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Single Cell Oil production: a new approach in biorefinery

Thesis by
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Single Cell Oil production: a new approach in biorefinery

by

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Penso che sia necessario educare le nuove generazioni al valore della sconfitta.
Alla sua gestione. All'umanità che ne scaturisce.
A costruire un'identità capace di avvertire una comunanza di destino, dove si può fallire e ricominciare senza che il valore e la dignità ne siano intaccati.
A non divenire uno sgomitatore sociale, a non passare sul corpo degli altri per arrivare primo. In questo mondo di vincitori volgari e disonesti, di prevaricatori falsi ed opportunisti, della gente che conta, che occupa il potere, che scippa il presente, figuriamoci il futuro, a tutti i nevrotici del successo, dell'apparire, del diventare.
A questa antropologia del vincente preferisco di gran lunga chi perde.
È un esercizio che mi riesce bene.
E mi riconcilia con il mio sacro poco.
(P. P. Pasolini)

ABSTRACT

A socially responsible economic growth, devoted to the future generations, requires long-term secure and available resources for industrial production, in terms of raw materials, energy and water. It should be environmentally friendly and a forward-looking financial system capable of future challenges with a global point of view. However, the present economic model based on the non-renewable fossil resources (oil, natural gas, coal, minerals) for energy and industrial production is the reason of energy instability, climate changes and therefore it cannot be considered sustainable.

In this context, biotechnological techniques, such as the biorefinery are becoming more attractive. The biorefinery consist in the sustainable transformation of biomass, such as plant, algae, yeast, bacteria, into a wide range of marketable products and, in the mean time, into energy. Oils derived from biomass sources are named Microbial Oils, Unicellular Oils or Single Cell Oils (SCOs). The sustainable production of Single Cell Oils (SCOs) has garnered recent attention. The goal is new strategies or the biochemical/microbial conversion processes in order to increase their productivity and competitiveness. The identified strategies include the metabolic and genetic engineering of microorganisms, new fermentation technologies, innovative process choices, low-value feedstock and the recycle of by-products. SCOs are produced by oleaginous microorganisms which are able to accumulate between 20% and up to 80% lipid per dry biomass in the stationary growth phase under nutrient limitations, e.g., nitrogen or phosphorus, with simultaneous excess of carbon source. Depending on the oleaginous microorganisms including bacteria, yeasts, microalgae or fungal species, fatty acid profile of SCOs can vary making them highly suitable for different industrial applications. The SCOs, obtained from plant and microbial sources, offer several advantages, including faster production, less labor, more season and climate flexibility, and easier scale-up.

The aim of this thesis is to explore the capacity, efficiency and productivity of oleaginous microorganisms, grown on agro-industrial lignocellulosic biomasses, to accumulate Single Cell Oils. This allow to reduce the environmental problem associated with the recycle of residues of various industrial processes allowing while increase the economic advantage linked to the SCO production. In order to make Single Cell Oil production more economical and sustainable, the experimental activities were aimed to achieve the following objectives:

- ✓ The optimization of operating conditions of enzymatic hydrolysis evaluating the effects of enzyme concentration, temperature and pH on the fermentable sugar production. The experimental tests were carried out to assess the viability of

innovative fermentation processes, i.e. in single stage or single reactor, with the aim to reduce the capital costs. It was found the synergism of enzymatic mix as well as the positive effects related to a lower temperature, suspended composition of hydrolysates, pH control and reaction time.

- ✓ The implementation of enzymatic hydrolysis and oleaginous fermentation in single reactor (SRF) that offers an useful option to integrate in a single reactor two different stages of the microbial oil production: the enzymatic hydrolysis of the pretreated lignocellulosic biomass and the microbial fermentation of the obtained fermentable sugar mixture. Specific glucose consumption rate (μ_s), lipid yield (Y_{lipid}) were calculated. The results suggest positive potential application of such process that still remains unexplored for Single Cell Oil production but demonstrate that they are suitable for biodiesel, bioplastic production or for other products of industrial interest.
- ✓ The investigation of synergistic effect of yeast-microalga mixed cultures, in order to find suitable operating conditions to improve SCO production in mixotrophic microbial cultures. To test the consortium in clearly defined conditions, a synthetic medium was developed that integrates necessary elements of known culture media for both organisms, with the use of pH control. Growth series were done in batch, under constant light, agitation and temperature, and monitored gas exchange. In the model system, symbiotic growth was observed of the consortium with synergistic effects on biomass yield. Similar results were obtained in a system using lignocellulosic hydrolysate, except for an increase in lag phase due the presence of inhibitors. Growth even in anaerobic conditions (N_2) confirmed synergistic interactions between the microalga and the oleaginous yeast. The performance of the consortium under different conditions is discussed in terms of growth rate, biomass production and lipid content of the biomass.
- ✓ The implementation of yeast-microalga mixed cultures in open pond, overcoming contamination problems. The yeast grew, reaching the maximum concentration just after few days. The rapid growth kinetic was expected for the yeast while the very low cell proliferation can be explained by the low quantity of organic carbon in the medium.

Keywords: Lignocellulosic biomass, Single Cell Oils (SCOs), steam explosion, enzymatic hydrolysis, oleaginous yeasts, microalgae, mixed cultures, FAME.

PREFACE

This thesis describes the most important results achieved during my Ph.D. study. The work was carried out mostly at Biochemical Engineering laboratory, Department of Chemical, Materials and Production Engineering (DICMaPI), University of Naples Federico II in the period from February 2014 to February 2017. Part of experimental activity was also conducted at the Bioscience and Territory Department, University of Molise in Isernia, Italy; at the Department of Biological Science at the University of Naples Federico II, Italy; and at INRA-LBE (Laboratoire de Biotechnologie de l'Environnement) in Narbonne, France. The thesis was submitted to the Department of Chemical, Materials and Production Engineering (DICMaPI), University of Naples Federico II on April 10, 2017 as part of the requirements of the academic degree of Doctor of Philosophy (Ph.D.) in Industrial Product and Process Engineering.

Gaetano Zuccaro
Napoli, April 10, 2017

CONTENT

LIST OF ABBREVIATIONS

15 FPU & 30 CBU: steam exploded *Arundo donax* hydrolysate using cellulase from *Trichoderma reesei* and β-glucosidase from *Aspergillus niger*, dose of 15 FPU/g cellulose and 30 CBU/g cellulose, respectively

15 FPU: steam exploded *Arundo donax* hydrolysate using only cellulase from *Trichoderma reesei*, dose of 15 FPU/g cellulose

30 CBU: steam exploded *Arundo donax* hydrolysate using only β-glucosidase from *Aspergillus niger*, dose of 30 CBU/g cellulose

A.U.: absorbance unit

Ac: sodium acetate

ADH: *Arundo donax* hydrolysate

BBM: Bold Basal Medium

BBM+G: Bold Basal Medium plus D-Glucose

Buffer pH 5.2: hydrolysate of steam exploded *Arundo donax* using buffer solution at pH 5.2

Buffer pH 6.2: hydrolysate of steam exploded *Arundo donax* using buffer solution at pH 6.2

CBU: Cellobiase Unit

CFU/mL: Colony Forming Unit/mL

Chl: *Chlorella emersonii 312/25*

Chla: *Chlamydomonas* sp.

EFAs: Essential Fatty Acids

FAME: Fatty Acid Methyl Ester

FPU: Filter Paper Unit

G: D-Glucose

G: glucose concentration

G_0: initial glucose concentration

GC: Gas Chromatography

G_{max}: maximum glucose concentration

HMF: hydroxymethylfurfural

HPLC: High Performance Liquid Chromatography

Lip: *Lipomyces starkeyi DBVPG 6193*

MP100: medium containing buffer MES hydrate (100 mM)

MES hydrate: 2-(N-Morpholino)ethanesulfonic acid hydrate

NaOH pH 5.2: hydrolysate of steam exploded *Arundo donax* using NaOH solution to adjust the pH 5.2

P_{CO2}: carbon dioxide fixation rate

PUFAs: Poly Unsaturated Fatty Acids

P_x: biomass productivity

SCOs: Single Cell Oils

SHF: Separate Hydrolysis and Fermentation

SRF Buffer pH 5.2: enzymatic hydrolysis and oleaginous Fermentation in Single Reactor keeping a pH value of 5.2 using a phosphate buffer solutions

SRF NaOH pH 5.2: enzymatic hydrolysis and oleaginous Fermentation in Single Reactor adding suitable amounts of 1 M NaOH or 0.1 M HCl to maintain a pH value of 5.2 under discontinuous conditions

SRF without pH control: enzymatic hydrolysis and oleaginous Fermentation in Single Reactor without pH control

SRF: enzymatic hydrolysis and oleaginous Fermentation in Single Reactor

SSF: Simultaneous Saccharification and Fermentation

UWRP Lip:m.c.: Urban Wastewater in Raceway Pond, using Lipomyces starkeyi and microalgal consortium ad inoculum

Without enzyme: steam exploded *Arundo donax* hydrolysate without enzyme dosage

Without pH control: hydrolysate of steam exploded *Arundo donax* without pH control

WSAELH Chla:Lip 1:1 dil 0, 2, 4: Wheat Straw subjected to Acid Hydrolysis plus Enzymatic hydrolysis on the Liquid fraction, using *Chlamydomonas:Lipomyces starkeyi* ratio of 1:1 and dilution factors of 0, 2, 4

WSAELH: Wheat Straw subjected to Acid hydrolysis plus Enzymatic Hydrolysis on the Liquid fraction

WSAESH Chla:Lip 1:1 dil 0, 2, 4: Wheat Straw subjected to Acid hydrolysis plus Enzymatic Hydrolysis on the Solid fraction, using *Chlamydomonas:Lipomyces starkeyi* ratio of 1:1 and dilution factors of 0, 2, 4

WSAESH: Wheat Straw subjected to Acid ydrolysis plus Enzymatic Hydrolysis on the Solid fraction

WSAH Chla:Lip 1:1 dil 0, 2, 4: Wheat Straw subjected to Acid Hydrolysis, using *Chlamydomonas:Lipomyces starkeyi* ratio of 1:1 and dilution factors of 0, 2, 4

WSAH: Weat Straw subjected to Acid Hydrolysis

YE: Yeast Extract

YEG: Yeast Extract and D-Glucose medium, which contains (g/L): KH_2PO_4 (3.0), Na_2HPO_4 (1.0), yeast extract (5.0), glucose (10.0), peptone (5.0)

$Y_{Glucose}$: Glucose Yield

Y_{lipid}: lipid yield

$Y_{x/s}$: Cell yield coefficient

μ_G: specific rate for glucose production

μ_s: specific glucose consumption rate

μ_x: specific growth rate

ν_G: glucose productivity

1. INTRODUCTION

1.1 A Global Redesign? Ambition of the "circular economy"

In recent years, several steps into the transition toward a biobased economy have been taken. The "circular economy" has been heralded as one of the most effective instruments for moving society towards a much needed 'resource revolution", with the aim to provide a better alternative to the dominant economic development model, so called "take, make and dispose" (*Ness, 2008*). In the last decade, three major disruptions to the circular economy were experienced: credit crisis of 2008; Green Fence Operation (GFO), when increased custom controls at the Chinese borders were implemented, enforcing strict quality criteria to imports in 2013; the ongoing collapse of crude oil prices. These crisis are major threats to the established resource recovery systems, but can also serve as an eye opener, forcing us to reflect upon the necessity for resource recovery (*Velis, 2015*).

Many adherents of the circular economy approach are strong proponents, on environmental and ethical premises, of material reuse and recycling (*Andersen, 2006*). It is rapidly landing on the world of waste and resources management, becoming a mainstream concept. The simple and straightforward question in recovering resources, such as biobased materials and energy, is "how?". In a circular economy, the resource loop would be closed, thus enhancing its reuse. Other products can be made from plant-based materials that biodegrade into fertilizer at end of their life. Extending this logic across the economy means a deep change in the basic structures of industrial systems. In terms of energy, redesigning industry at the system level would enable efficiency improvements and then potential savings. Part of energy needed for a circular economy would be provided by renewable sources. Efficient use of renewable sources for the production of both biobased products and bioenergy should be driven by well-developed integrated biorefining systems.

1.2 The definition for biorefinery

Biorefinery is the sustainable processing of biomass into a wide spectrum of marketable bio-products, such as food, feed, biomaterials, and chemicals, and bio-energy, such as fuels, power, and/or heat (*Cherubini, 2010*).

The assessment of biorefinery should also take into account the possible consequences related to the competition for food and biomass resources, the impact on water use and quality,

changes in land-use, soil carbon stock balance and fertility, net balance of gas emissions, impact on biodiversity, potential toxicological risks, and energy efficiency.

The impacts on international and regional dynamics, the end users and consumer needs, and the investment feasibility are also important aspects that need to be considered. For this reason the sustainability assessment is not an absolute number, but should be done in comparison to conventional systems providing the same products and services (*de Jong and Jungmeier, 2015*). New biorefinery concepts are, however, still mostly in R&D (Research and Development) phase or in pilot or small-scale demonstration state, and their industrial application is still far away. It is expected that these new concepts will be implemented in the market of different countries in the medium term (2015–2025) (*Wagemann et al., 2012*) although current economic conditions (relatively low oil prices, credit crisis, and recessions in parts of the global economy) might cause severe delays in the market of some biorefinery concepts.

Many different biorefinery concepts are being developed and implemented. Some of these are sometimes very complex, they use several feedstocks (e.g., algae, energy crops and wood chips from short rotation) to coproduce a wide spectrum of bio-products (e.g., bioethanol, phenols, Single Cell Oils, biodiesel) using technologies that still need to become commercial in the upcoming years. Among these, particular attention is paid to microbial cultures that can be established to convert lignocellulosic sugars or low-value hydrophobic substrates into Single Cell Oils.

1.3 Single Cell Oils

Oils derived from microbial sources are named microbial oils, unicellular oils or single-cell oils (SCOs). In particular, the term SCOs is used to denote oils of microbial origin. Its composition is similar to the oil composition of edible plants, animal oils and fats (*Kyle and Ratledge, 1992*; *Boswell et al., 1996*).

The potential of microbial oils and fats has been recognized since the last century. Before the 1980s, several scientists focused their attention on the biochemistry and metabolism accumulation of lipids in oleaginous microorganisms (*Botham and Ratledge, 1978*; *Botham and Ratledge, 1979*; *Gill et al., 1977*; *Ratledge and Hall, 1977*). In the subsequent 20 years, the biochemical processes and SCO production become one of the most popular research topic because it was discovered that SCOs can play a critical roles in human health (*Sijtsma and Swaaf, 2004*). Subsequently, the microbial screening for the SCO production became the mission of several scientists (*Papanikolaou et al., 2007*; *Wu et al., 2005*). The interest in SCO

production continuously increases and it is playing a important role in today's world due to its potential to solve the current energy crisis. Actually the mechanisms for Single Cell Oil accumulation by microorganisms are known and this allow to concentrate the researcher focus on finding low-cost and alternative feedstock and on improving the productivity of SCO production. It is obvious that SCOs will play a more critical role in the future, and low-cost substrates, such as lignocellulosic biomasses, will play a key role in the industrialization of SCO production (*Huang et al., 2013*). There are several models that describe the SCO accumulation in oleaginous yeast. Recently, Candrell and Walker (*2009*) proposed a logistic model to describe the growth profile; Fakas et al. (*2009*), using glycerol as a carbon source, have developed a model for SCO production considering the dependence of nitrogen source. It is therefore interesting to investigate this aspect partly described by Economou et al. (*2010*), matching the experimental data with mathematical model.

1.4 Properties of lignocellulosic materials

Biomass and biomass derived materials have been pointed out to be one of the most promising alternatives to fossil resources (*Keegan et al., 2013; Zhou et al., 2008*).

These materials are generated from available atmospheric CO_2, water and sunlight through biological photosynthesis. Therefore, this biomass has been considered to be the only sustainable source of organic carbon in earth and the perfect equivalent to petroleum for the production of bio-fuels and bio-fine chemicals with net zero carbon emission. In this context, lignocellulosic biomass, which is the most abundant and bio-renewable biomass on earth can play a critical role (*Somerville et al., 2010; Zhou et al., 2011*). It was estimated that 3.7×10^9 t of agricultural residues is produced annually as by-products by agricultural world-wide industries and that 1376×10^6 t cellulose and 848×10^6 t hemicellulose occur globally every year (*Bentsen et al., 2014*). Furthermore, it was estimated that one-third of the globally food produced for human consumption is wasted every year. The overall amount of food wasted corresponds to around 1.3×10^9 t (*Gustavsson et al., 2011*; *Gustavsson et al., 2013*). These estimation pointed out that lignocellulosic biomass is the most abundant carbon-neutral renewable source, which can decrease CO_2 emissions and atmospheric pollution. Thus, it is a promising alternative to limited crude oil, and it has a good potential for the biofuel, biomolecule and biomaterial production. Furthermore, the major component of lignocellulosic biomass, the cellulose, is considered the strongest potential candidate for the substitution of petroleum-based polymers owing to its ecofriendly properties like renewability, bio-compatibility and biodegradability (*Isikgor et al., 2015*).

Considering the economic point of view, lignocellulosic biomass can be produced quickly and at lower cost than other agriculturally biofuel feedstocks such as corn starch, soybeans and sugar cane. It is also significantly cheaper than crude oil (*Huber, 2008*). On the other hand, the development of the conversion of lignocellulosic biomass to fine chemicals and polymers still remains a big challenge for technical and economic obstacles (*Himmel et al., 2007*; *Zhou et al., 2011*) due to biomass recalcitrance that is the result to the terrestrial plant evolution, in particular the plant cell walls are becoming stronger and more efficient barriers against the intrusion and degradation from natural infections. Extensive research is currently being undertaken all over the world to address this problem (*Slavin et al., 2011*).

Biorefinery and biofuel technologies are developing to refine lignocellulosic biomass for the production of renewable oil and green monomers (*Stöcker, 2008*). In addition, the number of biorefinery-related pilot and demonstration plants has been increasing (*Cherubini and Strømman, 2011*). Actually, only few companies, such as Lignol, Verenium and Mascoma, are focusing their attention on the development of biorefining technologies for the production of advanced biofuels, biochemicals and biomaterials from non-food cellulosic biomass feedstocks (*Isikgor et al., 2015*). Lignocellulosic biomasses are characterized from micro and macrofibrils organized in crystalline structures. Those tight structures need to be pretreated in order to make their cellulose, hemicellulose, lignin and small amounts of pectin, protein and ash accessible to the subsequent hydrolysis step (*Galbe and Zacchi, 2002*).

The cellulose, hemicellulose and lignin amount is not constant between the lignocellulosic biomasses. It depends to the species, the age and growing conditions of biomass (*Carpita et al., 2001*; *Kuhad et al., 1997*). The cellulose is the most abundant constituent of plant cell structure, it is a polysaccharide characterized by a linear sequence of D-glucose molecules linked by β-1,4 glucosidic bonds with a high degree of polymerisation equal to 10000 or even higher. This structure results in the formation of intra and intermolecular hydrogen bonds, that characterize a specific property of crystallinity, even if some sources claim that the structure could be seen as amorphous (*Ding and Himmel, 2006*). In addition to being chemically stable and resistant to microbial degradation (*Ward and Moo-Young, 1989*), the cellulose fibrils are responsible for the great tensile strength of the cell wall (*Carpita and Gibeaut, 1993*). This structural and inherent integrity of cellulose is believed to play an important role in the recalcitrance of lignocellulosic biomass (*Ding and Himmel, 2008*). The hydrophobic surface involves the formation of water layer that prevents the diffusion of enzymes and degradation of the products on the surface (*Matthews et al., 2006*).

