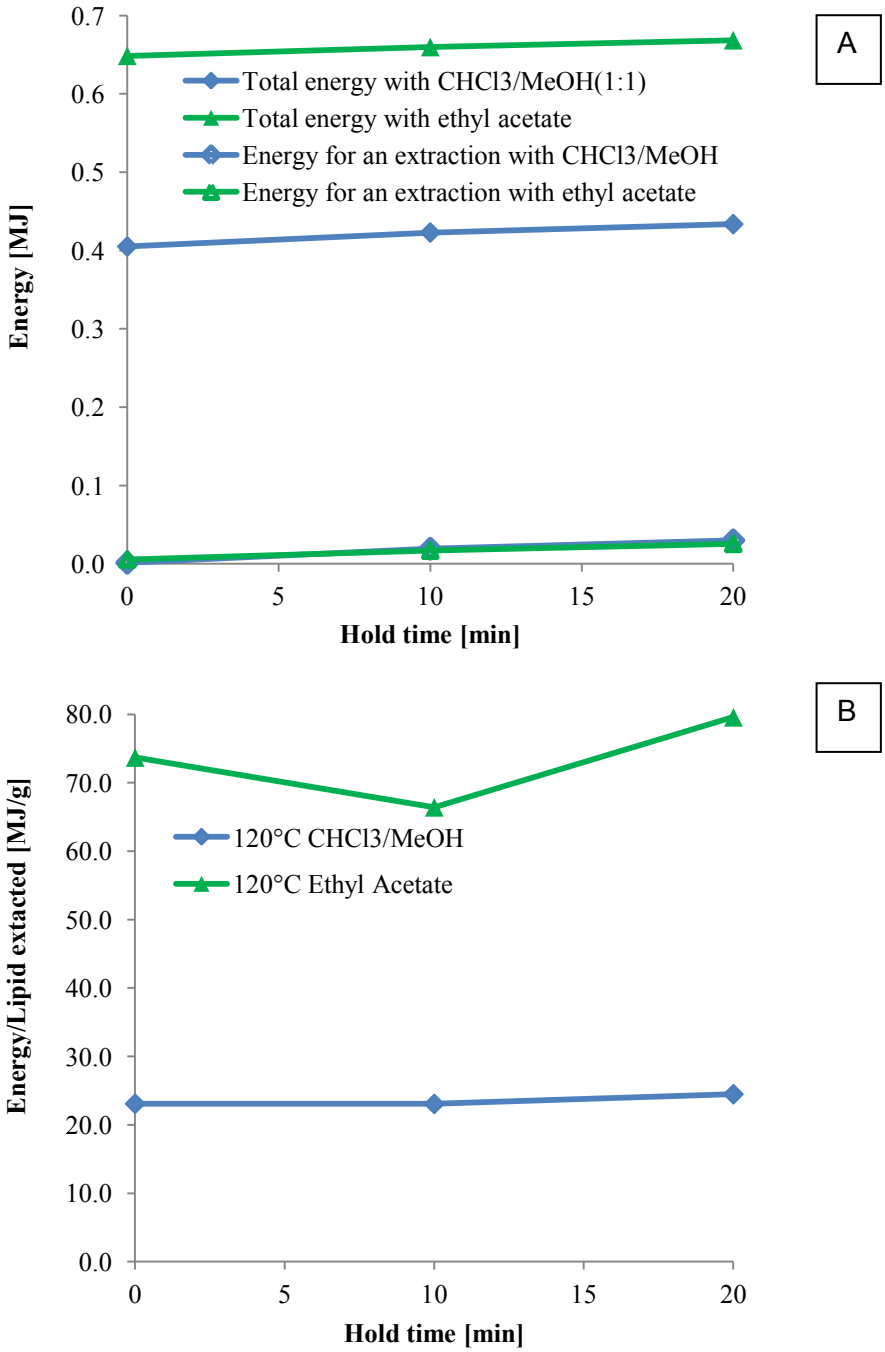


Fig. 5.34 Effect of the solvent. Energy [A]- Energy/lipid extracted [B]

Otherwise it is important to consider that methanol and chloroform are toxic. In a global estimation of energy calculation it would be important reflect also on the costs associated to the waste disposal and the impact factor on the environment using such solvents.

In Fig. 5.35A the graph of the energy used to lead a microwave extraction with MpB with $CHCl_3$:MeOH (1:1) and hexane and in 5.35B the energy/lipid extracted are presented.

Lipid extracted are the average of two experiments (as already specified in §5.3.5).

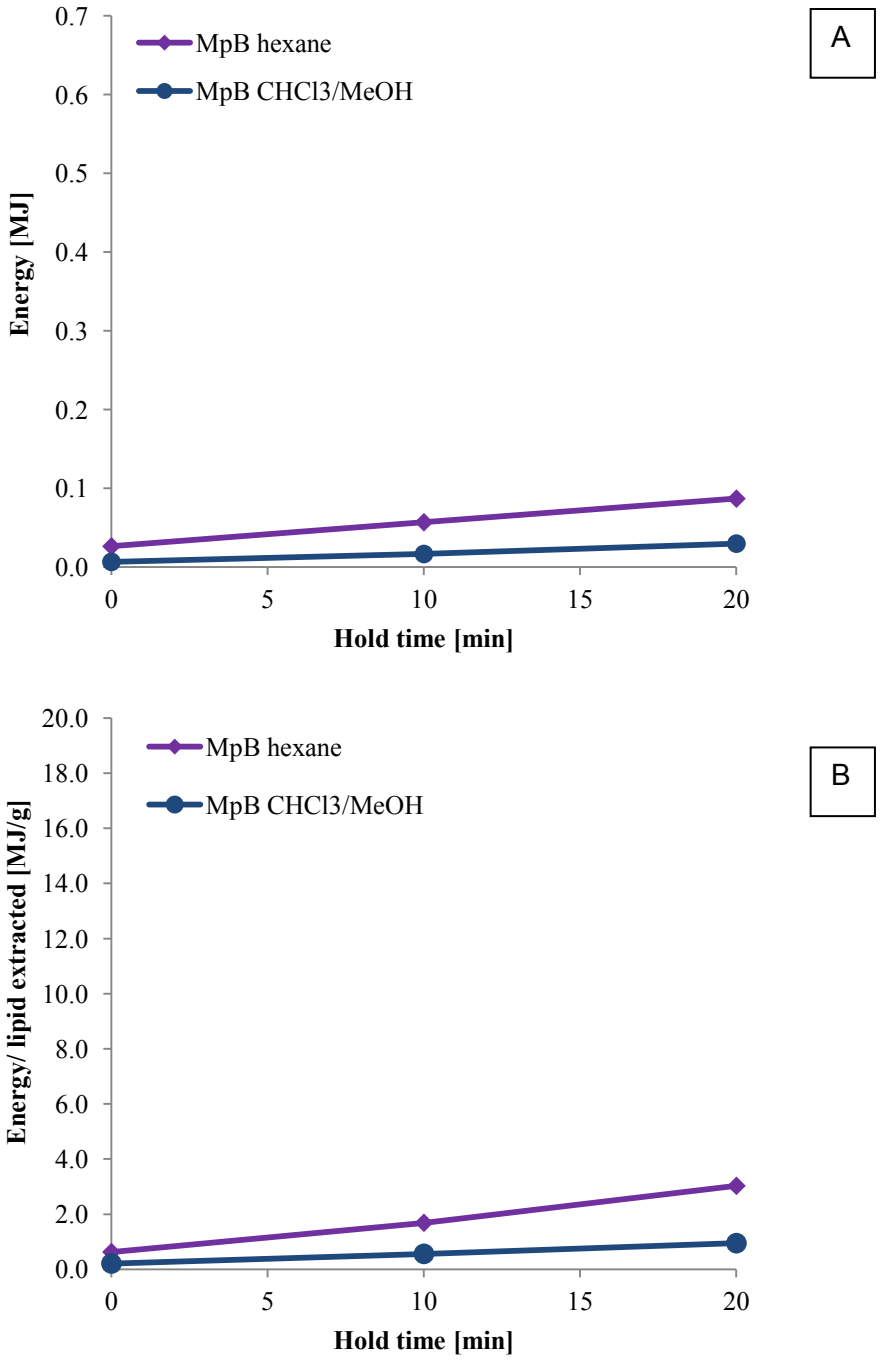


Fig. 5.35 Effect of the solvent. Energy [A]- Energy/lipid extracted [B]

According to the graph in Fig.5.35A, $CHCl_3$/MeOH (1:1) is at any time advantageous than hexane (respectively, from 0 minutes to 20 minutes, 0.0067 MJ, 0.0167 MJ and 0.0299 instead of 0.0265 MJ, 0.0570 MJ and 0.0870 MJ). The same can be said for graphs in Fig. 5.35B, where for 20 minutes the highest value is for hexane (3.025 MJ).

In Fig.5.36A the graphs considering also the energy associated to the production of the solvents were plotted. Due to its low energy demand of production, the total energy of an extraction with hexane is lower than that associated to $CHCl_3$/MeOH.

It is interesting to notice that, considering the embodied energies, the situation shown in Fig. 5.35B is different from Fig. 5.36B. In the latter, it is revealed that using hexane is advantageous for extractions with an hold time lesser than almost 10 minutes. After that, it is more convenient to use $CHCl_3$/MeOH (1:1). The most convenient values are related to hexane at 0 minutes (12.728 MJ/g) and 10 minutes (13.371 MJ/g) whereas at 20 minutes it is better to use $CHCl_3$/MeOH (1:1) (13.722 MJ/g).