Hemicellulose is the second most abundant polymer of plant cell structure. Unlike cellulose, hemicellulose has a random and amorphous structure, which is composed by several monomers including D-Glucose, D-galactose, D-mannose, D-xylose, L-Arabinose, D-glucuronic acid and 4-O-methyl-D-glucuronic acid. It is characterized by a degree of polymerisation lower than 200. Hemicelluloses are imbedded in the plant cell walls to form a complex network of bonds that provide structural strength by linking cellulose fibers into microfibrils and cross-linking with lignin.

The lignin is a complex network of phenyl propane units. It is the most important non-polysaccharide fraction of lignocellulosic biomass. The three characteristic lignin's monomers are: p-cumarilic alcohol, sinapyl alcohol and conferilic alcohol related by different ether bonds. The lignin also provides protection against chemical and microbial degradation (*Kuhad et al., 1997*) [Figure 1.1].

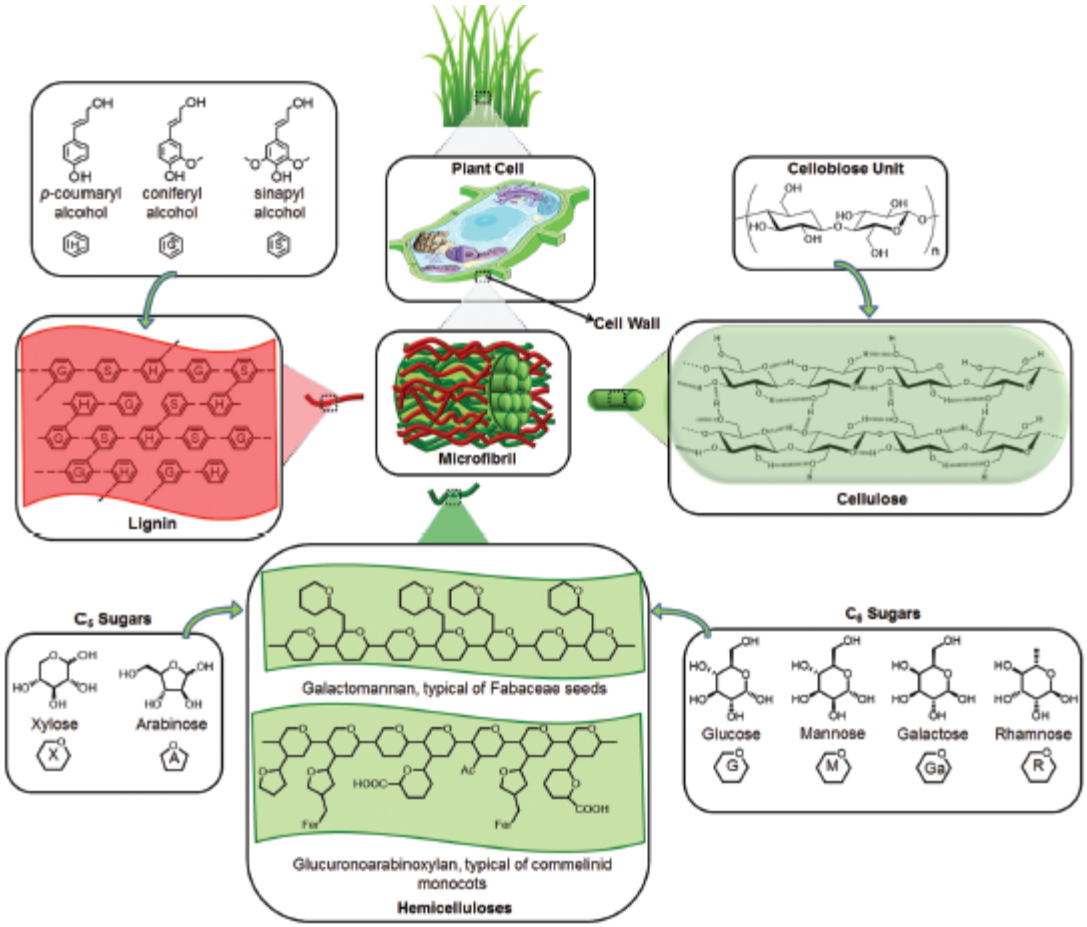

Fig. 1.1: The main components and structure of lignocellulose (SOURCE: *Isikgor et al., 2015*)

1.5 Pretreatment technologies

The lignocellulosic biomass is characterized by complexed structures and requires pre-treatment processes in order to make those structure accessible to the next step of enzymatic hydrolysis (*Galbe and Zacchi, 2002*). The choice of the pretreatment process must also prevent the carbohydrates degradation because the carbohydrates represent the most indispensable substrate for the metabolic activity of the microorganisms. The lignocellulosic biomass pretreatment is also required to avoid the formation of inhibitors directly related to lignin fraction. Thus, pretreatments are considered one of the most crucial steps since it has a large impact on all other steps in the conversion process. however, those pretreatment process are usually energy-expensive steps and significantly affect the cost of the overall process (*Agbor et al., 2011*; *Sun and Chen, 2002*). The pretreatment methodologies can be classified in physical, chemical-physical, chemical and biological. Some "physical" pre-treatment are mechanical shredding, pyrolysis and irradiation (*McMillan, 1994; Wyman 1996*), "chemical-physical" are steam explosion, AFEX (Ammonia Fiber Explosion), CO_2 and SO_2 explosion (*Alizadeh et al., 2005; Ballesteros et al., 2000; Dale et al., 1996; Sassner et al., 2005*), "chemical" are ozonolysis, acid and basic hydrolysis, oxidative delignification and organosolv (*Arato et al., 2005*; *Berlin et al., 2006*; *Karimi et al., 2006a; Karimi et al., 2006b; Schell et al., 2003)* while "biological" pretreatment require fungal and actinomycetes activity (*Fan et al., 1982; Wyman, 1996*). Table 1.1 shows the effects of several pre-treatment processes on the structure of lignocellulosic biomass.

Tab 1.1: Effects on chemical composition and on chemical/physical structure of different pretreatment processes (H, High; L, Low; ND, Not Determined) (SOURCE: *de Jong and Gosselink, 2014*)

Pretreatment	Sugar yield	Increases accessible surface area	Decrystallizes cellulose	Removes hemicellulose	Inhibitor Formation	Removes lignin	Alters lignin structure	Reuse of chemicals
Mechanical	L				ND			No
Steam explosion	H	H		H	H		L	
Liquid hot water	H	H	ND	H	H		L	No
Wet oxidation	H or L				ND			No
Dilute acid		H		H			H	
Concentrated acid	H	H	H	H	H	H	L	Yes
Lime (alkaline)		H	ND	L	L	H	H	Yes
Organosolv	H	H		H	H	H	L	Yes
AFEX/ARP		H	H	L	L	H	H	Yes
Ionic liquid		H	H	L	L	H	L	Yes
Supercritical fluid		H	H	H			L	

It is not possible define the best pre-treatment technology, but it is rather necessary reconcile the economic and chemical requirements with the industrial implementation (*de Jong and Gosselink, 2014*).

1.6 Enzymatic hydrolysis

The pretreatement of lignocellulosic biomasses is important to facilitate access to the crystalline structure by so-called hydrolytic enzymes, a range of cellulolytic fungi and bacteria. These microorganisms are being used for a number of industrial purposes, e.g. cotton processing, detergent enzymes and paper recycling.

Enzymatic hydrolysis of cellulose and hemicellulose is conducted under mild conditions (pH 4.5÷5.0 and 40÷50 °C) which ensure reduced corrosion problems, low energy consumption and low toxicity of the processed hydrolysates (*Taherzadeh and Karimi, 2007*).

It is a multi-step and heterogeneous reaction where the insoluble portion of the biomass is initially broken as a result of synergistic action of different enzymes (*Eriksson et al., 2002*; *Väljamäe and al., 2003*).

These can be divided into the following three types:

- Endoglucanases (EG), which hydrolyse internal *β*-1,4-D glucosidic linkages randomly in the cellulose chain;
- Cellobiohydrolases (CBH, also known as exoglucanases), which progress along the cellulose line and cleave off cellobiose units from the ends;
- *β*-glucosidases (BG), which hydrolyse cellobiose to glucose and also cleave off glucose units from cello-oligosaccharides.

The synergistic action of these enzymes is able to increase glucose conversion starting from lignocellulosic biomass, but is not neglegible the introduction of hemicellulosic enzymes, such as:

- Endo-1,4- β-D-xylanases to make xylan chain;
- 1,4-β-D-xylosidases to release xylose;
- Endo-1,4- β-D-mannanases to break internal bonds to obtain mannans;
- 1,4-B-D-mannosidase that cleave molecules of mannooligosaccaridi in mannose.

Cellulases and hemicellulases described above can be produced by bacteria such as *Clostridium*, *Ruminococcus*, *Streptomyces*, *Cellumonas*, *Bacillus* and *Ervinia* and fungi such as *Trichoderma*, *Penicillium*, *Fusarium* and *Humicola* (*Rabinovich et al., 2002*; *Sun and Cheng, 2002*). Among cellulases produced by various microorganisms, those derived from *Trichoderma reesei* or from *Trichoderma viride* have been widely studied and best

characterized. It is noted a stability of their enzymatic activity, a resistance to the presence of inhibitors, but reduced β-glucosidase activity. On the other hand, *Aspergillus* is able to overcome this inefficiency by *Trichoderma*. In many studies it has been observed a synergy between enzymes derived from two microbial strains to improve the efficiency of the hydrolysis process (*Itoh et al., 2003*; *Ortega et al., 2001*; *Tengborg et al., 2001*; *Wyman, 1996*) [Figure 1.2].

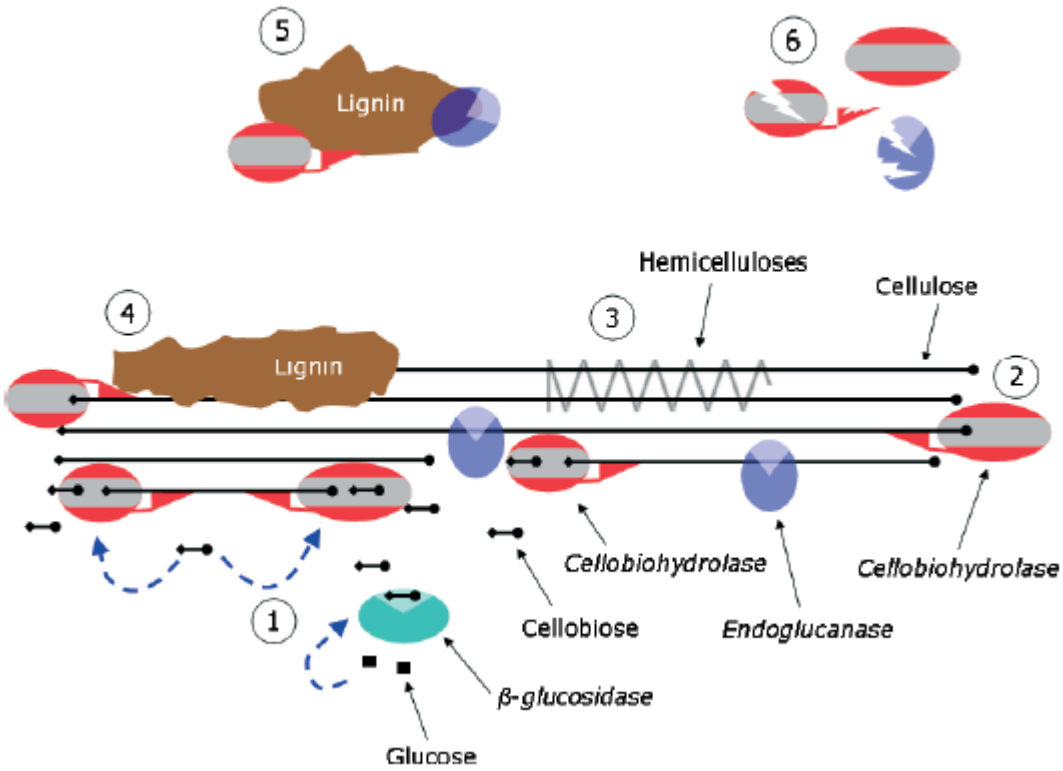

Fig. 1.2: Simplified diagram of lignocellulose hydrolysis showing synergism and limiting factors. Cellulose is symbolized straight lines. 1: Product inhibition of β-glucosidase (BG) and cellobiohydrolase (CBH) by glucose and cellobiose. 2: CBH hydrolysing from the end of a cellulose chain. 3 and 4: Hemicelluloses and lignin associated with or covering the microfibrils prevent the cellulases from accessing the cellulose surface. 5: Enzymes (both cellulases and hemicellulases) can be unspecifically adsorbed onto lignin particles or surfaces. 6*:* Denaturation or loss of enzyme activity due to mechanical shear, proteolytic activity or low thermostability (SOURCE: *Jørgensen et al., 2007*).

The enzymatic hydrolysis presents several obstacles that prevent the implementation on an industrial scale, the first is associated to enzyme cost but in recent years is much less incident for the intensification of research by, for example, Novozymes and Genecor aimed to reduce the use of these enzymes in order to minimize process operating costs (*Zhang et al., 2006*). Other parameters, already mentioned, such as pH, temperature and residence time of the process linked to other not secondary aspects such as substrate concentration which helps to

increase inhibition phenomena. The Equation (1.1) that allows to describe the inhibition phenomenon is associable to Michaelis-Menten equation:

$$v = \frac{k_{cat} E_0 S}{K_M \left(1 + \frac{I}{K_I}\right) + S} \tag{1.1}$$

$\frac{I}{K_I}$ is assumed constant, k_{cat} is the value of catalytic activity, E_0 represents the initial concentration of enzyme, S is substrate concentration, K_M is Michaelis-Menten constant, I inhibitor concentration and K_I represents the dissociation constant of enzyme. In case of product inhibition (β-glucosidase inhibition due to the presence of an excessive glucose concentration), the term $\frac{I}{K_I}$ becomes $\frac{P}{K_I}$ (P is the product and K_I the dissociation constant). If P increases due to the reaction catalyzed by enzyme, also the $\frac{P}{K_I}$ increases causing a decrease of reaction rate, strictly related to the increase of the product concentration.

Since industrial processes typically require high product concentrations, above mentioned inhibition phenomena significantly reduce reaction rate and the efficiency of enzymatic hydrolysis reactions in batch and continuous processes (*Andric et al., 2010*). One of actionable solutions to overcome this problem is to increase the ratio enzyme/substrate, however, contrary to the fulfillment of the objective to minimize operating costs or to add surfactants to change surface properties of cellulase, or to recover the enzyme fraction, once immobilized, through recycling mechanisms (*Tu et al., 2006*). All of these techniques for recycling and reducing enzyme adsorption have so far only been tested at laboratory scale. Furthermore, most of the studies do not include cost calculations to evaluate the feasibility of addition of different compounds to reduce enzyme binding or the costs of recycling. Therefore, the ability to scale up the techniques, the robustness and feasibility still needs to be demonstrated (*Jørgensen et al., 2007*).

Finally the development of methodologies that include the progressive glucose consumption produced during the hydrolysis step. This type of process is commonly called Simultaneous Saccharification and Fermentation (SSF) where hydrolysis and fermentation are located in a single reactor. This process has been considered as preferably process because of reduced operation all costs, lower enzyme requirement and increased productivity (*Alfani et al., 2000*). The drawback, still under study, is the ability to reconcile the cultivation requirements of yeasts that provide temperatures of 30÷37 °C and enzyme activity that is high at 40÷50 °C (*Olsson et al., 2006*; *Saha et al., 2005*; *Wingren et al., 2003*). SSF from lignocellulosic

biomass is commonly studied for bioethanol production (*Kim et al., 2008*; *Lynd et al., 2008*), however is still unexplored the use of SSF for single cell oil production (Liu et al., 2012). The reason is attributable to the different metabolic activity of oleaginous yeasts that produce lipids in intracellular spaces of solid phase, and to the difficulty of oxygen supply due to the increase of culture medium viscosity especially in case of high concentrations of processed biomass (*Ageitos et al., 2011*; *Beopulos et al., 2011*; *Liu et al., 2012*).

1.7 Oleaginous microorganisms

1.7.1 Oleaginous yeasts

The growing importance of Single Cell Oil applications in nutraceutical and pharmaceutical field, and the possibility to use microbial oils also for biodiesel production has created scientific appeal aimed to obtain alternative ways such as offered by oleaginous yeasts.

Oleaginous yeasts are not influenced by climate variability, they grow in presence of different substrate sources as hexose and pentose sugars with high growth rates (*Chi et al., 2010*). Generally, they have the ability to accumulate Single Cell Oils more than 20-25% of their weight (*Ratledge and Wynn, 2002*; *Beopoulos and Nicaud, 2012*) but not more than 65% of their dry weight in specific growth conditions (*Angerbauer et al., 2008*).

Oleaginous yeasts are attracting an increasing interest for their growth requirements that are the effect of poor request of nitrogen source and the ability to trigger a cascade of reactions leading to intermediate compounds formation such as Acetyl-CoA (*Li et al., 2007*; *Papanikolaou and Aggelis, 2011*) and accumulation mechanisms (*Nagamuna et al., 1985*) that are derived from tricarboxylic acid cycle (TCA cycle) that takes place in mitochondria of eukaryotic microorganisms [Figure 1.3].

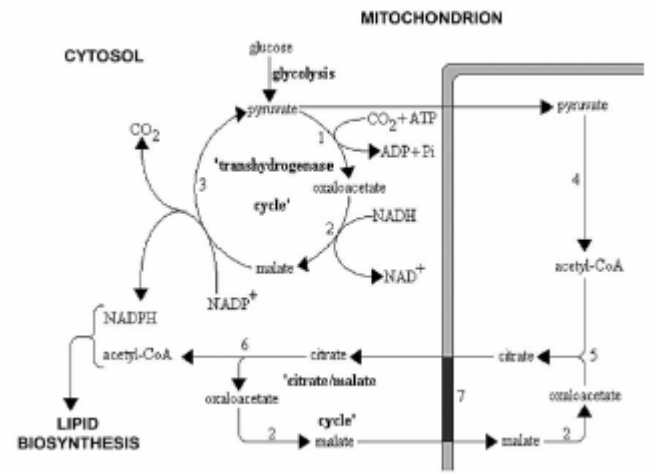

Fig. 1.3: Citrate/malate cycle and the transhydrogenase cycle (TCA) are acetyl-CoA and NADPH precursors for lipogenesis in oleaginous microorganisms (SOURCE: *Ratledge, 2004*)

The eukaryotic microorganisms, oleaginous and not oleaginous, share the same biosynthetic pathway, however, exists a fundamental difference that distinguishes them in the presence of a carbon source excess. In a medium with abundant carbon source and limiting amounts of nitrogen, when all the nitrogen source has been consumed, oleaginous microorganisms utilise carbon source for lipid synthesis resulting in an excess of triacylglycerols; while not oleaginous microorganisms convert carbon source in polysaccharides (glycogen, glucans, mannans, etc.) and are not predisposed to accumulate lipids (maximum up to 10-20%). As above mentioned, since the biosynthetic pathway of fatty acids is the same, in oleaginous microorganism, the reason is related to the production of acetyl-CoA in the cytosol as a precursor for FAS (Fatty Acids Synthetase) and to the production of NADPH, which is used as a reducing agent in the synthesis of fatty acids (*Ratledge, 2004*).

Fatty acid biosynthesis in almost all organisms, is directed to C16 and C18 saturated fatty production that are modified by a sequence of enzymes (elongase and desaturase), able to insert progressively a defined range of unsaturations. In oleaginous yeasts, most fatty acids are commonly represented by oleic acid (C18:1, n-9), linoleic acid (C18:2, n-6), palmitic acid (C16:0) and palmitoleic acid (C16:1), as well as by C18:3 or alpha-linolenic acid that, in general, represent less than 10% of the total (*Xu et al., 2012*).

It has been proven in recent years that yeasts such as *Rhodotorula glutinis* (*Easterling et al., 2009*), *Rhorosporidium toruloides* (*Li et al., 2007*), *Trichosporon fermentans* (*Zhu et al., 2008*) and *Lipomyces starkeyi* (*Angerbauer et al., 2008*; *Zhao et al., 2008*) have a potential production also because they can be cultured in simpler media made with low cost substrates (*Huang et al., 2013*). A further important advantage to use such oleaginous yeasts comes from the ability to produce lipids under aerobic conditions from residual substances, without adding expensive nutrients [Table 1].

Tab 1.2: Oleaginous yeast species for single-cell oil production from cellulosic sugars (SOURCE: *Abghari and Chen, 2014*)

Oleaginous yeast species	Substrate	Comments	Reference
***Cryptococcus* sp.**	Glucose and corncob hydrolysate	Batch and fed-batch to reach lipid content and productivity of more than 61% w/w and 1 g/L/day, respectively	*Chang et al., 2013*
Cryptococcus curvatus	Oligocelluloses and oligoxyloses	Batch to reach lipid content and coefficient of more than 30% and 0.17 g/g sugar, respectively	*Gong et al., 2014*
Lipomyces starkeyi	Corncob acid hydrolysate	Batch to reach lipid content and yield of 47% and 8.1 g/L, respectively	*Huang et al., 2014*
Lipomyces starkeyi	Co-fermentation of cellobiose and xylose	Batch to reach lipid content and coefficient of 55% and 0.19 g/g sugar, respectively	*Gong et al., 2012*
Rhodosporidium toruloides	Jerusalem artichoke	Batch and fed-batch to reach lipid content and yield of 43.3-56.6% and 17.2-39.6 g/L, respectively	*Zhao et al., 2010*
Rhodotorula graminis	Undetoxified corn stover hydrolysate	Batch to reach lipid productivity and lipid content of 0.21 g/L/h and 34% w/w, respectively	*Galafassi et al., 2012*
Trichosporon coremiiforme	Corncob acid hydrolysate	Batch to reach lipid content and yield of 37-40% and 7.7-9.8 g/L, respectively	*Huang et al., 2013b*
Trichosporon cutaneum	Diluted acid pretreated and biodetoxified corn stover	Simultaneous saccharification and fermentation to reach lipid yield of 3.03-3.23 g/L	*Liu et al., 2012*
Yarrowia lipolytica	Sugarcane bagasse and rice bran hydrolysate	Batch to reach lipid content and yield of 48-58.5% and 5.16-6.68 g/L, respectively	*Tsigie et al., 2012*

The main lipid molecules present in yeasts are triacylglycerols (TAGs) and steril-esters (SE) and are accumulated in the cells during stationary growth phase. TAGs have a distribution of the acyl substituents very similar to the plant oils, in particular the central position is occupied almost exclusively by an unsaturated acyl group. Since these molecules are without positive or negative charge, they can't be part of cell membranes, however, they are sequestered in hydrophobic particles called lipid particles (LP) or lipid bodies. The lipids are accumulated in the form of micro droplets, and composed almost entirely of triacylglycerols. Their extraction implies other lipid fractions (phospholipids, sterols, steril-esters and other) associated to the cell membrane and the presence of free fatty acids due to and uncontrolled lipolysis which occurs during extraction process (*Cordisco, 2009*).

Lipid accumulation implies three consecutive stages: synthesis, storage and mobilization. These processes require the interaction of various cellular components, in particular the plasmic membrane, the lipid particles (LP) and the endoplasmic reticulum (ER) (*Cordisco, 2009*). The LP biogenesis is still not completely known, but one of the most accepted models is so-called "budding model", which assumes the LP formation from the endoplasmic

reticulum membrane [Figure 1.4]. According to this model, specific enzymes synthesize neutral lipids, such as TAG and SE, which accumulate in the phospholipid bilayer of ER membrane. This accumulation continues until it comes off a mature LP (*Ratledge, 2004*; *Czabany et al., 2007*; *Courchesne et al., 2009*).

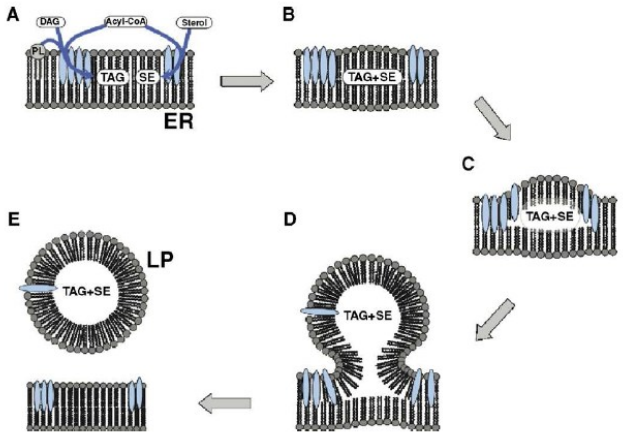

Fig. 1.4: LP formation from endoplasmic reticulum (ER) trough budding model (SOURCE: *Ratledge, 2004*)

1.7.2 Microalgae

Microalgae are not the newly known microorganisms for human beings, since they are widely used for decades as the feedstock for traditional applications in cosmetic, pharmaceutical and nutrition sectors. In addition, a variety of bio-active substances, such as carotenoids, polysaccharides and β-carotene, can be produced. The forms of microalgal products include tablets, capsules, liquids, pure molecules with high values such as fatty acids, pigments and stable isotope biochemicals, and cosmetics found in face and skin care products, such as anti-aging creams, refreshing or regenerant care products, emollient and anti-irritant in peelers (*Koller et al., 2012*; *Samarakoon et al., 2012*).

Microalgae are prokaryotic or eukaryotic photosynthesis microorganisms that can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure. Examples of prokaryotic microorganisms are Cyanobacteria (*Cyanophyceae*) and eukaryotic microalgae are for example green algae (*Chlorophyta*) and diatoms (*Bacillariophyta*) (*Li et al., 2008a*; *Li et al., 2008b*). In particular, microalgae may assume many types of metabolisms, such as photoautotrophic, heterotrophic, mixotrophic and photoheterotrophic (*Mata et al., 2010; Zhao et al., 2012*), offering the highest yields of lipids, mainly made of triglycerides (*Chen et al., 2009*). These are composed of saturated and unsaturated fatty acids with 12–22 carbon atoms, some of them of ω-3 and ω-6 families. At present, the research has

focused on the photoautotrophic production of microalgal oil using light as energy source, but there are significant drawbacks associated with photoautotrophic algal cultures for Single Cell Oil production. First, it is difficult to solve the contradiction between accumulation of biomass and lipid synthesis during the microalgal life cycle (*Liu et al., 2011*). Second, light attenuation is unavoidable for photoautotrophic cultures from lab to pilot scale, and it may significantly reduce productivity (*Wilhelm and Jakob, 2011*).

Many microalgae strains such as *Chlorella sorokiniana* (*Chen et al., 2013*), *Chlorella saccarophila* (*Herrera-Valencia et al., 2011*), *Nannochloropsis sp.* (*Jiang et al., 2011*; *Wahidin et al., 2013*; *Chiu et al., 2009*), *Neochloris oleoabundans* (*Li et al., 2008c*), *Cladophora fracta* (*Demirbas, 2009*), *Chlorella protothecoides* (*Demirbas, 2009*; *Xiong et al., 2008*), *Chlorella vulgaris* (*Liu et al., 2008*) and *Chlamydomonas sp.* (*Ho et al., 2014*; *Nakanishi et al., 2014*) could supply Single Cell Oil production.

As above explained, microalgae cultures can have different types of metabolisms that are distinguished in autotrophic, heterotrophic, mixotrophic and phototrophic. In autotrophic condition, microorganisms obtain energy absorbing light energy for CO_2 reduction and they imply O_2 release. Most of algae belong to this category and require minimal amount of organic compounds for growth, such as vitamins. Heterotrophy it means that are required organic carbon sources to support the growth. Some microalgae are able to assimilate organic carbon as a source of energy to grow in dark mode. Heterotrophic growth solves technical and physiological problems related to the presence and distribution of light and CO_2 associated to autotrophic growth. Therefore, it offers the possibility of increasing cell concentration and productivity. However, mixotrophic growth is defined as a growth, where CO_2 and organic carbon source are assimilated simultaneously, using photosynthetic metabolism and cell respiration. Normally, cell growth rate in mixotrophy is approximately the sum of growth rate in heterotrophic and autotrophic mode, and the advantages of heterotrophic growth, such as high concentration and productivity, are also applicable to mixotrophic growth.

Finally, photoheterotrophy also known as photo assimilation or photo metabolism, describes a metabolism in which the presence of light is required to use organic compounds as a carbon source.

Not only the carbon (in form of CO_2 and organic source) is required for the microalgae metabolism, but also vitamins, salts, other nutrients (nitrogen and phosphorus) and the balance of following parameters such as oxygen, carbon dioxide, pH, temperature, and light intensity (*Chojnacka et al., 2004*).

The pathway of triglyceride synthesis in microalgae consists of three steps (*Huang et al., 2010*):

1) formation of acetyl-CoA in the cytoplasm (acetyl-CoA is the initiator, its formation takes place in the chloroplast, where it is formed an intermediate, glyceraldehyde phosphate (GAP), that is transferred to the cytoplasm and subsequently consumed. After the export of GAP from chloroplast to cytoplasm, the carbon source is directed to the sugar synthesis (that represent the main storage products in the cytoplasm of plant cells) or oxidation through the glycolytic pathway to pyruvate. Therefore, a part of the exogenous glucose is directly converted into starch and the rest is oxidized via glycolysis);

2) elongation and desaturation of carbon chains of fatty acids (elongation depends mainly on the reaction of two enzyme systems that include acetyl-CoA and requires the collaboration of malonyl-CoA [Figure 1.5]. Desaturation of carbon chain occurs further elongation of carbon chain takes place to produce long-chain fatty acids which are unusual in normal plant oils);

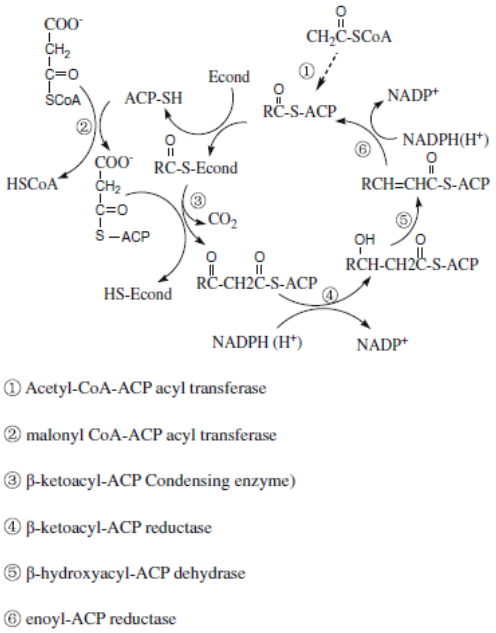

Fig. 1.5: Fatty acid synthesis (SOURCE: *Shen and Wang, 1989*)

3) triglyceride biosynthesis of triglycerides (generally, L-α-phosphoglycerol and acetyl-coA are two major primers. The reaction steps are shown in Figure 1.6);

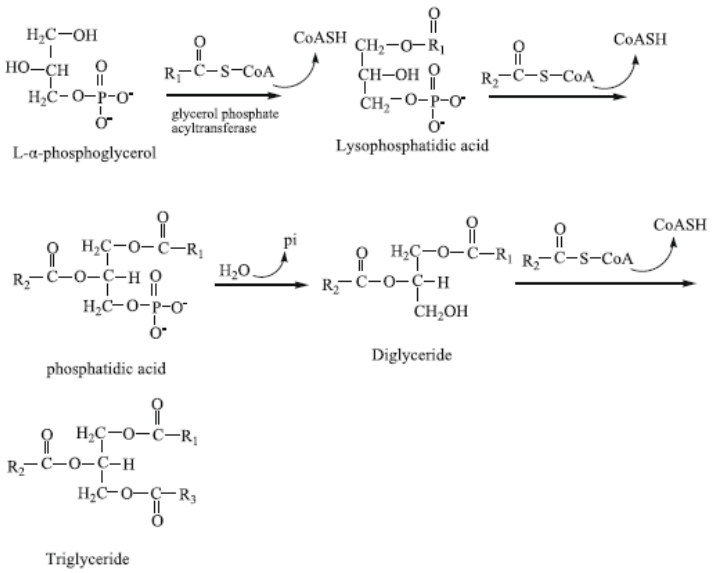

Fig. 1.6: Triglyceride biosynthesis (SOURCE: *Huang et al., 2010*)

1.8 Cultivation strategies

The efficiency of the cultivation systems depends on several factors, including the minimization of operating and plant costs.

As concerns oleaginous fermentations from lignocellulosic biomasses to SCO production, there are different processes which can be designed in several ways. The common is to separate hydrolysis and fermentation step (Separate Hydrolysis and Fermentation, SHF). The major advantage of SHF method is that is possible to carry out hydrolysis (40÷50 °C) and fermentation (30÷37 °C) at their own optimum conditions. The main drawback of SHF is the inhibition of hydrolytic enzymes activity by released sugars. A new perspective is offered by combining enzymatic hydrolysis of pretreated lignocelluloses and fermentation in a single step or in a single reactor. A further advantage is that the sugars produced by hydrolysis are immediately consumed by fermenting microorganisms, and possible substrate inhibition of the enzymes during the hydrolysis is limited (*Wingren et al., 2003*). This process has been mainly studied for bioethanol production. The major advantage compare to SHF is that the released glucose is immediately consumed by fermenting microorganisms and the reduction in material cost since only one reactor is needed. A possible limitation to the applicability of the processes in single reactor is the different requirements of hydrolytic enzymes and oleaginous yeasts in terms of operating temperature and pH. Therefore, for the industrial scale, new improvements in enzyme technology (e.g. thermostable cellulases and higher inhibitor tolerance) are required (*Viikari et al., 2007*). Only few papers have been devoted to the enzymatic hydrolysis coupled with oleaginous yeast growth in a single reactor. Liu et al.

(*2012*) have studied integration of lipid production and enzymatic hydrolysis of corn stover, using diluted acid pretreated. The lipid concentration obtained were 3.03 g/L and 3.23 g/L using *Trichosporon cutaneum* yeast cultivated in 5 L and 50 L stirred-tank reactor respectively. Gong et al. (*2013*) have described Simultaneous Saccharification and enhanced lipid production of glucose and cellulose by *Criptococcus curvatus*. When cellulose was loaded at 32.3 g/L, lipid yield reached 0.20 g/g of cellulose. Gong et al. (*2013*) have demonstrated that *Criptococcus curvatus* can be utilize either oligocelluloses or oligoxyloses as the sole carbon source for microbial oil production.

An interesting perspective, yet not adequately deepened by the research community, is represented by mixed cultures of microorganisms common in natural ecological system. When using a mixed culture, two or more microorganisms are synchronously cultivated within the same medium, where these microorganisms can mutually exploit complementary metabolic activities to survive, grow and reproduce (*O'Reilly and Scott, 1995*).

In the literature review little information on Single Cell Oil production is available regarding microalga and oleaginous yeast mixed cultures. Many methods and techniques, such as the use of bioreactors, the use of low-cost carbon substrates like industrial wastes or hydrolysates of lignocellulosic biomasses have been developed to reduce the cost of SCO production and identify a convenient solution to achieve those targets.

AIM OF THE THESIS

The research activity has been developed according to the general plan and, in particular, to the respect of the goal that was to develop a new approach in biorefinery focused on new cultivation strategies. The initial study was, therefore, concentrated in particular on the optimization of different process parameters (temperature, pH, enzyme dosage) related to the enzymatic hydrolysis, with the aim of assessing the applicability in enzymatic hydrolysis and oleaginous fermentation in a single reactor (SRF). During the experimental activity, it has been investigated the influences of environmental factors as initial pH, but also inhibitory effects of organic acids, furans from hexoses decomposition and phenols from lignin decomposition, released during hydrolysis and fermentation. Thus, this new process has offered an opportunity for an integrated process to reduce time and costs for Single Cell Oil production from lignocellulosic biomasses.

One of the objectives was also to investigate the symbiotic interactions of yeast and microalga mixed cultures to confirm that both microorganisms could significantly enhance biomass and lipid production. In theory, there are synergistic effects on gas, substance exchange and pH adjustment in the mixed culture system of oleaginous yeasts and microalgae based on the mutually beneficial relationship. In yeast-microalga mixed cultures, microalgae could act as an O_2 generator for the yeast while the yeast provided CO_2 to microalgae and both carried out production of lipids. It was also evaluated using lignocellulosic hydrolysates. Finally, the combined growth of yeast and microalgae using urban wastewater as substrate was studied and the lipid biomass concentration during the culture growth phases was monitored. Most of the experimentations on the combined yeast and microalgal cultures reported in literature remained in little scale or in laboratory controlled conditions, while in this work the cultivation was conducted in a 200 L raceway pond operating outdoor.

2. MATERIALS AND METHODS

2.1 Pretreatment of lignocellulosic material

Arundo donax (giant reed) is a perennial, rhizomatous grass, classified as energy crop (*Angelini et al., 2005*), with different characteristics such as high dry biomass yield (30÷40 ton ha^{-1} $year^{-1}$) (*Angelini et al., 2009*; *Mantineo et al., 2009*), the ability to grow in marginal lands (*Nassi et al., 2011*), with reduced input of water (*Lewandoski et al., 2003*), a high content in cellulose and hemicellulose (about 60%) (*Komolwanich et al., 2014*; *Shatalov and Pereira, 2013*) that make this interesting crop for bioethanol, biodiesel and bio-polymer production (*Williams and Biswas, 2010*; *Pirozzi et al. , 2010*).

It has been pretreated in a continuous pilot plant for the steam explosion (mod. StakeTech System Digester) located at ENEA–Trisaia Research Centre (Rotondella, Matera, Italy).

Steam explosion of biomass is a pretreatment process that opens up the fibers, and makes the biomass polymers more accessible for subsequent processes, i.e. fermentation and hydrolysis.

It is considered to be one of the most important. Its attractive features, in comparison to autohydrolysis, pulping, and other methods, include the potential for significantly reducing the environmental impact, the investment costs, and the energy consumption (*Avellar and Glasser, 1998*).

The biomass has been steam exploded, processing 150-200 kg/h of dry biomass, to which water was added to raise the intrinsic humidity up to 50%. The pretreatment was carried out at 210 °C for 4 minutes. The severity factor (*SF*) was determined to be 3.84 according to the following Equation 2.1 (*Garrote et al., 1999*):

$$SF = \log(R) = \log\left(t \times e^{\frac{T-100}{14.75}}\right) \quad (2.1)$$

where t is pretreatment time and T is pretreatment temperature.

2.2 The hydrolysis of lignocellulosic biomass

2.2.1 Arundo donax hydrolysate

Steam exploded *Arundo donax* was mixed with distilled water at pH 5.2, to obtain a solution with 5% w/v solid content and it was treated with commercial enzymes purchased from Sigma-Aldrich consisting of cellulase from *Trichoderma reesei ATCC 26921* and β-glucosidase from *Aspergillus niger*.

Enzyme complexes used for the biomass pre-treatment usually consist of 1,4-β-D-glucanohydrolases (endoglucanases), 1,4-β-D-glucan cellobiohydrolases (exoglucanases), and β-D-glucoside glucohydrolase (β-glucosidase or cellobiase). A mix of two enzymes with cellulolytic activity was used to reduce the operating costs. The reason was also related to the choice of steam explosion as pretreatment because is able to separate hemicellulose from cellulose and lignin.