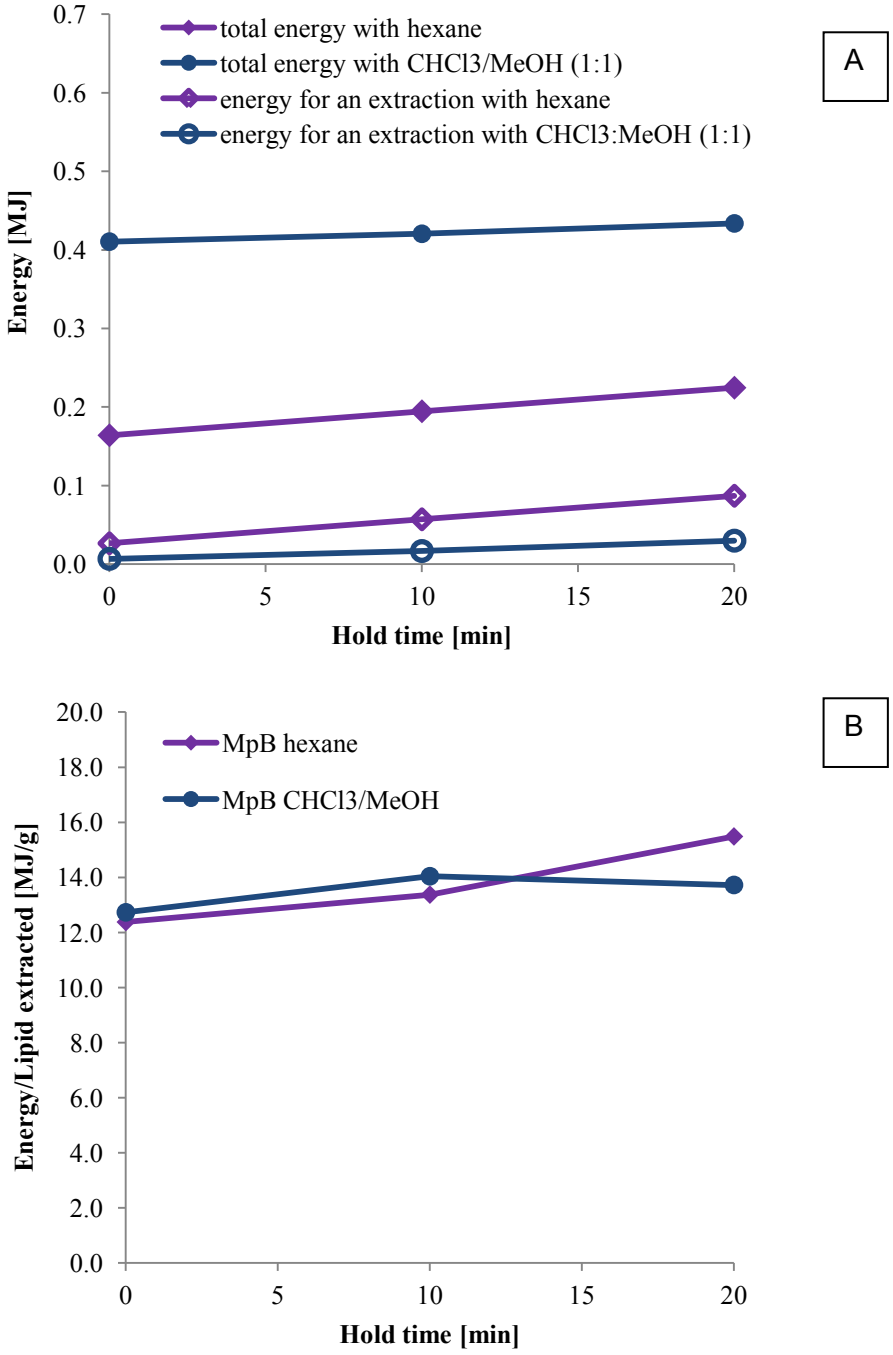


Fig. 5.36 Effect of the solvent. Energy [A]- Energy/lipid extracted [B]

5.7 DIRECT TRANSESTERIFICATION WITH LIPOMYCES STARKEY

5.7.1 CONVENTIONAL HEATING

Samples of Ls has been used to lead direct transesterification with conventional heating.

In Fig. 5.37 biomass profile has been shown, obtained via spectrophotometry. Dilutions of (1:10) have been made after the first 24 hours.

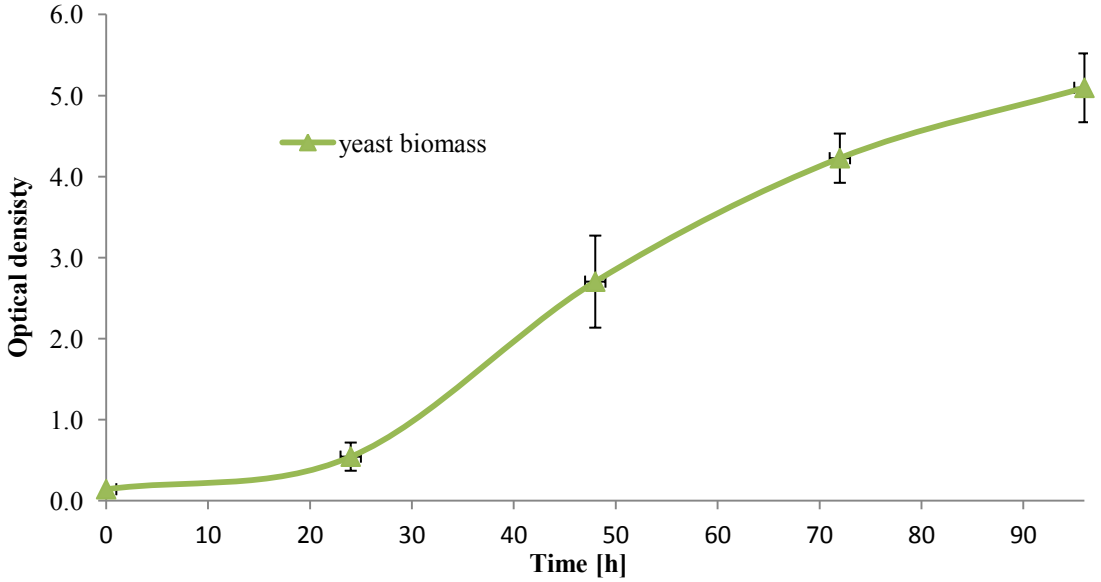


Fig. 5.37 Yeast biomass profile of Ls

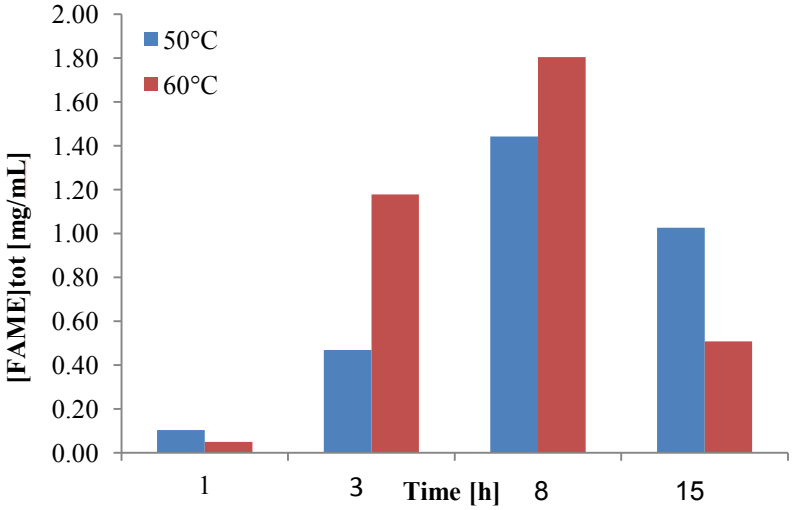


Fig. 5.38 Effect of temperature and time on FAME yields. Reaction conditions: 1:20 biomass:MeOH, 4g/L NaOH

In Fig. 5.38, results of preliminary tests (1 test for each time) are shown. The aim of those tests were to understand if it is possible to lead a direct transesterification with conventional heating at temperatures equal to 50 °C and 60 °C.

The total concentration of fatty acid methyl esters $[FAME]_{tot}$ is calculated as follows:

$$[FAME]_{tot}=\sum[FAME]_i \quad (5.3)$$

Where $[FAME]_i$= Concentration of each fatty acid methyl ester individuated [mg/mL]

The best results were been obtained at 60°C after 8 h of reaction. Substances were identified via gas-chromatography by comparison of their retention times with those of standards of FAMEs and the concentrations of each ester were calculated by means of molar attenuation coefficients obtained after calibration lines.

In the following Table 5-6 the fatty methyl esters analyzed are listed:

Table 5-6 Fatty acid methyl esters

	Fatty acids methyl esters
C14:0	Methyl Myristate
C16:0	Methyl Palmitate
C18:0	Methyl Stearate
C18:1	Methyl Oleate
C18:2	Methyl Linoleate
C18:3	Methyl Linolenate
C20:0	Methyl Arachidate

The results paved the way to the possibility of leading a direct transesterification.

5.7.2 MICROWAVE HEATING

5.7.2.1 Numerical dosimetry for microwave exposure

Before starting with the tests of direct transesterification with microwaves, numerical dosimetry was carried out to determine the optimal exposure conditions of yeast's samples to an electromagnetic field at a frequency of 2.45 GHz and an intensity able to increase the sample temperature from the ambient to about 50 °C. These optimal conditions (position and orientation) can provide high efficiency (in terms of absorbed power respect incident power) and homogeneity of electromagnetic field distribution into the sample.

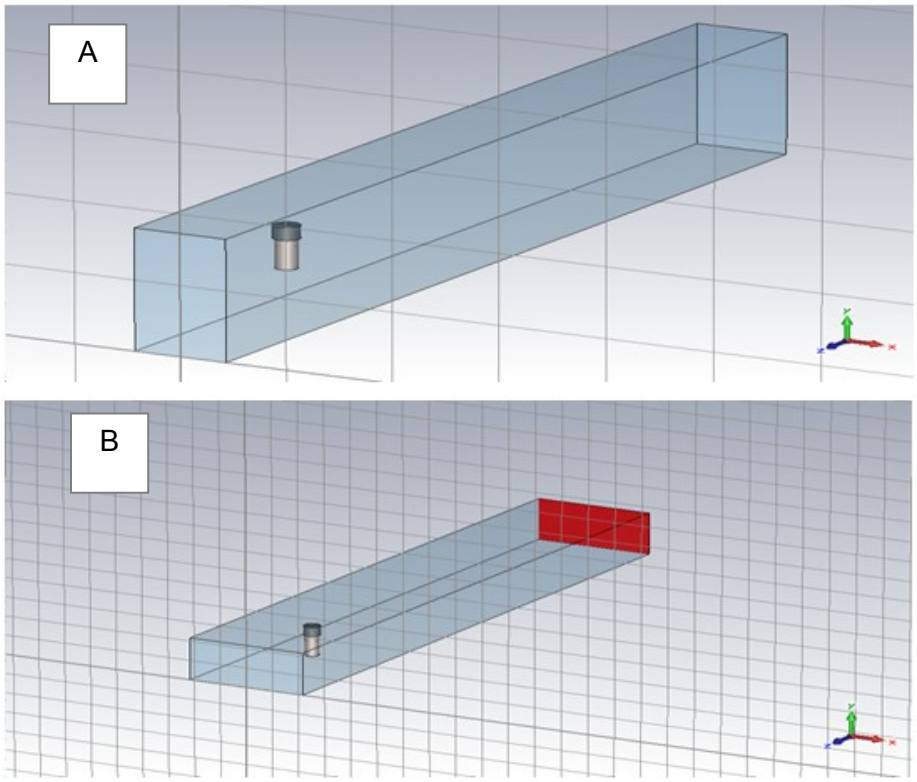


Fig. 5.39 Configuration 1 [A] – Configuration 2 [B] 3D-Model (CST-Microwave studio)

For this purpose, the simulation platform CST - Microwave Studio was employed. It allowed to define a 3D model of the biological sample and the applicator used for the exposition (Fig. 5.39A and B).

Samples were simulated as methanol in a glass container with a polypropylene cap and modeled as perfectly cylindrical.

Table 5-7 Relative permittivity, electrical conductivity and density

Material	ε'	σ [S/m]	ρ [kg/m^3]
Glass vial	4.82		
Polypropylene cap	2.2		
Methanol at 25°C	23.44	1.719	792

Dielectric properties (relative permittivity and electric conductivity) of the methanol and the basic catalysts NaOH and KOH dissolved in methanol were measured by means of an open-ended coaxial probe. The results are reported in the following paragraph. Dielectric properties of other materials (sample holders) were gathered from the literature. The software CST - Microwave Studio allows to calculate specific absorption rate:

$$SAR = Pa/m \quad [W/Kg] \tag{5.4}$$

being Pa the absorbed power [W] and m the mass [Kg] of the sample.