Cellulase activity was measured following the NREL filter paper assay (*Adney and Baker, 1996*; *Ghose 1987*) and reported in Filter Paper Units per milliliter of solution (FPU/mL). β-glucosidase activity was measured using the method described by Wood & Bhat (*1988*) and reported in cellobiase units (CBU). Enzymatic hydrolysis was performed in flask with 200 mL of slurry. Different hydrolysis tests were carried out to explore different process conditions. Initially, only cellulase from *Trichoderma reesei ATCC 26921* (15 FPU/g of cellulose) was introduced as control. Tests were carried out also to evaluate synergistic and inhibition phenomena, using cellulase and β-glucosidase with different dosages. As just mentioned, it was decided to introduce cellulase from *Trichoderma reesei ATCC 26921* (15 FPU/g of cellulose) and β-glucosidase from *Aspergillus niger* (30 CBU/g of cellulose) (*Gong et al., 2013*). To evaluate combined effects, fermentable sugar conversion and enzymatic hydrolysis yield, cellulase and β-glucosidase were introduced with a concentration equal to 7.5, 15, 30 FPU/g of cellulose and 15, 30, 60 CBU/g of cellulose, respectively.

Tests were performed to evaluate inhibition effects, introducing progressively a higher β-glucosidase concentration 30, 60, 90, 120, 180 CBU/g of cellulose combined with a concentration of cellulase of 15 FPU/g of cellulose.

The tests were conducted initially in batch mode with 200 mL of slurry (5% w/v of steam exploded *Arundo donax*), at 50 °C, initial pH 5.0, for 72 hours with agitation speed of 160 rpm (MINITRON, Infors HT, Switzerland).

The temperature has an important effect on the enzymatic activity. In fact, denaturation phenomena are caused by the decline of natural enzyme configuration above 60 °C (*Parameswaran et al., 2011*).

Tests were carried out to combine process conditions of enzymatic hydrolysis with oleaginous fermentation in terms of temperature and pH, with the aim to perform the tests in a single stage or in a single reactor. The selected temperatures were 30 °C and 40 °C in addition to 50 °C, already explored, and with optimal conditions obtained in previous tests.

Finally, steam exploded *Arundo donax* was suspended in different media in terms of composition and pH. Subsequent tests were performed under following conditions:

• without pH control (**without pH control**);
• with phosphate buffer (0.05 M) at pH 5.2 (**Buffer pH 5.2**);
• with phosphate buffer (0.2 M) at pH 6.2 (**Buffer pH 6.2**);
• with NaOH solution to pH 5.2 (**NaOH pH 5.2**).

The temperature was 40 °C, the speed rotation 160 rpm, the time 72 hours and the chosen optimum enzyme dosage as obtained from previous tests. The pH was monitored and adjusted using NaOH and HCl in the sample NaOH pH 5.2. All the tests were carried out, at least, in duplicate.

2.2.2 Wheat straw hydrolysate

Hydrolysis was also conducted on no pretreated wheat straw in powder. Acid hydrolysis was performed, using the following parameters: H_2SO_4 1% v/v, 121 °C and 21 min.

The acid hydrolysate was then subjected to enzymatic hydrolysis, treating the solid fraction and the liquid fraction using: cellulase from *Trichoderma longibrachiatum* (1% w/w), xylanase from *Trichoderma longibrachiatum* (1% w/w), 50 °C, 72 h, and MES buffer 100 mM and NaOH 0.1 M, where required to bring the pH to a value equal to 5.0. Hydrolysates were employed for the next stage of fermentation after vacuum pump filtration.

The samples were classified as:

- Wheat Straw subjected to Acid Hydrolysis (**WSAH**);
- Wheat Straw subjected to Acid Hydrolysis plus Enzymatic hydrolysis on the Liquid fraction (**WSAELH**);
- Wheat Straw subjected to Acid Hydrolysis plus Enzymatic hydrolysis on the Solid fraction (**WSAESH**).

2.3 Separate hydrolysis and fermentation (SHF) and enzymatic hydrolysis and oleaginous fermentation in single reactor (SRF) of steam exploded Arundo donax

After the optimization of process parameters related to enzymatic hydrolysis, the subsequent stage of oleaginous fermentation was considered.

Lipomyces Starkeyi DBVPG 6193 was used as oleaginous yeast, purchased from the Culture Collection of Dipartimento di Biologia Vegetale (University of Perugia, Italy). The strain was maintained at 5 °C on a YPD solid medium with the following composition (g/L): yeast extract (10), peptone (20), D-glucose (20), agar (20). Prior to fermentation, yeast was grown in a 100 mL Erlenmeyer flasks with an initial volume of 50 mL which contained (g/L):

KH_2PO_4 (3.0), Na_2HPO_4 (1.0), yeast extract (5.0), glucose (10.0), peptone (5.0). The pH was adjusted to 5.5÷6 and, prior to inoculation, the pre-culture broth was sterilized at 121 °C for 21 min. Culture media for Single Cell Oil production were inoculated with 5% v/v of pre-culture media. The incubation of the preculture media were carried out at 30 °C, 160 rpm for 48 hours (Minitron HT Infors, Switzerland) and microbial biomass analyzed through turbidometry at 600 nm up to 1 A.U. (Absorbance Unit) and then used as inoculum. Tests in Separate Hydrolysis and Fermentation (SHF) and enzymatic hydrolysis and Fermentation in Single Reactor (SRF) were conducted using 500 mL aerobic flasks.

SRF experiments were performed under non-sterile conditions to preserve protein and carbohydrate integrity, preventing the Maillard reaction (*Rosenthal et al., 1996*) as well to retain the most likely parameters for industrial applications. Suspension of steam-exploded *Arundo donax* (5% w/v) was used in an Erlenmeyer flask with a working volume of 200 mL. A commercial mixture containing 15 FPU/g of cellulose of Cellulase from *Trichoderma reesei* and 30 CBU/g of cellulose of Cellobiase from *Aspergillus niger* (Sigma-Aldrich) were employed. After 48 h, a suspension of yeast cells *(Lipomyces Starkeyi*) was added to the same flask using 5% v/v concentration.

The pH control was carried out during the experimental tests to limit the pH fluctuations. The enzymatic hydrolysis of the lignocellulosic materials usually leads to a pH reduction, whereas the oleaginous fermentation causes a pH increase.

Consequently, the experimental tests in enzymatic hydrolysis and Fermentation in Single Reactor (SRF) were performed following three different protocols as regards pH control:

(a) without pH control (**SRF without pH control**),

(b) keeping a pH value of 5.2 using a phosphate buffer solutions (**SRF Buffer pH 5.2**);

(c) adding suitable amounts of 1 M NaOH or 0.1 M HCl to maintain a pH value of 5.2 under discontinuous conditions (**SRF NaOH pH 5.2**).

The test in Separate Hydrolysis and Fermentation (SHF) were performed in the same culture conditions observed for the tests just described, except the separation of hydrolysis and fermentation steps. The highest pH values (5÷6) were observed when adopting a semicontinous control (c). In the presence of a phosphate buffer solution (b), the measured pH was in the range 4.5-5.0. When no pH control was adopted (a), the lowest values of pH were observed, as the pH of the medium dropped below 4.

In a typical SRF test, all the enzymes were added at the beginning and the biomass was hydrolysed at 40 °C and 160 rpm (Minitron, Infors HT, Switzerland). After 48 hours, temperature and stirring rate were changed to 30 °C and 160 rpm, respectively, and yeast cells

(*Lipomyces Starkeyi DBVPG 6193)* were inoculated. All the tests were carried out, at least, in duplicate.

2.4 Mixed oleaginous yeast-microalga cultures

In an attempt to improve lipid yields, it was evaluated synergistic effect of microalga-oleaginous yeast mixed cultures. The tests were conducted, initially, with the aim to find optimum culture conditions for microalga strains. The seed culture was pre-cultivated onto 100 mL of BBM medium at in an incubator shaker at a shaking speed and continuous illuminated by 150 µmol m^{-2} s^{-1} cool-white fluorescent lamps.

Chlorella emersonii 316/25, *Chlorella protothecoides 165*, *Chlorella saccarophila 042, Viridiella fridericiana 035*, *Chlorella zofingiensis 252, Chlorella sorokiniana 317* were achieved from the ACUF collection of the Department of Biological Science at the University of Naples, Federico II (http://www.biologiavegetale.unina.it/acuf.html). These algae have only minimal requirements to the medium for the growth. There is the need of inorganic ions and a minimal quantity of organic compounds, such as vitamins. Carbon, nitrogen and phosphorus are the most important nutrients for the autotrophic algal growth (*Becker et al., 1986*). Bold Basal medium (*Bold, 1949*) is an artificial freshwater medium, which is practical for growing green algae. Bold Basal Medium (BBM) supplemented with $NaNO_3$ (40 mg/L) as nitrogen source was adopted. The medium was autoclaved for 20 minutes. The final pH should be 6.8. The BBM medium contained the following components: $CaCl_2 \cdot 2H_2O$ ($1.70 \cdot 10^{-4}$ M), KH_2PO_4 ($1.29 \cdot 10^{-3}$ M), EDTA anhydrous ($1.71 \cdot 10^{-4}$ M), KOH ($5.52 \cdot 10^{-4}$ M), K_2HPO_4 ($4.31 \cdot 10^{-4}$ M), NaCl ($4.28 \cdot 10^{-4}$ M), $MgSO_4 \cdot 7H_2O$ ($3.04 \cdot 10^{-4}$ M), $NaNO_3$ ($2.94 \cdot 10^{-3}$ M), H_3BO_3 ($1.85 \cdot 10^{-4}$ M), $FeSO_4 \cdot 7H_2O$ ($1.79 \cdot 10^{-5}$ M), H_2SO_4 ($1.79 \cdot 10^{-5}$ M), $ZnO_4 \cdot 7H_2O$ ($3.07 \cdot 10^{-5}$ M), $MnCl_2 \cdot 4H_2O$ ($3.07 \cdot 10^{-5}$ M), MnO_3 ($4.93 \cdot 10^{-6}$ M), $CuSO_4 \cdot 5H_2O$ ($6.29 \cdot 10^{-6}$ M), $Co(NO_3)_2 \cdot 6H_2O$ ($1.68 \cdot 10^{-6}$ M). The concentration of inoculum was equal to 10% v/v.

The flasks were incubated at 30 °C and under light intensity around 100 µmol photons m^{-2} s^{-1}. All chemicals were purchased from Sigma-Aldrich. *Chlorella emersonii 316/25* and *Lipomyces starkeyi DBVPG 6193* were incubated from BBM and YEG agar slant, respectively, to 500 mL Erlenmeyer flasks containing 100 mL seed media. The culture flasks were inoculated, separately and simultaneously, to achieve the initial cell density of $5.5 \cdot 10^5$ Cells/mL for both microorganisms. The cultures performed containing both inorganic and organic substrates and were grown in:

- BBM medium (**BBM**);
- BBM plus D-Glucose (10 g/L) (**BBM+G**);

- Yeast Extract and D-Glucose medium, which contains (g/L): KH_2PO_4 (3.0), Na_2HPO_4 (1.0), yeast extract (5.0), glucose (10.0), peptone (5.0) (**YEG**).

The pH of media was adjusted to 6 and prior to inoculation, the culture broth were sterilized at 121 °C for 21 min.

In the last step steam exploded *Arundo donax* was used. *Arundo donax* hydrolysate (**ADH**) was inoculated using *Lipomyces starkeyi DBVPG 6193* and microalga strains, *Chlorella emersonii 316/25*, simultaneously. The initial cell density was $5.5 \cdot 10^5$ Cells/mL for both microorganims. All the tests were carried out, at least, in duplicate with the same operating conditions as above mentioned.

For the experimental activity performed at INRA-LBE (Laboratoire de Biotechnologie de l'Environnement) in Narbonne (France), *Chlamydomonas* sp. was used. It is a model green alga (*Harris, 2001*) that can grow mixotrophically, using various carbon sources and then it is studied for its behavior in different operating conditions. For example it can uptake acetate, which is then incorporated into citric cycle (*Johnson & Alric, 2012*).

The aim of this experimental activity was to verify the differences of kinetic parameters related to different growth conditions. The cultures were performed in 100 mL of MP or enriched MP medium with glucose, yeast extract and sodium acetate, added using the concentrations as explained subsequently. All the tests were carried out at 24 °C in an incubator shaker and continuous illuminated by 100 μmol m^{-2} s^{-1} cool-white fluorescent lamps. The **MP100** medium contained the following components (per liter):

Buffer MES hydrate (100 mM), Beijerinck solution 40x (25 mL), KPO_4 (1 M), Hutner's trace elements (1 mL).

After the autoclaving, the pH was set to 6.25, as obtained from previously optimization (data not shown). Tests were conducted using enriched MP100 medium, adding D-Glucose (**G**) (10 g/L) and Yeast extract (**YE**) (0.5 g/L) inoculated by *Chlamydomonas* sp. (**Chla**), *Lipomyces starkeyi DBVPG 6193* (**Lip**) and both strains (**Chla:Lip**):

- **MP100+G+YE Chla**;
- **MP100+G+YE Lip**;
- **MP100+G+YE Chla:Lip 1:1, 2:1, 3:1**.

Preliminary tests were carried out to evaluate the best *Chlamydomonas* sp./*Lipomyces starkeyi DBVPG 6193* ratio. The cultures were inoculated by 5% v/v of the seed precultures. The same operating conditions were used for enriched MP100 medium adding also sodium acetate (**Ac**) (1.5 g/L). The samples were marked as:

- **MP100+G+YE+Ac Chla**;
- **MP100+G+YE+Ac Lip**;
- **MP100+G+YE+Ac Chla:Lip 1:1, 2:1, 3:1**.

Sodium acetate was added to verify the ability of yeast and microalga to metabolise an alternative carbon source represented by the organic acid and to simulate realistic growth conditions in presence of lignocellulosic biomass hydrolysates. All the tests were carried out, at least, in duplicate.

In order to verify the growth results in mixed cultures, two different vials were performed, where O_2 was replaced by N_2 and marked as:

- **MP100+G+YE Chla:Lip 1:1 N_2**;
- **MP100+G+YE+Ac Chla:Lip 1:1 N_2**.

In the last step wheat straw was used (**WS**). The final aim was focused on growth conditions applicable also with lignocellulosic biomass hydrolysates or other low cost feedstocks. The samples were previously processed using acid (**A**) and acid plus enzymatic hydrolysis (**AE**). Several different dilution factors (0, 2, 4) were used to verify inhition effects due to phenols and furans. Finally, the cultures were inoculated by *Lipomyces starkeyi DBVPG 6193* (**Lip**) and microalga strain, *Chlamydomonas* sp. (**Chla**), separately and simultaneously (**Chla:Lip 1:1**) with the same inoculum concentrations of 5% v/v. All the tests were carried out, at least, in duplicate. The samples were labeled as:

- **WSAH Chla:Lip 1:1 dil 0, 2, 4** (Wheat Straw subjected to Acid Hydrolysis, using *Chlamydomonas*:*Lipomyces starkeyi* ratio of 1:1 and dilution factors of 0, 2, 4);
- **WSAELH Chla:Lip 1:1 dil 0, 2, 4** (Wheat Straw subjected to Acid Hydrolysis plus Enzymatic hydrolysis on the Liquid fraction, using *Chlamydomonas*:*Lipomyces starkeyi* ratio of 1:1 and dilution factors of 0, 2, 4);
- **WSAESH Chla:Lip 1:1 dil 0, 2, 4** (Wheat Straw subjected to Acid Hydrolysis plus Enzymatic hydrolysis on the Solid fraction, using *Chlamydomonas*:*Lipomyces starkeyi* ratio of 1:1 and dilution factors of 0, 2, 4).

2.5 Combined yeast and microalgal consortium in pilot scale raceway pond for urban wastewater treatment and potential SCO production

In order to monitore the growth conditions of oleaginous yeast (*Lipomyces starkeyi DBVPG 6193*) and microalgal consortium in mixed cultures using urban wastewater as different low cost feedstock, the experimental activity was carried out in a raceway pond.

The goal of these tests was focused also on the decrease of capital costs strictly related to the operating conditions (no sterile) and to the reactor design (raceway pond).

The tests were carried out in a 200 L raceway pond operating outdoor with one single-loop open channel and semi-circular end-walls. The pilot plant was installed on the roof of the Department of Bioscience and Territory, University of Molise, Pesche (IS), Italy [Figure 2.1]. It operated during the month of July with an average natural light intensity of approximately 600 μmol m^{-2} s^{-1} and natural light/dark cycles. A four-blade paddle wheel driven by a motor engine working at 6 rpm supported the mixing of the culture media. The culture media consisted in untreated urban wastewater, half diluted with tap water for a total volume of 150 L.

The microalgal consortium, used as inoculum, was obtained from the urban wastewater treatment plant of Isernia (Italy) and maintained in laboratory controlled condition using BBM medium in 1 L flask in agitation (150 rpm) to maintain the biomass in suspended condition, under a continuous light of 1500 Lux (Cool White Fluorescent Lamps) at 25 °C. Optical microscope analysis showed that inoculum was composed by cyanobacteria, diatoms and microalgae (mostly *Scenedesmus* sp. and *Chlorella* sp.).

Lipomyces starkeyi DBVPG 6193 was added as oleaginous yeast following the culture conditions as already described [Chapter 2, § 2.3]. Several physiological studies relating to growth and lipid production by *Lipomyces starkeyi* was reported in literature but urban wastewater was tested as growth medium for this microorganism (*Tapia et al., 2012*). The microalgal consortium and *Lipomyces starkeyi* inoculum concentration was 3% and 1.5% v/v, respectively. The cultivation was carried out for 14 days in batch mode. The sample was labeled as:

- **UWRP Lip:m.c** (Urban Wastewater in Raceway Pond, using Lipomyces starkeyi and microalgal consortium ad inoculum).

To identify bacteria, mould and fungus colonies, culture media were used to inoculate Petri dishes with following media:

- ✓ Brain Heart Infusion agar (BHI);
- ✓ Mannitol Salt agar (MSA);
- ✓ Mac Conkey agar (MAC);
- ✓ Sabouraud broth (SAB).

Fig. 2.1: Pilot scale raceway pond installed on the roof of the Department of Bioscience and Territory, University of Molise, Pesche (IS), Italy

2.6 Analytical methods

Measurements of pH were made by a inoLab® Multi 740 Multimeters pH-meter (WTW). The biomass concentration was monitored with a Shimadzu UV6100 and Thermo Scientific Helios Epsilon spectrophotometer (Japan) and by measuring turbidity of liquid samples at 600 nm. Dry cell weight (DCW) or microbial biomass (g/L) were determined by filtering 2-3 mL of culture over pre-weight PES filters (0.45 μm; Sartorius Biolab, Germany). The retained biomass on filters was washed, dried at 105 °C for 24 hours and then stored in a desiccator before being weighed. The individual cell counts of yeast and microalga was determined with a haemocytometer. When the microorganisms were cultured in SRF, turbidometric measurements could not be carried out due to the darkness of the medium. Consequently, the total count of microorganisms was carried out by sequential diluition in Petri dish containing YPD agar medium (peptone 10 g/L, yeast extract 10 g/L, glucose 20 g/L, agar 15 g/L) (Sigma-Aldrich). The cell proliferation was measured as CFU/mL (Colony Forming Unit/mL).

After centrifugation and filtration with 0.2 μm cut-off filters, the liquid sample was analysed for residual substrate content (glucose) and soluble fermentation products (VFA, alcohols). Glucose was measured using an enzymatic kit (Sigma Aldrich). Reducing sugars and pentose sugars were measured by Nelson-Somogyi assay (*Sadarivam and Manickam, 1996*) and Fluoroglucinol (*Douglas, 1981*) colorimetric assay, respectively. Glucose was also analyzed by HPLC (LC2010, Shimadzu, Japan), equipped with a refractive index detector (RID-20A, Shimadzu, Japan).

The total concentration of phenolic compounds was determined using Folin-Ciocalteu (*Singleton et al., 1999*), using cathecol as standard. A simple method based on UV spectra was followed for the estimation of total furans (furfural and hydroxymethylfurfural) in the

hydrolysates (*Martinez et al., 2000*). UV spectra were recorded on a spectrophotometer using 1 cm cells.