Assuming negligible the power lost on the walls of the applicator, SAR is proportional to conductivity and density of the sample as well as to the intensity of the electric field. SAR is related to the increase of the temperature induced by the absorption of electromagnetic energy too:

$$SAR = c\,\Delta T/\Delta t \quad [W/Kg] \tag{5.5}$$

where c is the specific heat of the solution (0.601 cal/g·°C at 15-20 °C (Perry *et al.*, 1984, which corresponds to 2515 J/Kg·°C), ΔT is the temperature increase [°C] due to RF exposure evaluated over the time interval Δt [s].

The optimum exposure condition obtained in terms of the best trade off between efficiency and homogeneity of the SAR distribution is when the sample is placed inside the rectangular waveguide at 18.3 cm from the short and on the base of the base wall.

In Fig. 5.40 the temperature profile in the adopted conditions (SAR= 50.3 W/kg) is reported for an exposure to microwave equal to 1 h.

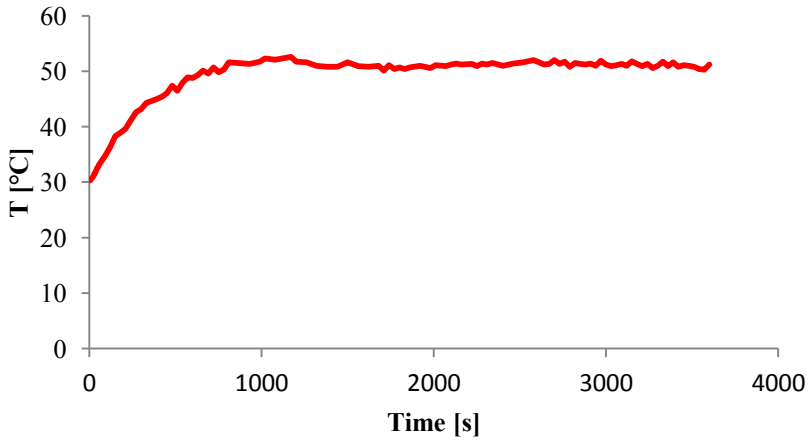


Fig. 5.40 Temperature profile in a waveguide, for an exposure of 1 h at T= 50 °C

5.7.2.2 Permittivity runs

Permittivity is one of the most important properties useful to understand the characteristics of the medium subjected to microwave exposition.

Tests were conducted on the methanol and the basic catalysts NaOH and KOH dissolved in methanol by means the open-ended coaxial probe technique. The reflection coefficient at the probe tip immersed into the sample is measured by means of a network vector analyzer. The temperature was again controlled in a non perturbative way by a fiber optic thermometer.

The graphs related to the real and imaginary part of the permittivity $\varepsilon = \varepsilon' - i\varepsilon''$ as a function of frequency are reported in the following figures. Referring to the methanol at a temperature of 25 °C they were compared with that reported in literature (Fig. 5.41 A and B).

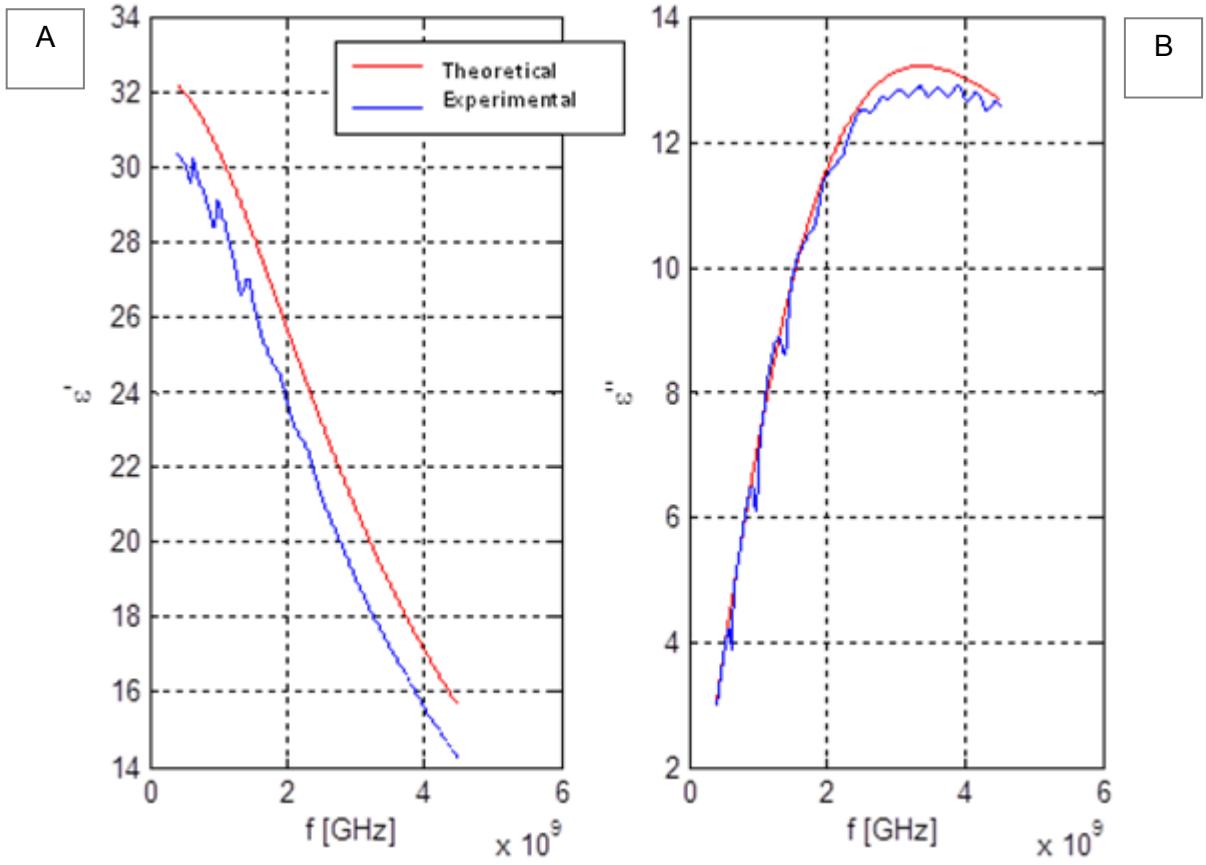


Fig. 5.41 Graphs related to Ɛ'[A] and Ɛ''[B] referring to methanol at T=25 °C

The real part represents the reflection of the microwaves by the sample, while the imaginary part gives an indication on the absorption. The results are in agreement with literature data (Romeo *et al.*, 2011).