COD was analysed by a colorimetric method using Hach vials. The TOC measurements were carried out with a TOC-VCSH/CSN (Shimadzu, Japan), upon suitable dilution of a culture medium sample. The TOC values were obtained subtracting the IC (inorganic carbon) value from the TC (total carbon) value.

For volatile organic acids, alcohols and carbohydrates were also used the following methods:

- ✓ Volatile organic acids and ethanol were determined by GC analysis, using a Shimadzu GC-17A equipped with a FID detector and a capillary column with a PEG stationary phase (BP20, 30 m by 0.32 mm i.d., 0.25 μm film thickness, from SGE). Samples of 1 μL were injected with a split-ratio of 1:10. Helium was fed as carrier gas with a flow rate of 6.5 mL/min. Injector and detector temperatures were set to 320 °C and 250 °C, respectively. Initial column temperature was set to 30 °C, kept constant for 3 min, followed by a ramp of 10 °C/min till 140 °C, then kept constant for 1 min.
- ✓ VFA (Volatile Fatty Acids) were also analyzed by HPLC (LC2010, Shimadzu, Japan), equipped with a refractive index detector (RID-20A, Shimadzu, Japan).
- ✓ Concentrations of organic acids, alcohols and carbohydrates were measured by HPLC (Dionex Ultimate 3000) with a refractive index detector (Waters R410). Samples were first centrifuged at 12,000 g for 15 min and then supernatants were filtered with 0.2 μm syringe filters. HPLC analysis were performed at a flow rate of 0.7mL/min on an Aminex HPX-87H, 300 x 7.8 mm (Bio-Rad) column at a temperature of 35 °C. H_2SO_4 at 4 mM was used as the mobile phase.

NH_4^+, NO_3^- and PO_4^{3-} content were measured in the soluble phase by ion chromatograph (ICS 3000, Dionex) equipped with pre-columns and separation columns CG 16 and CS16 (3 mm ø) for cations and AG15 and AS15 (2 mm ø) for anions, respectively. The eluents used for this analysis were methanesulphonic acid (MSA) 25 mM at 0.35 mL/min for cations and KOH 10 mM at 0.3 mL/min for anions at initial time and MSA 40 mM at 24 min and KOH 74 mM at 28 min. The column temperature was set at 35 °C. To obtain the soluble fractions, the samples were centrifuged at 14,000 rpm for 10 min (Eppendorf 5424).

Lipid estimation was performed with sulfo-phospho-vanillin assay (*Mishra et al., 2014*).

Lipids were extracted by a method adapted from Bligh and Dyer (*Bligh and Dyer, 1959*). The samples were stirred in a $CHCl_3/CH_3OH$ mixture (2:1 w/v) over 24 hours, and the oleaginous biomass was filtered off and washed with additional $CHCl_3$. This procedure was repeated three times. The solvent was then removed by evaporation under N_2 stream.

The total lipid concentration was estimated by gravimetric method. To calculate the lipid concentration of the cells, the cells were dried to a constant weight with an oven at 80 °C. The lipids extracted were subjected to transesterification reaction in a stirred container at 60 °C for 10 min, using NaOH (1% w/v) or HCl (1% w/v) as catalysts and using methanol as reagent. The samples were dried by N_2 stream and subsequently 1 mL of heptane was added for the analysis. The fatty acid compositions of the FAME were analyzed using gas chromatography (GC). The GC (GC-MS 2010, Shimadzu, Japan) was equipped with a flame ionization detector and an Omegawax 250 (Supelco) column (30 m x 0.25 mm I.D., 0.25 µm). Helium was used as carrier gas (flow rate: 30 mL/min). The samples were initially dissolved in 1 mL of heptane and 1 µL of this solution was loaded onto the column. The temperature of the column was kept at 50 °C for 2 min, then heated to 220 °C at a rate of 4 °C/min, and finally kept constant for 2 minutes. Methyl decanoate was used as internal standard. The peaks of each methyl ester was identified by comparing the retention time with the peak of the pure standard compound.

Gas measurement for carbon dioxide (CO_2), nitrogen (N_2) and oxygen (O_2) was performed using Perkin Elmer® Clarus 480 Gas Chromatograph, Waltham, USA. 200 µL of each sample was injected in a capillary R-Q column separating CO_2 from other gases. The remaining gases were separated by another capillary Rt-Molsieve 5Å column. The temperature of the injector was 250°C and the thermic conductivity detector was at 150 °C and the vector gas was Argon (350 kPa, 34 mL/min). Calibration was performed on this machine with a standard gas with 25% CO_2, 0.1% H_2S, 0.5% O_2, 10% N_2 and 64.4% CH_4 in composition. Biogas measurement was performed for measuring gas composition in the reactors once a week after steady state.

A fluorometer (AquaFluorTM; Handheld Fluorometer/Turbidimeter; Turner Designs) was used to measure the content of in vivo chlorophyll a in the untreated samples. Therefore the excitation light of the fluorometer passes through the medium and causes the Chlorophyll a inside the cells to fluoresce. This signal allows estimating the concentration of Chlorophyll a in the sample.

3. RESULTS AND DISCUSSION

3.1 Process parameter optimization of enzymatic hydrolysis

It is believed that improvements in enzyme technology are the key parameter to an economically viable lignocellulosic Single Cell Oil production (*Horn and Eijsink, 2010*). It has also been proven that enzyme loading has an high effect on glucose yield (*Schell et al., 1999*) and in terms of operating costs. It makes enzyme concentration to an interesting topic to further study.

The first tests of enzymatic hydrolysis were conducted to assess the synergistic action of cellulase and β-glucosidase with their separate use, in order to verify the theoretical hypothesis (*Wilson, 2008*) of new site formation for hydrolytic attack, the presence of stereospecific enzymatic activity and the formation of enzyme-enzyme complex (*Wood et at., 1990*; *Woodward, 1991*; *Boisset et al., 2000*).

Figures 3.1, 3.2, 3.3 show profiles of glucose, reducing and pentose sugar concentration derived from different hydrolysis processes carried out through combined and separated use of cellulase from *Trichoderma reesei ATCC 26921* and β-glucosidase from *Aspergillus niger*. The samples, containing 5% w/v of steam exploded *Arundo donax*, were kept in agitation at 160 rpm, 50 °C for 72 hours (*Gong et al., 2013; Zuccaro et al., 2014*). The sample in absence of both enzymes was chosen as control (**without enzyme**).

Enzyme dosages were expressed as FPU/g of cellulose for cellulase and CBU/g of cellulose for β-glucosidase (FPU [=] Filter Paper Unit, CBU [=] Cellobiase Unit) and decided after previous experimental activity (data not shown).

The samples were labeled as:

- **without enzyme**;
- **15 FPU** (only cellulase from *Trichoderma reesei*, dose of 15 FPU/g cellulose);
- **30 CBU** (only β-glucosidase from *Aspergillus niger*, dose of 30 CBU/g cellulose);
- **15 FPU & 30 CBU** (cellulase from *Trichoderma reesei* and β-glucosidase from *Aspergillus niger*, dose of 15 FPU/g cellulose and 30 CBU/g cellulose, respectively).

Figures 3.1, 3.2 show maximum glucose and reducing sugar concentrations for the sample containing only β-glucosidase (**30 CBU**), 2.6 g/L and 5 g/L, respectively, lower than the values observed in the sample with only cellulase (**15 FPU**), where the same maximum values were 5 g/L and 16 g/L, respectively. These results may be justified by different hydrolytic action of cellulase and β-glucosidase. The first one breaks polymeric chains in specific and random positions. It removes mono (glucose) and dimers (cellobiose) from the cellulosic

chain (*Howard et al., 2003*), and it is the only enzyme able to hydrolyse highly crystalline cellulose. Finally, the resulting molecules of cellobiose are degraded by β-glucosidase, which catalyses the hydrolysis of the resulting dimers in two molecules of glucose. β-glucosidase is very important, since cellobiose inhibits cellulase activity. The values of pentose sugar concentration in **15 FPU** and **30 CBU**, were very low and justified, probably, by the decrease of hemicellulosic fraction due to the separation from lignin and cellulose due to the previous steam-exploded pretreatment of *Arundo donax* [Figure 3.3].

Synergistic action of both enzymes, cellulase and β-glucosidase (**15 FPU & 30 CBU**), allows the increase of glucose and reducing sugar concentration, 5.5 g/L and 20 g/L, respectively [Figure 3.1, 3.2], if compared to the samples, **15 FPU** and **30 CBU**, above mentioned. It was confirmed that β-glucosidase activity carried out only after fermentable sugar production due to cellulase activity.

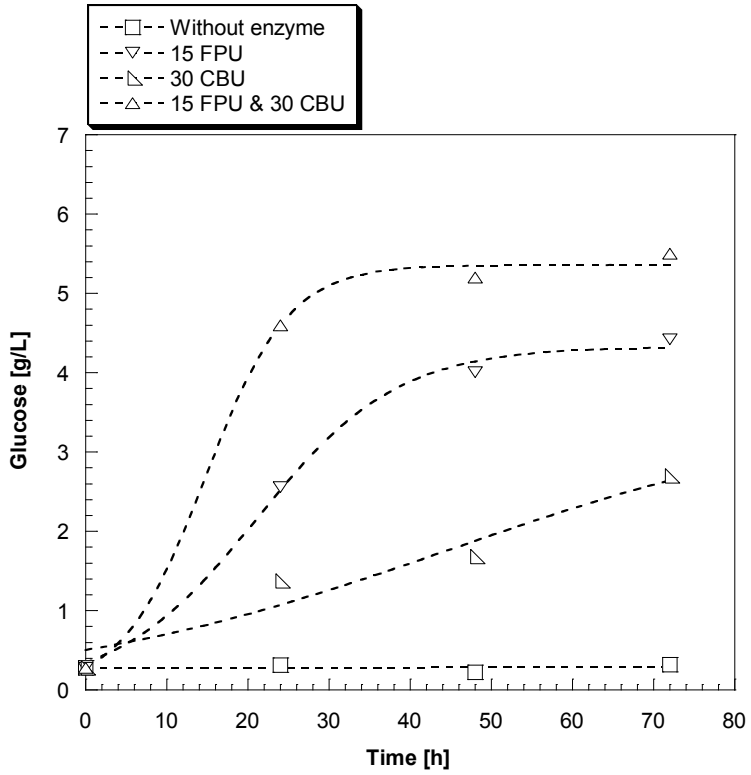

Fig. 3.1: Glucose profiles related to steam exploded *Arundo donax* hydrolysates (T=50°C, 160 rpm, 72 h)

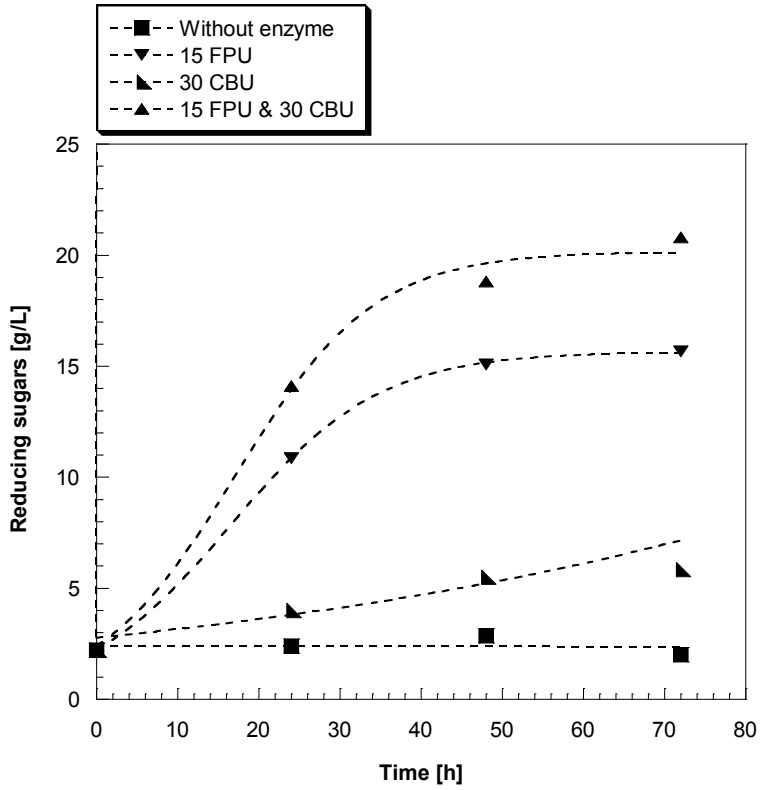

Fig. 3.2: Reducing sugar profiles related to steam exploded *Arundo donax* hydrolysates (T=50°C, 160 rpm, 72 h)

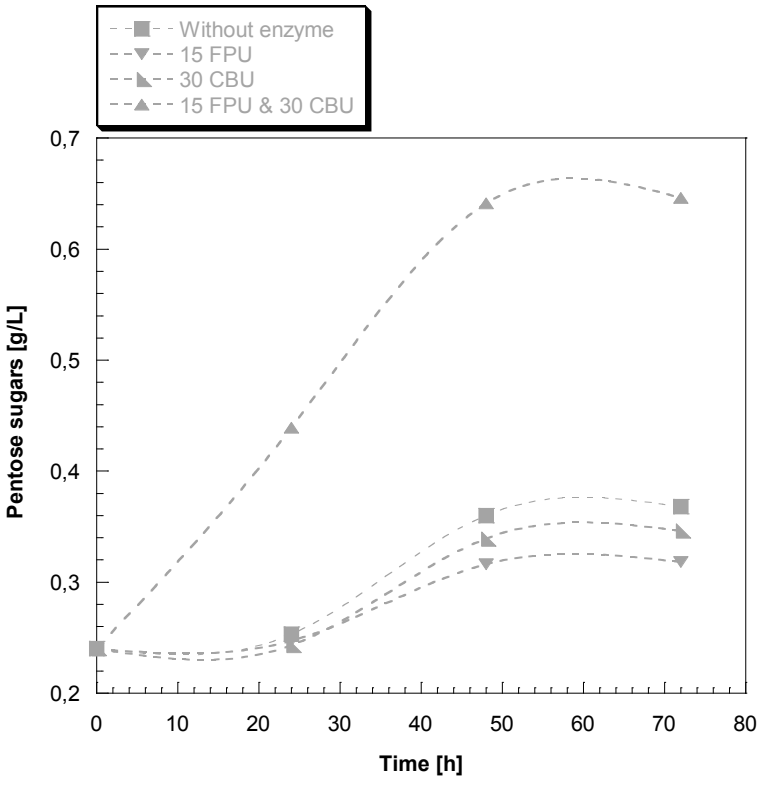

Fig. 3.3: Pentose sugar profiles related to steam exploded *Arundo donax* hydrolysates (T=50°C, 160 rpm, 72 h)

Table 3.1 shows glucose yield ($Y_{Glucose}$) (*Ask et al., 2012*; *Chien-Tai and Meyer, 2014*). The values were calculated according to Equation 3.1. Theoretical glucose ($w_{Theoretical\ Glucose}$) was related to cellulose content in *Arundo donax* (*Pirozzi et al., 2015*).

$$Y_{Glucose}[g\,g^{-1}] = \frac{m_{Glucose}}{m_{Theoretical\ Glucose}} \tag{3.1}$$

The experimental results reported in Figure 3.1, 3.2 and 3.3 were fitted according to the following Equation (3.2) [Figures 3.1, 3.2, 3.3; (dashed line)]:

$$G[gL^{-1}] = \frac{G_0\, e^{\mu_G t}}{1-\frac{G_0}{G_{max}}(1-e^{\mu_G t})} \tag{3.2}$$

where G is the concentration of glucose (g/L), G_0 is the initial glucose concentration (g/L), G_{max} is the maximum concentration of glucose (g/L) and μ_G is the specific rate for glucose production during enzymatic hydrolysis.

The agreement between the experimental data and the result of the interpolating relationship was satisfactory [Figures 3.1, 3.2, 3.3] and confirmed the presence of inhibition phenomena. Table 3.1 also reports the parameter of productivity (ν_G) assessed as ratio between the glucose concentration (g/L) and the process time (h).

Tab. 3.1: Kinetic parameters for steam exploded *Arundo donax* hydrolysates (T=50°C, 160 rpm, 72 h)

	μ_G [h^{-1}]	$Y_{Glucose}$ [$g\ g^{-1}$]	ν_G [$g\ L^{-1}\ h^{-1}$]
Without enzyme	4.62×10^{-4}	0.020	0.004
30 CBU	0.039	0.172	0.037
15 FPU	0.116	0.283	0.061
15 FPU & 30 CBU	0.195	0.379	0.082

It was observed an increase in specific rate of glucose production (μ_G) from 0.039 h^{-1} for the sample with only β-glucosidase (**30 CBU**) to 0.116 h^{-1} with only cellulase (**15 FPU**) and 0.195 h^{-1} with cellulase and β-glucosidase (**15 FPU & 30 CBU**) [Table 3.1]. These results demonstrated that the combined use of cellulase and β-glucosidase (**15 FPU & 30 CBU**) increased μ_G in comparison to the sample with only cellulase (**15 FPU**) of 40.5% and with only β-glucosidase (**30 CBU**) of 80%; and $Y_{Glucose}$ of 25% and of 54.5%, respectively.

In a attempt to verify the impact of enzyme dosage on glucose yield related to the enzymatic hydrolysis of steam exploded *Arundo donax* and on inhibition phenomena related to combined use, tests were carried out under the following operating conditions:

- 7.5 FPU/g of cellulose and 15 CBU/g of cellulose (**7.5 FPU & 15 CBU**);
- 15 FPU/g of cellulose and 30 CBU/g of cellulose (**15 FPU & 30 CBU**);
- 30 FPU/g of cellulose and 60 CBU/g of cellulose (**30 FPU & 60 CBU**);

each sample was characterized by CBU/FPU ratio equal to 2.

Figure 3.4 shows an increase of glucose concentration from 4.5 g/L in **7.5 FPU & 15 CBU** to 6.2 g/L in **30 FPU & 60 CBU**. The increase of glucose production was 37% compared to an increase of enzyme dosage of 4 times. Probably, it is related to inhibition phenomena that prevent a progressive glucose production during the enzymatic hydrolysis. This consideration may be confirmed by the values of glucose production rate, shown in Table 3.2. μ_G was 0.189 h^{-1} for the sample **7.5 FPU & 15 CBU** and 0.186 h^{-1} for **30 FPU & 60 CBU**, that confirmed the inability to increase μ_G beyond a certain value even by increasing the enzyme dosage.

Figures 3.5 and 3.6 report reducing and pentose sugar profiles with different trends if compared to glucose [Figure 3.4]. Increasing enzyme loading could overcome the inhibition due to lignin. It is likely that the specific adsorption sites for acting enzymes are more likely to be occupied, but site concentration remains the same at the beginning of hydrolysis. For example, Hu et al. (*2014*) reported no clear correlation between the amount of residual lignin and the positive effect of hydrolytic enzymes observed on the enzymatic hydroysis of steam and organosolv pretreated pine. Consequently, more research on enzyme interactions with lignin is necessary to better utilize enzymatic synergism, to avoid the lignin inhibition and enahnce the enzyme saccharification yield.

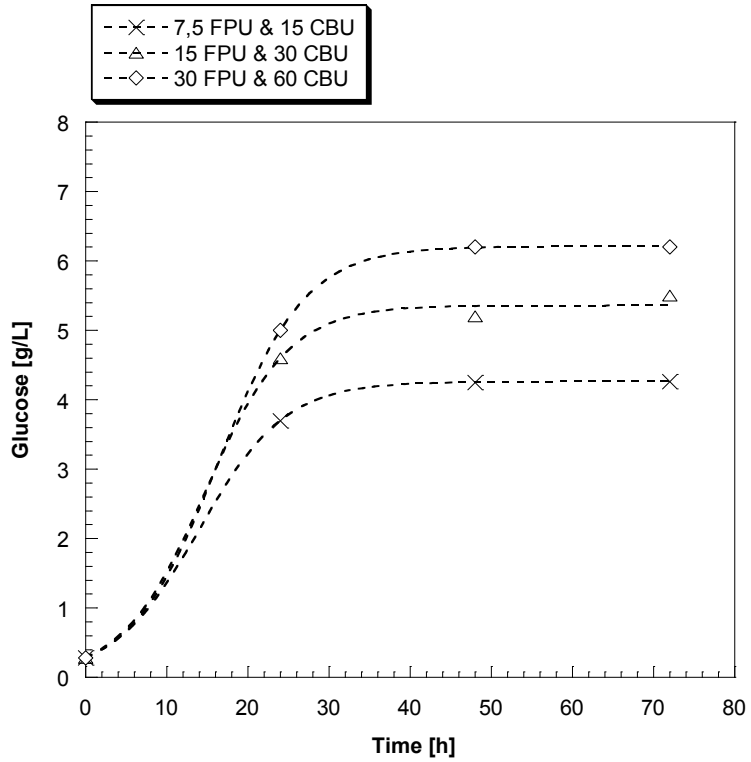

Fig. 3.4: Glucose profiles related to steam exploded *Arundo donax* hydrolysates (T=50°C, 160 rpm, 72 h, CBU/FPU=2)

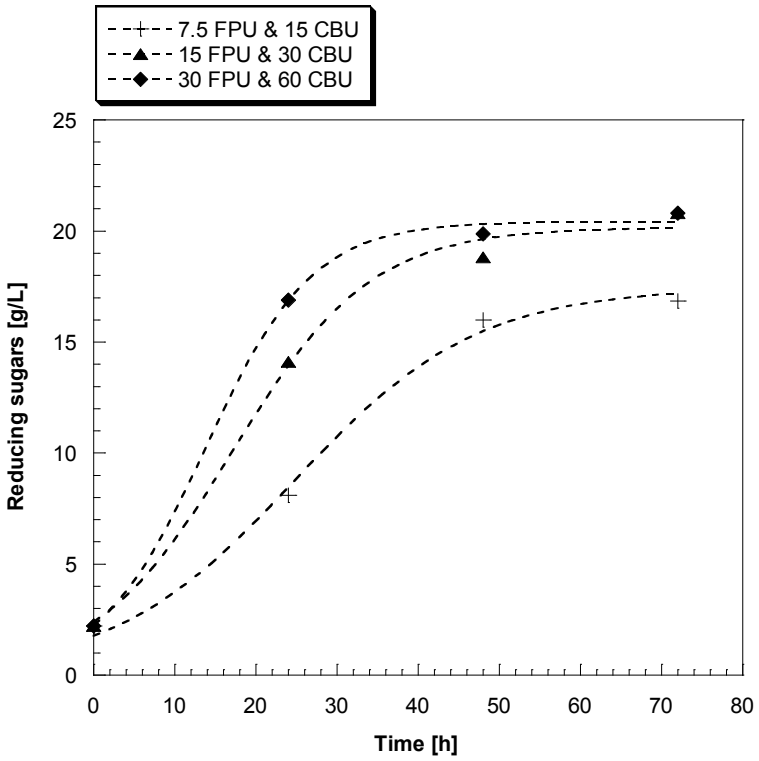

Fig. 3.5: Reducing sugar profiles related to steam exploded *Arundo donax* hydrolysates (T=50°C, 160 rpm, 72 h, CBU/FPU=2)

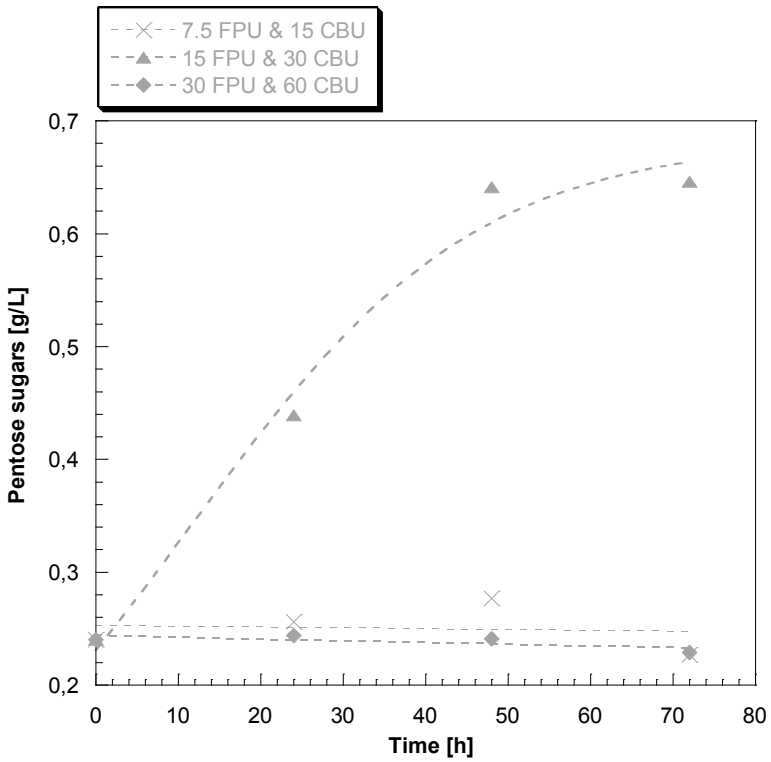

Fig. 3.6: Pentose sugar profiles related to steam exploded *Arundo donax* hydrolysates (T=50°C, 160 rpm, 72 h, CBU/FPU=2)

Table 3.2 also shows the values of $Y_{Glucose}$ and ν_G. It was possible to observe and increase of 31% from **7.5 FPU & 15 CBU** to **30 FPU & 60 CBU** for both values.

Tab. 3.2: Kinetic parameters for steam exploded *Arundo donax* hydrolysates (T=50 °C, 160 rpm, 72 h, CBU/FPU=2)

	μ_G [h^{-1}]	$Y_{Glucose}$ [$g\ g^{-1}$]	ν_G [$g\ L^{-1}\ h^{-1}$]
7.5 FPU & 15 CBU	0.189	0.273	0.059
15 FPU & 30 CBU	0.195	0.379	0.080
30 FPU & 60 CBU	0.186	0.397	0.086

Figures 3.7 and 3.8 show the maximum glucose and reducing sugar concentration as a function of enzyme (cellulase, FPU) dosage.

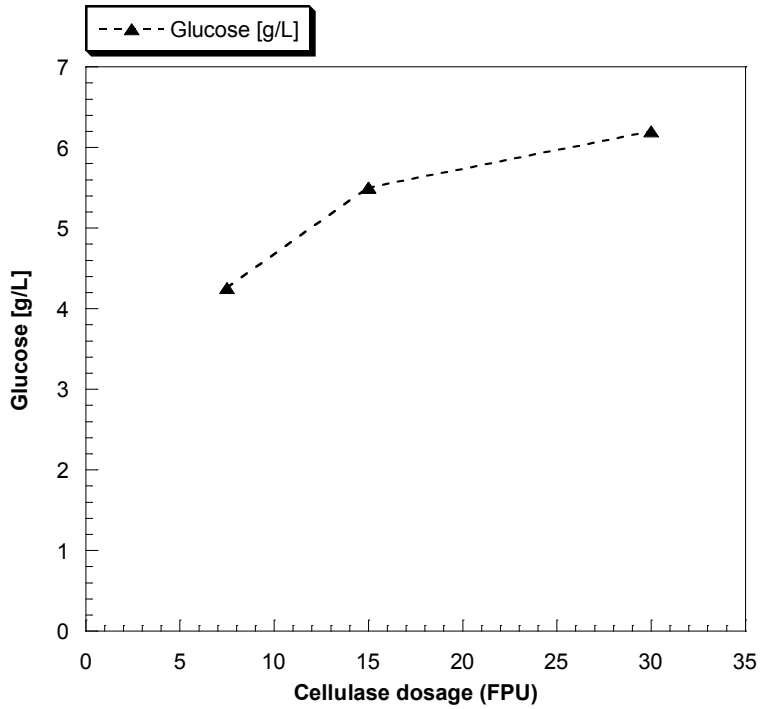

Fig. 3.7: Maximum glucose concentration as a function of enzyme dosage (T=50°C, 160 rpm, 72 h, CBU/FPU=2)

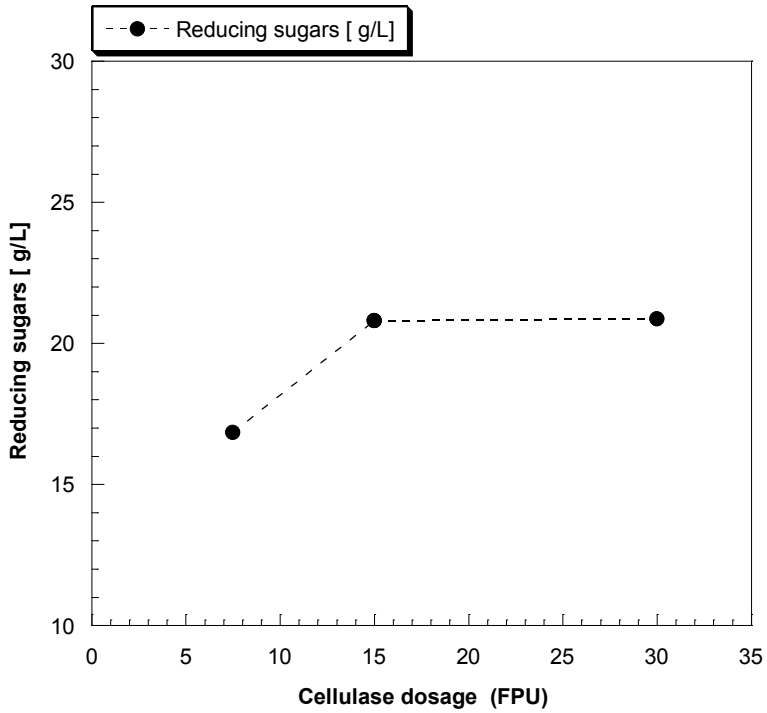

Fig. 3.8: Maximum reducing sugar concentration as a function of enzyme dosage (T=50°C, 160 rpm, 72 h, CBU/FPU=2)

Therefore, it was supposed that the remaining glucose production is related to the β-glucosidase activity rather than cellulase.

Experimental evidence suggested the presence of a residual unconverted cellobiose, from which to obtain additional glucose. Therefore, the next experimental work was performed to increase the ratio of β-glucosidase and cellulase, from sample containing 15 FPU/g of cellulose and 30 CBU/g of cellulose, only by increasing the concentration of β-glucosidase from *Aspergillus niger* and an unchanged value of cellulase from *Trichoderma reesei*.

Figure 3.9 reports that an increase in β-glucosidase concentration (CBU) involves an increase in glucose production, confirming the hypothesis of residue and unconverted cellobiose. Glucose concentration increased from 5.9 g/L in the sample **15 FPU & 30 CBU** to 7.5 g/L in **15 FPU & 180 CBU**.

The possibility to operate with a higher β-glucosidase dosage should be, however, compared to the increase of glucose yield.

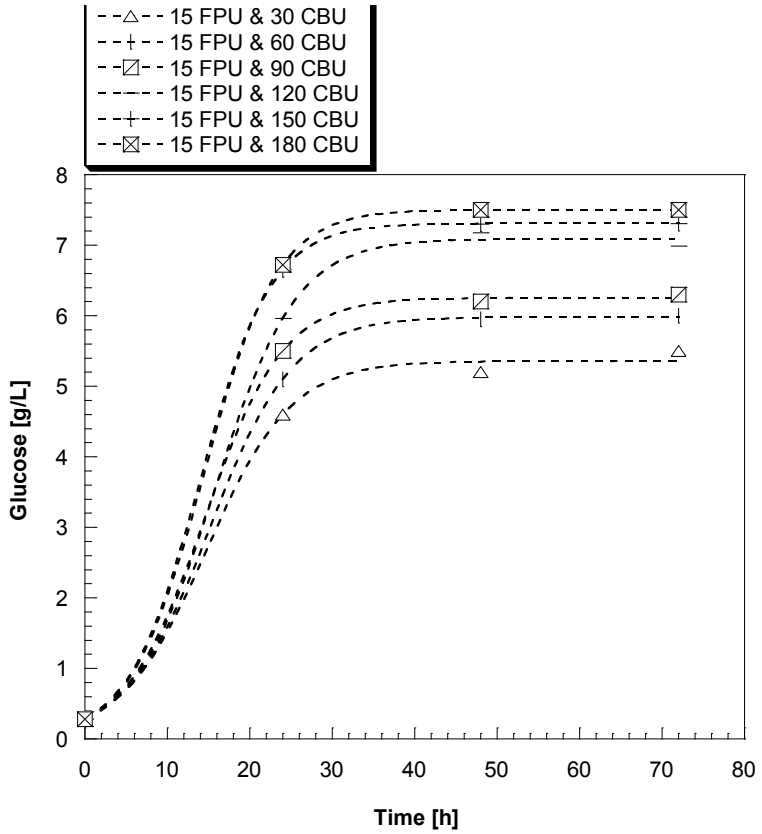

Fig. 3.9: Glucose profiles related to steam exploded *Arundo donax* hydrolysates (T=50°C, 160 rpm, 72 h, CBU/FPU 2÷12)

The lack of a significant increase is attributable, however, to an inhibiton phenomenon related to the cellulolytic enzymes, which reduces the yield and the conversion rate during the hydrolysis (*Gan et al., 2003*).

Surfactants could improve the enzymatic hydrolysis of lignocellulose by preventing irreversible and non-productive adsorption of enzymes on lignin, with non-ionic surfactants (e.g., Tween 20, Tween 80 and PEG) and anionic surfactants (e.g., lignosulfonate) particularly effective (*Börjesson et al., 2007, Wang et al., 2013*). The addition of a surfactant during the enzymatic hydrolysis of lignocellulosic biomass can influence the adsorption and desorption behavior of enzymes, i.e., the interactions between lignin and surfactant significantly reduce the non-productive adsorption of enzymes on lignin which thus increases the hydrolysis yield (*Eriksson et al., 2002a*). Therefore an increase, almost 25%, does not justify the use of high enzyme concentrations in view of an operating cost optimization and for a potential industrial implementation of this process (*Lynd et al., 2008*). Table 3.3 shows the relative kinetic parameters.

Tab. 3.3: Kinetic parameters for steam exploded *Arundo donax* hydrolysates (T=50°C, 160 rpm, 72 h, CBU/FPU 2÷12)

	μ_G [h^{-1}]	$Y_{Glucose}$ [g g^{-1}]	ν_G [g L^{-1} h^{-1}]
15 FPU & 30 CBU	0.195	0.379	0.082
15 FPU & 60 CBU	0.199	0.384	0.083
15 FPU & 90 CBU	0.210	0.403	0.087
15 FPU & 120 CBU	0.203	0.448	0.097
15 FPU & 150 CBU	0.231	0.468	0.101
15 FPU & 180 CBU	0.225	0.480	0.104

In an attempt to improve operating parameters of the enzymatic hydrolysis and, at the same time, to find operating conditions for oleaginous yeast cultivation to realize a process in a single reactor, the effect of temperature reduction on the catalytic activity of the enzymatic mix, used in previous step, were explored. In this regard, tests were performed at 40 °C in comparison to 50 °C, using cellulase from *Trichoderma reesei*, 15 FPU/g of cellulose; β-glucosidase from *Aspergillus niger*, 30 CBU/g of cellulose. Figures 3.10 and 3.11 show a comparable glucose and reducing sugar conversions also at 40 °C that suggested the possibility to use steam exploded *Arundo donax* also as feedstock for the enzymatic hydrolysis with a lower temperature. This results were promising for the future processes as above mentioned.

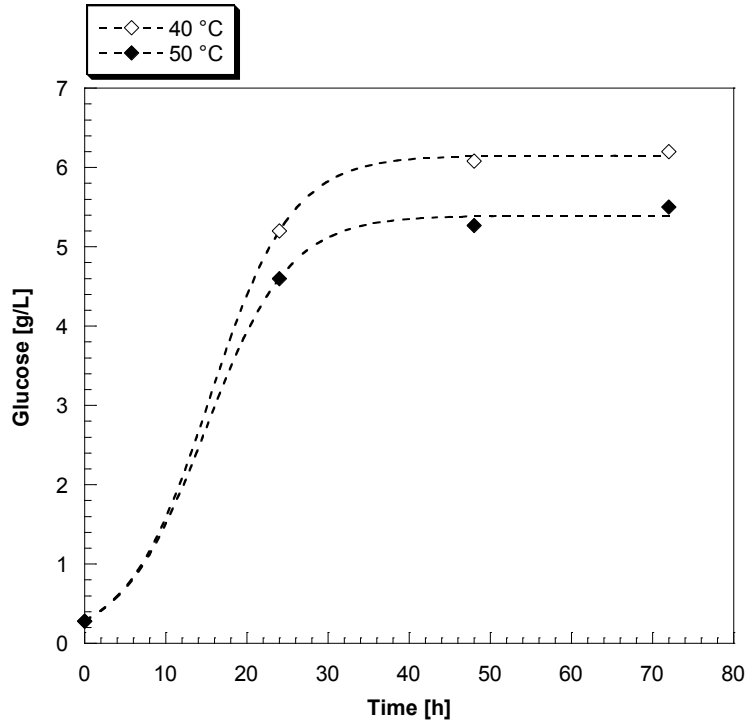

Fig. 3.10: Glucose profiles related to steam exploded *Arundo donax* hydrolysates (T=40÷50°C, 160 rpm, 72 h, 15 FPU & 30 CBU)

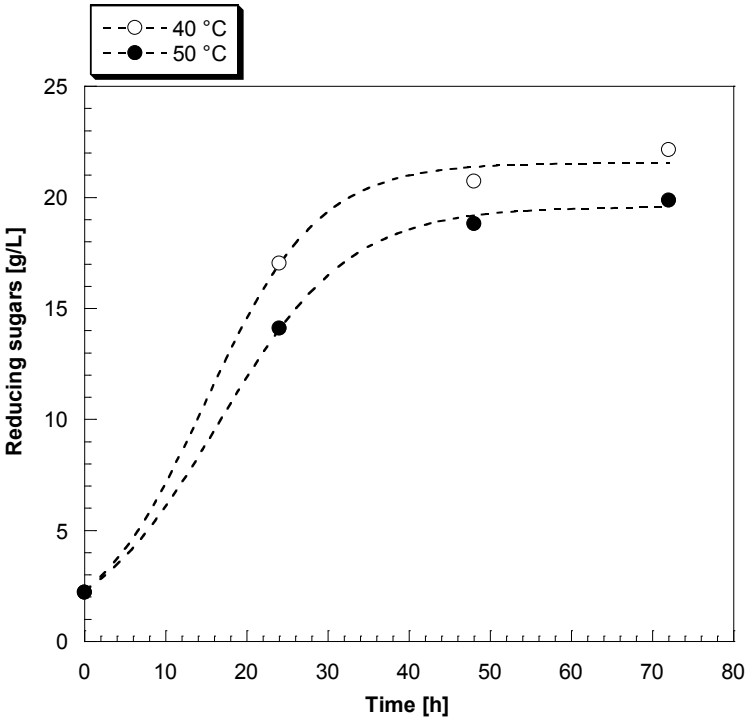

Fig. 3.11: Reducing sugars profiles related to steam exploded *Arundo donax* hydrolysates (T=40÷50°C, 160 rpm, 72 h, 15 FPU & 30 CBU)

Glucose yield ($Y_{Glucose}$) and specific rate for glucose production (μ_G), shown in Table 3.4, were calculated according to Equations 3.1 and 3.2.