Following tests were carried out with the basic catalysts NaOH and KOH dissolved in methanol. For these tests it was not possible to make a comparison with the literature data and the results were reported in Fig. 5.42 A and B and compared with those of only methanol.

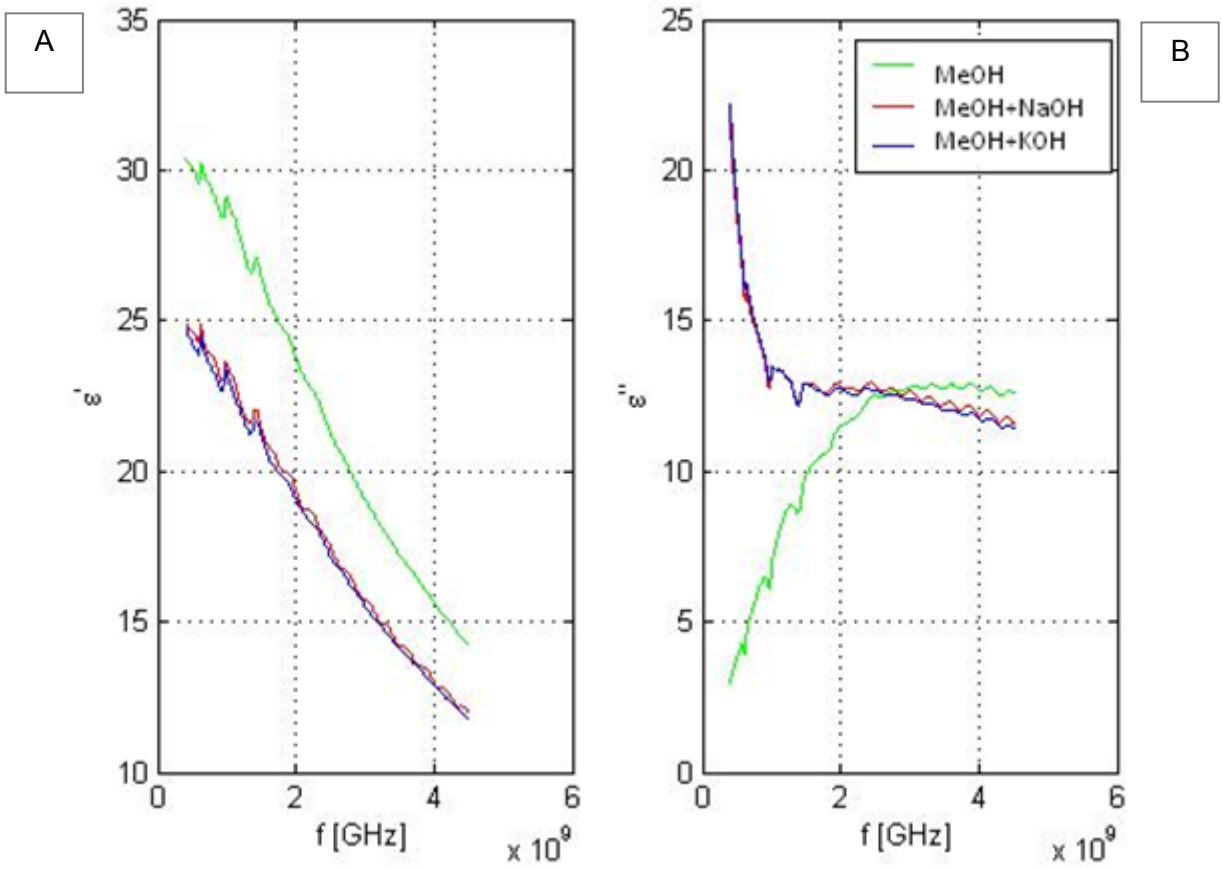


Fig. 5.42 Graphs related to Ɛ'[A] and Ɛ''[B] referring to methanol, methanol + NaOH and methanol + KOH

As regards Ɛ', the trend of curves with the salts is similar to that of methanol alone. On the contrary the conductivity of salts influence the Ɛ' behavior at low frequencies, however there is an intersection of the curves at about 2.45 GHz, the working frequency that it is typically adopted for microwave heating. This feature allows to consider a similar behavior of the three solutions when exposed at 2.45 GHz microwaves.

5.7.3 COMPARISON BETWEEN CONVENTIONAL AND MICROWAVE HEATING

Following tests were conducted to investigate the differences between the results obtained heating the samples with conventional heating or with microwaves.

Temperature chosen was 50 °C and the time of reaction 3 h.

The yields in terms of fatty acid methyl esters are calculated considering the amount of lipids in the dry sample of yeast obtained after an extraction using Bligh&Dyer's method (1959). These tests are made only to have a calculation basis.

Gravimetrically it has been found that almost 13% of the biomass are lipids.