The values in Table 3.4 demonstrated an increase of $Y_{Glucose}$ at 40 °C equal to 23%, but not in terms of μ_G that had the same trend in the first exponential part where specific rate for glucose production was evaluated.

Tab. 3.4: Kinetic parameters for steam exploded *Arundo donax* hydrolysates (T=40÷50°C, 160 rpm, 72 h, 15 FPU & 30 CBU)

15 FPU & 30 CBU	μ_G [h^{-1}]	$Y_{Glucose}$ [$g\ g^{-1}$]	ν_G [$g\ L^{-1}\ h^{-1}$]
40 °C	0.197	0.427	0.096
50 °C	0.195	0.379	0.082

A further aspect of inhibition phenomena was monitored during the experimental activity related to by-product formation. Acetic acid equal to a maximum value of 2.5 g/L for processed hydrolysate at 40 °C compared to 2 g/L at 50 °C, was monitored. Phenols were also measured with a final concentration of 0.6 g/L for all samples. Finally, furan concentration was about 0.1 g/L at 50 °C and 0.08 g/L at 40 °C.

A significant influence on the hydrolysis step wasn't detected. However, these compounds could represent an inhibitory effect on the metabolic activity of oleaginous yeasts during the subsequent fermentation. Two mechanisms, e.g., have been proposed to explain the inhibitory effect of weak acids: uncoupling and intracellular anion accumulation (*Russell, 1992*). HMF has been reported to be converted at a lower rate than furfural, which might be due to lower membrane permeability, and cause a longer lag-phase in growth (*Larsson et al., 1998*). The main conversion product was 5-hydroxymethyl furfuryl alcohol (*Palmqvist and Hahn-Hägerdal, 2000*), suggesting similar mechanisms for HMF and furfural inhibition.

The tests carried out during this experimental activity suggested an optimum temperature of 40 °C that was able to promote a higher conversion of fermentable sugars, but also to allow a lower formation of inhibitory products compared to that observed at 50 °C.

The subsequent experimental step was conducted to evaluate the impact of pH fluctuation in partial acid conditions (4.8÷5.8) (*Zhao et al., 2009*). Acid pH would facilitate both hydrolysis and fermentation processes (*Calvey et al., 2016*).

For this purpose tests were performed under the following operating conditions [Chapter 2, § 2.2] :

- **Without pH control**;
- **Buffer pH 5.2**;
- **Buffer pH 6.2**;
- **NaOH pH 5.2**.

Daily, a pH check was carried out and if necessary adjusted with NaOH (1M) or HCl (0.1M) solutions. The hydrolysis step was conducted at 40 °C, 160 rpm and 72 hours using enzyme mix that consist of cellulose from *Trichoderma reesei* (15 FPU/g of cellulose) and β-glucosidase from *Aspergillus niger* (30 CBU/g of cellulose) as a results of optimal conditions evaluated in the previous steps. Figures 3.12 and 3.13 show glucose and reducing sugar conversions. The highest conversion was obtained for the sample **NaOH pH 5.2**. The pH was costant for all samples except for that without pH control where pH decreased until 4.3 (data not shown). Figures 3.12 and 3.13 confirm also that **Buffer pH 6.2** is the worst choice to do enzymatic hydrolysis for its reduced glucose and reducing sugar conversion. A slight decrease of pH, as shown in **Without pH control** (data not shown), didn't contribute to reduce significantly glucose and reducing sugar concentration, if compared to **Buffer pH 5.2**, where pH value was constant. Pentose sugar concentration was monitored and was not significantly different from 0.4÷0.6 g/L for all samples (data not shown).

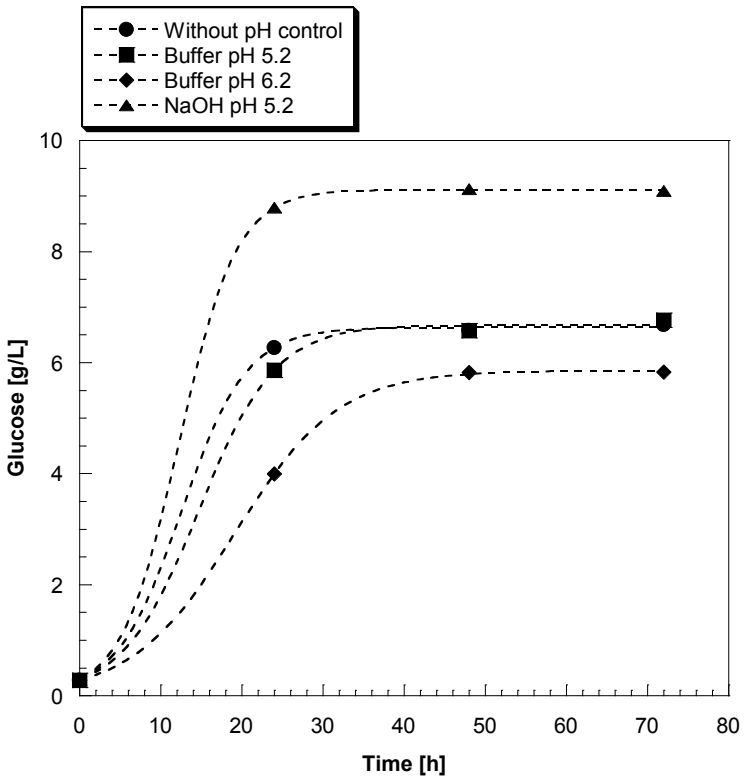

Fig. 3.12: Glucose profiles related to steam exploded *Arundo donax* hydrolysates (T=40 °C, 160 rpm, 72 h, 15 FPU & 30 CBU)

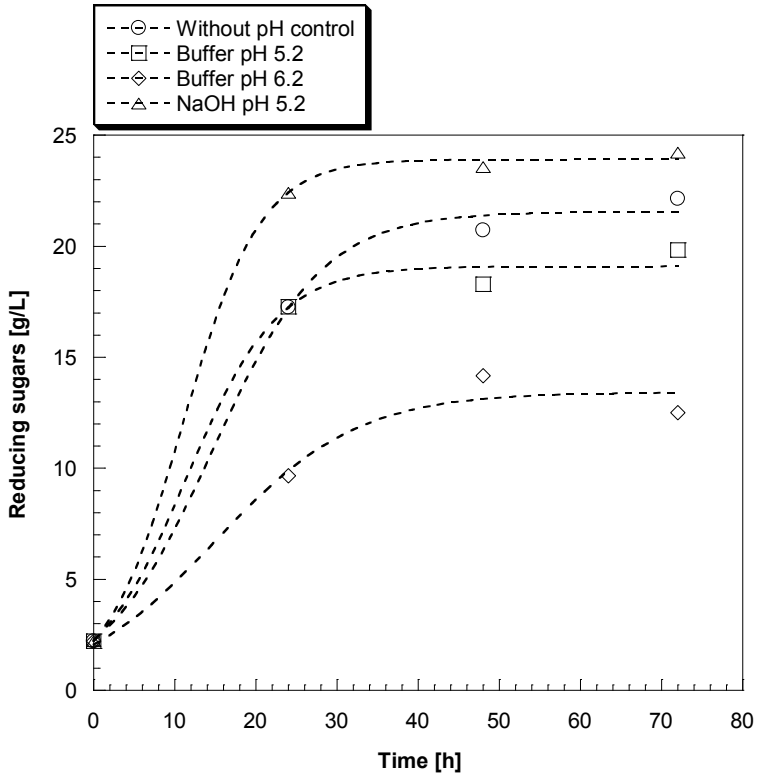

Fig. 3.13: Reducing sugars profiles related to steam exploded *Arundo donax* hydrolysates (T=40 °C, 160 rpm, 72 h, 15 FPU & 30 CBU)

Table 3.5 reports the values of kinetic parameters calculated following Equations 3.1 and 3.2. The highest μ_G and $Y_{Glucose}$ for **NaOH pH 5.2** were 0.281 h^{-1} and 0.627 g g^{-1}, respectively. For **Buffer pH 5.2**, μ_G and $Y_{Glucose}$ values were 0.212 h^{-1} and 0.466 g g^{-1}, respectively. The percentage decreased in terms of μ_G, $Y_{Glucose}$ and ν_G were 24.5, 25.6 and 26.1%, respectively. Therefore, **Buffer pH 5.2** offered the chance to avoid the daily control of pH and the exploitation of buffering capacity also during the fermentation.

Tab. 3.5: Kinetic parameters for steam exploded *Arundo donax* hydrolysates (T=40 °C, 160 rpm, 72 h, 15 FPU & 30 CBU)

	μ_G [h^{-1}]	$Y_{Glucose}$ [g g^{-1}]	ν_G [g L^{-1} h^{-1}]
Without pH control	0.248	0.461	0.139
Buffer pH 5.2	0.212	0.466	0.093
Buffer pH 6.2	0.157	0.401	0.081
NaOH pH 5.2	0.281	0.627	0.126

The presence of acetic acid and other volatile organic acids was monitored. In the sample **Without pH control,** a negative effect in terms of acetic acid production was observed that

reached 2.7 g/L at the end of the test. In **NaOH pH 5.2** and **Buffer pH 5.2**, instead, the same value was equal to 1.43 g/L and 2 g/L, respectively.

Phenols had not shown a substantial change in different samples. The constant values could be associated to the release during biomass pretreatment. Their amount was about 0.5÷0.6 g/L. The amount of furans was approximately 0.1÷0.12 g/L for all samples.

These compounds could represent an inhibition factor on the metabolic activity of oleaginous microorganisms. In that case, it could be required to apply detoxification techniques such as overliming, steam stripping (*Hsu, 1996*; *McMillan, 1994*) or washing. This procedure, however, results of sugar loss of 15% (*Chundawat et al., 2007*).

The experimental tests of enzymatic hydrolysis were carried out to find operating conditions to assess the applicability of innovative fermentation processes, i.e. in single stage or single reactor, with the aim to reduce the capital costs.

It was possible to detect the synergism of cellulase from *Trichoderma reesei* and cellobiase from *Aspergillus niger*, as well as the positive effects related to a lower temperature (40 °C) as well as the suspended composition of hydrolysates to control pH fluctuations and reaction time in which it was observed a maximum conversion in 48 hours.

3.2 Separate Hydrolysis and Fermentation (SHF) and Enzymatic hydrolysis and oleaginous Fermentation in Single Reactor (SRF) of steam exploded *Arundo donax*

In a preliminary phase, tests of separate hydrolysis and fermentation were planned to simulate a classic process of SHF (Separate Hydrolysis and Fermentation) and consequently to evaluate the benchmarks with non-classical cultures of enzymatic hydrolysis and oleaginous Fermentation in Single Reactor (SRF).

The fermentation tests were conducted starting from hydrolysate, obtained from the optimal operating conditions resulting from enzymatic hydrolysis tests [Chapter 3, § 3.1].

Culture medium was inoculated with 5% v/v of *Lipomyces starkeyi DBVPG 6193* pre-culture grown in YPD broth at 30 °C for 72 hours, at 160 rpm [Chapter 2, § 2.3].

Figure 3.14 shows a complete glucose conversion within 48 hours, associated to a pentose sugar consumption that continued with a lower conversion rate. Microbial biomass profile was symmetrical to glucose profile except for the final stretch where the residual increase was associated to the residual fraction of pentose sugar conversion. Phenols (0.56 g/L) were related to lignin fraction and they were released during the pretreatment, not related to the enzymatic hydrolysis and fermentation that were conducted at low temperatures to allow their

production. However, their concentration was not able to promote inhibition phenomena for metabolic activity of oleaginous microorganism (*Lipomyces starkeyi DBVPG 6193*).

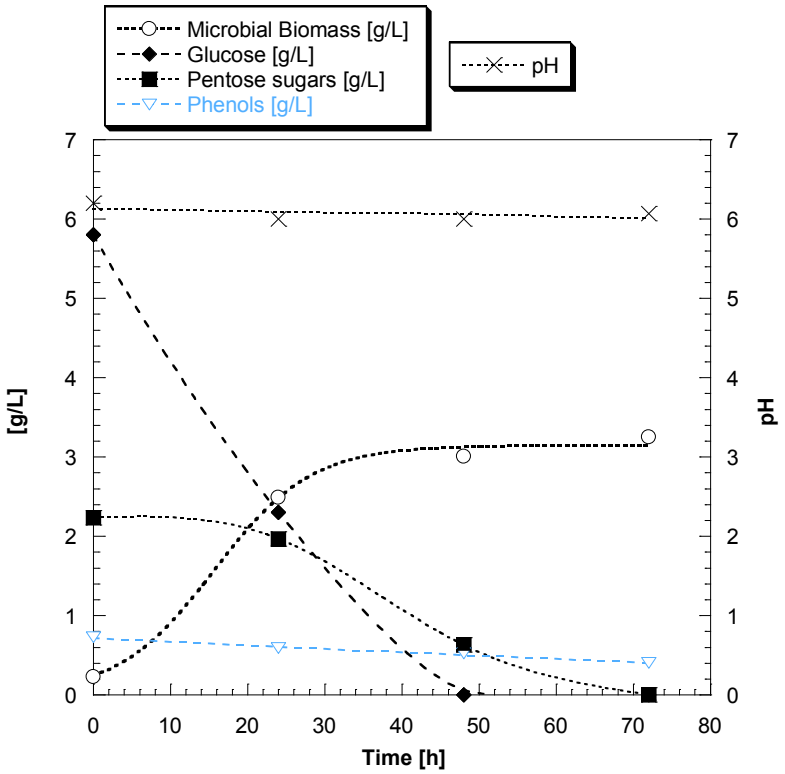

Fig. 3.14: pH, microbial biomass and metabolites profiles of steam exploded *Arundo donax* and *Lipomyces starkeyi* in **SHF** process (T=30 °C, 160 rpm, 72 h)

The growth in a batch system follows the Equation 3.3:

$$\frac{dx}{dt} = \mu x \qquad (3.3)$$
$$t = 0;\ x = x_0$$

μ is specific growth rate (h^{-1}), assuming that at t=0, biomass concentration is equal to x_0 and integrating the differential Equation 3.3, it was obtained the following Equation 3.4:

$$x = x_0\, e^{\mu t} \qquad (3.4)$$

The profile of glucose concentration *s(t)* (g/L) can be determined following Equation 3.5:

$$s = s_0 * e^{-\mu t} \qquad (3.5)$$

The metabolic activity of *Lipomyces starkeyi* was initially characterized by a short lag phase and subsequently by an exponential trend, finally characterized by a zero-order kinetic that proceeed until complete glucose conversion. The transition could be due to a limitation of nitrogen source.

Following experimental activity included the search of operating conditions able to lead the enzymatic hydrolysis and oleaginous fermentation in a single reactor (SRF) in order to reduce capital costs and risk of contaminations (*Angerbauer et al., 2008*). However, mixing residual lignin with yeasts made yeast recirculation very difficult. In addition, since the optimal temperature for yeast growth and enzyme activity differ, the operating conditions used in SRF cannot be optimal for both enzymes and oleaginous yeast.

Figures 3.15, 3.16, 3.17 show the profiles of reducing sugar, glucose, pentose sugar concentrations and other by-products (ethanol, volatile organic acids, phenols and furans) during SRF. Reducing sugar and glucose conversion were monitored. In **SRF Buffer pH 5.2** and **SRF NaOH pH 5.2** the latter values were significantly higher than in **SRF without pH control**, justified by the pH variability, that was considered an inhibitory factor for oleaginous yeast fermentation. The pH values show a progressive acidification, until 3.0, in **SRF without pH control** (data not shown). In presence of pH control through buffer solution, **SRF Buffer pH 5.2**, this value has not undergone significant fluctuations, further minimized in **SRF NaOH pH 5.2**.

As shown in Figures 3.15, 3.16, 3.17 during the enzymatic hydrolysis from 0 h to 48 h, the initial reducing sugars and glucose increased in all the samples. It was observed an increase of reducing sugars, up to 15.16 g/L, and glucose up to 6.25 g/L, for **SRF Buffer pH 5.2** and an increase of reducing sugars, up to 19.67 g/L, and glucose, up to 6.96 g/L, for **SRF NaOH pH 5.2**. Figures 3.16 and 3.17 show also complete reducing sugar and glucose conversion during oleaginous fermentation (48÷144 h), that follows the first stage of enzymatic hydrolysis. Acetic acid production was also found. In **SRF without pH control**, an increase of acetic acid concentration was observed, while, in **SRF buffer pH 5.2** and **SRF NaOH pH 5.2** samples was observed its decrease.

Acetic acid production is usually associated to the deacetylation of hemicellulose chains. It occurs significantly during the hydrolysis of xylan-rich lignocellulosic raw materials such as hardwoods, and in herbaceous energy crops such as *Arundo donax*. At high concentrations, however, negative effects are observed on the specific growth rate, increasing also the timing of lag phase (*Taherzadeh et al., 1997*; *Verduyn et al., 1990*).

According to Hu et al. (*2009*) also a 7 g/L of acetate concentration can result an improvement of the lipid content of 68% using *Rhodosporidium Toruloides,* but, at low pH, weak acids prevail in undissociated form, being more hydrophobic and prone to crossing the membrane by simple passive diffusion. The anion of weak acids have been shown to be key factors in acid toxicity (*Abbott et al., 2007*; *Stratford et al., 2013*) and a cause of low value of viable-cells. In general, yeast cells exposed to an increase of acid concentration exhibited significant decrease in specific glucose consumption rate.

The microbial growth wasn't detected using turbidimetry but using CFU/mL (Colony Forming Unit/mL) count. The maximum value of $1.84 \cdot 10^8$ CFU/mL was observed for **SRF NaOH pH 5.2**, significantly higher if compared to **SRF without pH control,** equal to $1.00 \cdot 10^6$ CFU/mL.

Phenols are a heterogeneous group of compounds originated from a partial degradation of lignin (*Ando et al., 1986*). Phenol concentration, almost 0.6 g/L for all samples, was released during first 24 hours. After 96 hours of fermentation, a slight decrease in phenol concentration was observed [Figures 3.15, 3.16, 3.17], but was not able to produce inhibitory effects on microbial growth. Furan concentration profiles show in Figures 3.15, 3.16, 3.17 did not appear to produce inhibitory effects on the performance of microbial growth. SRF process was performed without sterilisation and in absence of auxiliary nutrients. Both inhibitory compounds in suspension might be related to steam explosion pretreatment.

Ethanol production was also monitored during SRF process, in order to evaluate the ability of oleaginous yeasts to metabolize part of fermentable sugars not only for lipid accumulation, but also for alcoholic fermentation. It is known that the ethanol synthesis in presence of lignocellulose hydrolysates follows quite complex pathways. As a matter of facts, in addition to direct use of glucose, ethanol production from cellobiose has been demonstrated (*Vanrooyen et al., 2005*; *Gurgu et al., 2007*). Ethanol production was affected by pH shift. In fact, as shown in Figures 3.16, 3.17, a greater ethanol production was observed in **SRF NaOH pH 5.2**, due to a higher fermentable sugar concentration used for ethanol production and the depletion of dissolved oxygen concentration that could induce this phenomenon.

As noted in *Y. lipolytica* by Workman et al. (*2013*), oxygen limiting conditions create cofactor imbalances, particularly an excess of NADH, which are restored by the reduction of sugars into polyols or by the reduction of pyruvate into ethanol or butanediol.

The ethanol yield ($Y_{ethanol}$) was based on total glucose available, as calculated using the following Equation 3.6:

$$Y_{ethanol}\,[g\,g^{-1}] = \frac{m_{ethanol}}{m_{total\ glucose}} \quad (3.6)$$

Ethanol yield was 0.19 g/L total glucose [Equation 3.6] in **SRF NaOH pH 5.2**, which was lower than reported for alcoholic fermentations where the yield is instead of 20 g/g dry biomas (*Zhang et al., 2014*) or about 0.6 g/g fermentable sugars (*Ask et al., 2012*). Ask et al. (*2012*) obtained a final ethanol concentration 14.1 g/L. In the experimental activity, already described, it was obtained a maximum value of 3 g/L.