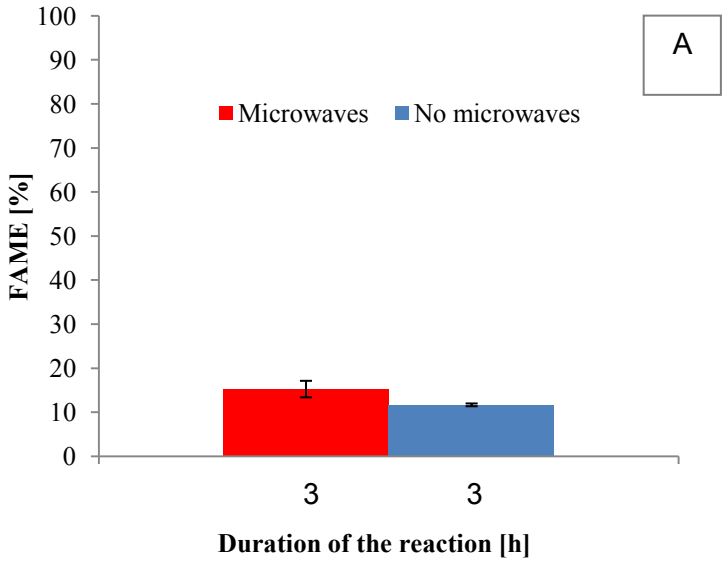
Results are shown in Fig. 5.43A and summarized in Table 5-8.

FAME are calculated as follows:

FAME [%]=FAMEtot/Lipids [mg/mg] (5.4)

Table 5-8 Results of the direct transesterification

Sample	t [h]	[FAME]tot [mg/mL]	FAME [%]	St.dev.
Microwaves	**3**	4.0	15.3	1.87
No microwaves	**3**	3.0	11.7	0.32



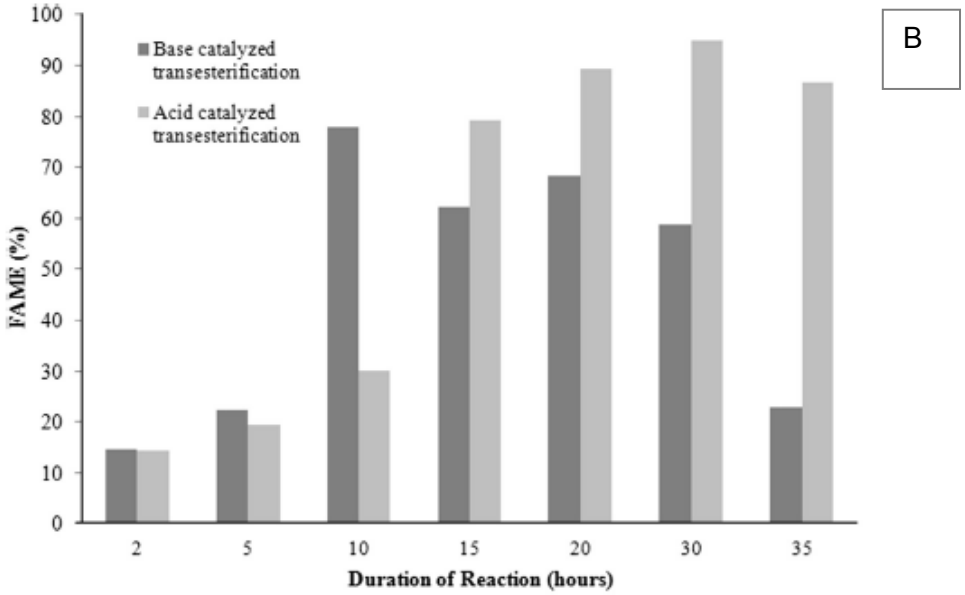


Fig. 5.43 FAME yields after 3h of reaction using NaOH) catalyst under the same operating condition with and without microwaves. Data points show the averages from duplicate analyses [A]

FAME yields from Thliveros *et al.*, 2014. Data points show the averages from duplicate analyses and the standard deviation <1%.[B]

Results obtained are comparable with the data illustrated by Thliveros *et al.* (2014). Results related to the microwave heating were higher in comparison to conventional heating, as shown in Fig. 5.43A and B.

To understand the lipid profile, the yield [%] in Fig. 5.44 A and A' is given by:

$$FAME_i = [FAME]_i/[FAME]_{tot} *100\ [\%] \quad (5.5)$$

Where $[FAME]_i$ is the concentration of each fatty acid methyl ester [mg/mL] and $[FAME]_{tot}$ is the concentration of all esters individuated [mg/mL]

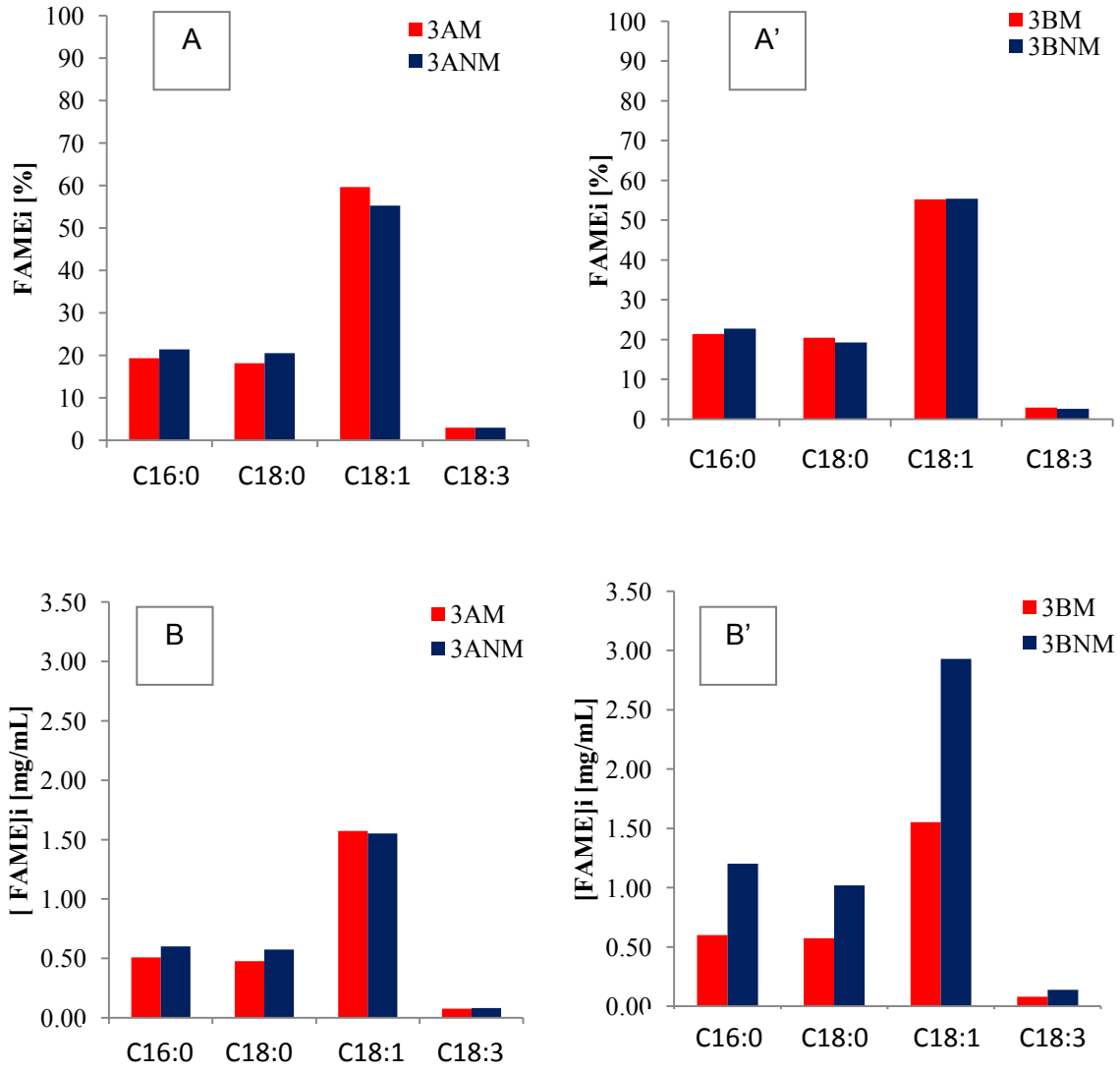


Fig. 5.44 Lipid profile [A][A'] and concentration of FAME [B] [B'] after 3h of reaction
3AM-3BM= samples subject to microwaves
3ANM-3BNM=samples subject to conventional heating

Fatty acids profiles obtained are partially comparable with those by Wild *et al.,* (2010), due to the highest percentage of (C18:1) and a relatively high percentage of (C16:0).

6. SUMMARY AND CONCLUSIONS

In this thesis some experiments were conducted on *Lipomyces starkeyi* and *Metschnikowia pulcherrima* in order to investigate the possibility of obtaining microbial oils and biodiesel.

The first results concerning the application of pulsed electric fields on Ls samples gave some indications about the ideal concentration of the cell suspension (12.5 mg/mL) and PI (0.005 mM) to obtain clear imagines by means of a confocal microscope. Furthermore, using a treatment of 800 V, 2 Hz, 40 pulses of 2 ms it has been possible to notice differences between a sham and an electroporated sample. The latter showed a higher fluorescence, attributable to the access of the dye to the inner part of the cell, probably due to the effects of the application of pulses.

Further applications are needed to understand the role of electroporation on yeast cell's wall and to confirm the results obtained. It is of pivotal importance to recognize if the cells are irreversibly disrupted by pulsed electric fields or not. If the cells are only temporarily electroporated, the variable time must be investigated.

The results obtained are promising for future applications of this technique as a pretreatment for the extraction of lipids, even if other tests are needed.

Concerning the conventional lipid extraction on MpA, it has been found that at room temperature the best solvent is the mixture $CHCl_3$:MeOH (1:1), with 20.6% of lipid extracted after 4 h. Raising the temperature to 120 °C can decrease the time of extraction (23.4% after 20 minutes of extraction). Using a molar ratio of (2:1) between $CHCl_3$ and MeOH has been useful only until 10 minutes on extraction.

Concerning the microwave extraction, preliminary tests were made to investigate the absorption of several solvents. $CHCl_3$:MeOH (1:1) is the fastest solvent to heat up whereas hexane is the slowest due to its non-polarity. MIBK, 2Me-THF and Limonene/EtOH (1:1) are also promising green solvents due to their rapidity to become warm, but the latter has got a very high boiling point.

The best results obtained in terms of lipid extracted with MpA are those related to $CHCl_3$:MeOH (1:1) at 120 °C already at 0 minutes of hold time and with a yeast loading of

0.1 of biomass. Also with MpB biomass the ideal solvent was $CHCl_3$:MeOH (1:1). It is interesting to notice that using 2Me-THF at 80 °C is better than 120° C.

^1H NMR data demonstrated the similarity of microbial lipid profile to vegetable oils.

Energy calculations were done both with MpA and MpB biomass, in the first case investigating variables such as effect of temperature, solvent and yeast loading.

The most interesting results showed that according to the energetic point of view, hexane could be a good solution using MpB biomass, due to the low energy costs associated to its production. However its high toxicity would require its replacement with greener alternative solvents. Energy calculations about conventional heating are needed.

Direct transesterification reaction with Ls samples inside a waveguide showed encouraging results. Further experiments are required to better investigate the possibility of increasing the yield in terms of FAME, optimizing the culture broth. On the subject of the direct transesterification in a microwave reactor on MpA samples, ^1H NMR spectra showed the absence of tryglycerides peaks and this result is comparable with biodiesel spectra in literature.

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