Morikawa et al. (*2014*) studied biological co-production of ethanol and biodiesel from wheat straw using SSF for ethanol production and hemicellulosic hydrolysate to produce microbial lipids in two separated processes. Mass balance showed that 0.9 g lipids and 10.1 g ethanol can be produced from 100 g wheat straw. The experimental data obtained according to Equation 3.7 for lipid yield and Figures 3.16 and 3.17 for ethanol concentration showed almost 2.4 g lipids and 6 g ethanol for both **SRF Buffer pH 5.2** and **SRF NaOH pH 5.2**, respectively. The exploitation of ethanol and Single Cell Oil co-production was feasible, though more optimization should be performed to improve economical and mass balance of the process.

As a matter of facts, negative effects on specific growth rate and lag phase have been observed at acetic acid concentrations higher than 3 g/L (*Taherzadeh et al., 1997*; *Verduyn et al., 1990*), though at lower concentrations acetic acid may increase ethanol yield instead of biomass yield due to the uncoupling of ATP utilization from growth (*Narendranath et al., 2001*). However, oleaginous yeasts are particularly tolerant to acetic acid inhibition (*Narendranath et al., 2001*).

Furfural and HMF directly inhibit alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH), and aldehyde dehydrogenase (ALDH) (*Almeida et al., 2007*). Both furfural and HMF are known to inhibit the alcoholic fermentation also in oleaginous yeasts (*Palmqvist et al., 2000*).

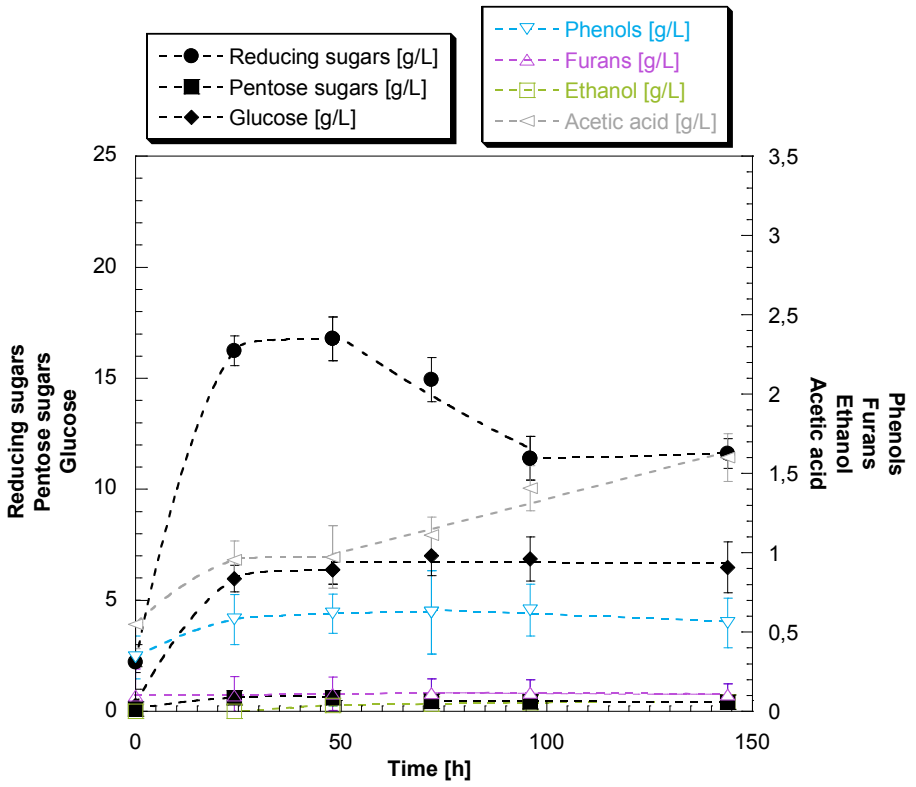

Fig. 3.15: Concentration profiles during SRF process (**SRF without pH control**) (T=40÷30 °C, 160 rpm, 144 h)

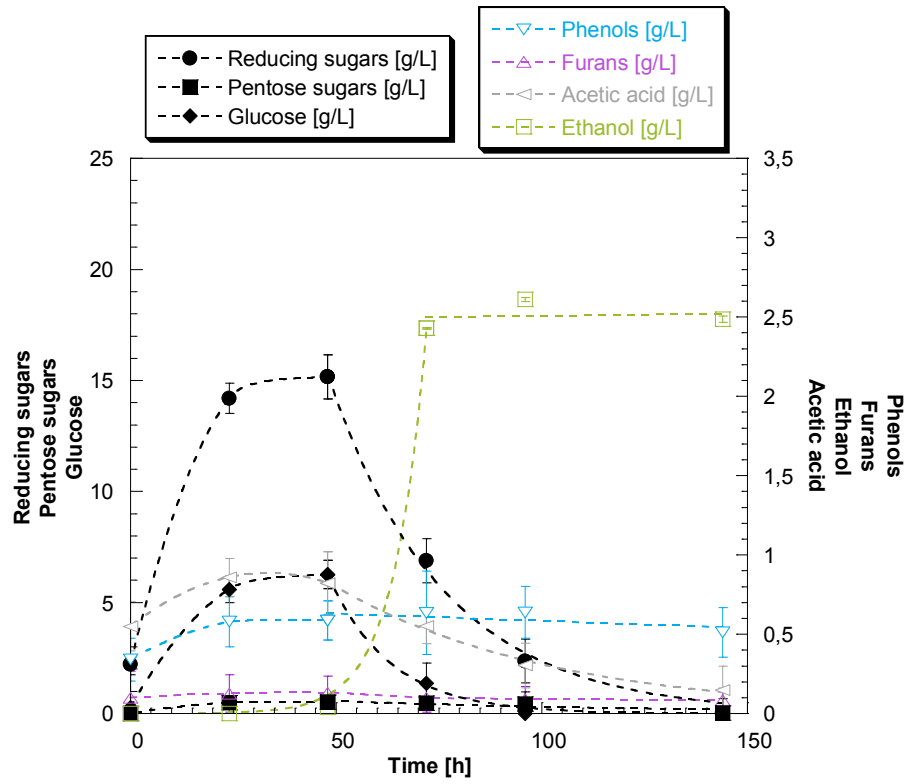

Fig. 3.16: Concentration profiles during SRF process (**SRF Buffer pH 5.2**) (T=40÷30 °C, 160 rpm, 144 h)

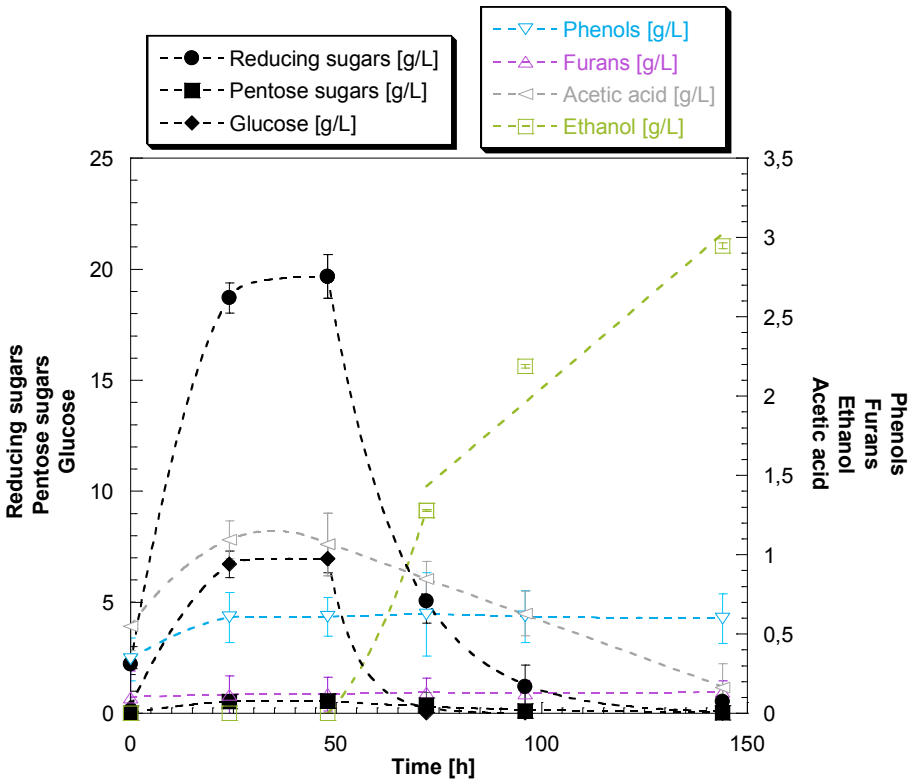

Fig. 3.17: Concentration profiles during SRF process (**SRF NaOH pH 5.2**) (T=40÷30 °C, 160 rpm, 144 ore)

Specific glucose consumption rates (μ_s) [Table 3.6] were calculated and increased from 0.00 h^{-1} for **SRF without pH control** to 0.066 h^{-1} for **SRF Buffer pH 5.2** and 0.138 h^{-1} for **SRF NaOH pH 5.2**. The value for **SRF NaOH pH 5.2** was comparable to **SHF**. This result suggested positive potential application of such process in single reactor that still remains unexplored for microbial oil/Single Cell Oil production.

Tab. 3.6: Kinetic parameters in **SHF** e **SRF** processes

	μ_s [h^{-1}]
SHF	0.159
SRF without pH control	0.000
SRF Buffer pH 5.2	0.066
SRF NaOH pH 5.2	0.138

Lipid yield (Y_{lipid}), according to Equations 3.7, lipid productivity (ν_{lipid}) and lipid accumation (*[Lipid]*) were evaluated.

$$Y_{lipid}\ [g\ g^{-1}] = \frac{m_{lipid}}{m_{total\ glucose}} \tag{3.7}$$

Tab. 3.7: Lipid yield, lipid productivity and lipid concentration in **SHF** and **SRF** samples

	Y_{lipid} [g g^{-1}]	ν_{lipid} [g L^{-1} day^{-1}]	*[Lipid]* [g L^{-1}]
SHF	0.26	0.38	1.48
SRF without pH control	0.05	0.10	---
SRF Buffer pH 5.2	0.21	0.34	1.36
SRF NaOH pH 5.2	0.16	0.26	1.08

As shown in Table 3.7, highest lipid yield (Y_{lipid}) in SRF cultures was obtained using buffer at pH 5.2 (**SRF Buffer pH 5.2**). This result is comparable to lipid yield in SHF. For this reason SRF process using *Lipomyces starkeyi*, as oleaginous yeast, and *Arundo donax*, as lignocellulosic biomass, can be considered feasible. In **SRF Buffer pH 5.2**, lipid productivity was equal to 0.34 g L^{-1} day^{-1}, promising value in comparison to lipid productivity calculated by Huang et al. (*2014*) for corncob hydrolysate treated with dilute sulfuric acid (0.74 g L^{-1} day^{-1}) in separated reactors. Gong et al. (*2014*) studied simultaneous saccharification and enhanced lipid production using *Cryptococcus curvatus*. The productivity was 4.69 g L^{-1} day^{-1} and the lipid content was 9.8 g/100 g raw corn stover. Gong et al. (*2012*) calculated lipid coefficient as gram lipid produced per gram substrate consumed. The value for Co-fermentation of cellobiose and xylose by *Lipomyces starkeyi* for lipid production was almost 0.2 g g^{-1}. Lipid concentration (*[Lipid]*) equal to 1.36 g/L for **SRF Buffer at pH 5.2** and 1.08 g/L for **SRF NaOH pH 5.2** was lower than observed by Huang et al. (*2014*) and Matsakas et al. (*2014*), 8.1 and 6 g/L, who used corncob hydrolysate treated with dilute sulfuric acid and dried sweet sorghum in separated reactors, respectively. Gong et al. (*2014*) obtained up to 11 g/L in simultaneous saccharification and lipid production in presence of extra nutrients.

Figures 3.18 and 3.19 show the distribution of fatty acids in alkaline and acid catalysis. In Figure 3.18, the experimental data indicated a prevalence of mid-chain fatty acids with 16 and 18 carbon atoms. In **SRF Buffer pH 5.2**, the most abundant was oleic acid (C18:1), followed

by palmitic acid (C16:0), equal to 43.92 and 34.59%, respectively. Smaller amounts of stearic acid (C18:0) and arachidic acid (C20:0) were also present. However, a balanced proportion between saturated and unsaturated fatty acids was observed, that encourages the use as automotive fuel (*Benjumea et al., 2011*). For instance palmitic and oleic acids have been established as main biodiesel components and provide highest cetane response (*Knothe, 2008*), corresponding with those of soybean oil and jatropha oil, which are used as feedstock for biodiesel production. Similar fatty acid composition of lipids obtained from *Lipomyces starkeyi* was found when the yeast was cultivated on starch (*Wild et al., 2010*), mixtures of glucose and xylose (*Zhao et al., 2008*) and various mixtures of glucose, cellobiose and xylose (*Gong et al., 2012*).

Therefore, the exploitation of Single Cell Oils for other purposes was studied by Simopoulos (*2008*) and Bharathiraja et al. (*2017*) and related to the possibility of ensuring mono or polyunsaturation, known as EFAs, essential fatty acids of ω-6 and ω-9 families; chemical mediators that, for example, allow to fight the emergence of diseases such as atherosclerosis (*Simopoulos, 2008*) and PUFAs as components of thrombocytes, neuronal and muscle cells, cerebral cortex as well as the immunocompetent cells (*Bharathiraja et al., 2017*).

This issue of the ratio of unsaturated to saturated fatty acids in the lipids is of great interest for industrial production of Single Cell Oil. For this reason, it was decided to test acid catalyzed reaction. Acid catalysis could be proposed in order to overcome limitation due to high free fatty acid concentration (exceed 1% wt). However, acid-catalyzed system is not desirable choice for commercial applications due to slow reaction rate, high reaction temperature, environmental and corrosion problems.

Figure 3.19 show an increase of palmitic acid (C16:0) if compared to alkaline catalyzed reaction in **SRF Buffer pH 5.2**, while a decrease of monounsaturated fatty acid (C18:1).

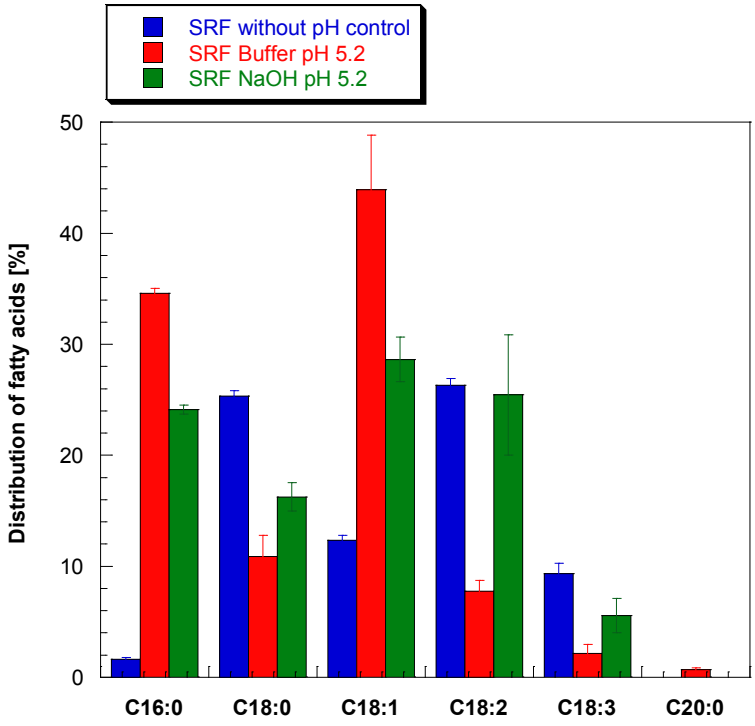

Fig. 3.18: Fatty acid distribution (% w/w) with alkali-catalysis in **SRF**

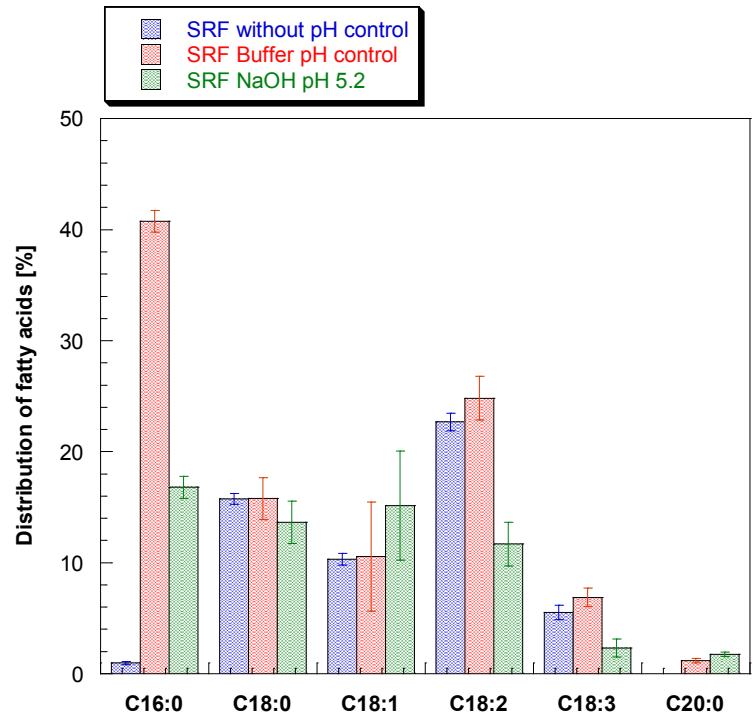

Fig. 3.19: Fatty acid distribution (% w/w) with acid-catalysis in **SRF**

3.3 Microalga-oleaginous yeast mixed cultures to improve Single Cell Oil production

The following experimental activity was focused on innovative scenario for Single Cell Oil production, exploiting the synergistic effects of microalga-oleaginous yeast mixed cultures. Oleaginous yeasts have a great growth rate in aerobic condition where in large amount of O_2 must be required and CO_2 be emitted (*Li et al., 2007*). Microalgae are considered as another attractive source due to their high lipid content and CO_2 reduction efficiency (*Eugenia, 2012*; *Xiong et al., 2010*). The carbon limitation has restricted the locations where algal cultures can be economically feasible, because the use of CO_2-rich flue gas is only possible in the vicinity of power plants (*Beal et al., 2015*).

In theory, there are synergistic effects on gas, substance exchange and pH adjustment in the mixed culture system of oleaginous yeasts and microalgae based on the mutually beneficial relationship. In mixed cultures, microalgae could act as an O_2 generator for the yeast while the yeast provided CO_2 to microalgae and both carried out lipid production.

At present, research has focused on the photoautotrophic production of microalgal oil using light as energy source, but there are significant drawbacks associated with photoautotrophic algal cultures for oil production. First, it is difficult to solve the contradiction between accumulation of biomass and lipid synthesis during the microalgal life cycle (*Liu et al., 2011*). Second, light attenuation is unavoidable for photoautotrophic cultures from lab to pilot scale, and it may significantly reduce productivity (*Wilhelm and Jakob, 2011*).

The following strains, achieved from the ACUF collection of the Department of Biological Science at the University of Naples-Federico II, were used for experimental activity to find optimal culture conditions:

- *Chlorella protothecoides 165;*
- *Chlorella saccarophila 042;*
- *Chlorella emersonii 316/25;*
- *Viridiella fridericiana 035;*
- *Chlorella zofingiensis 252;*
- *Chlorella sorokiniana 317.*

Bold Basal Medium (BBM), constant light and agitation were chosen. All cultures were performed in batch mode. Figure 3.20 shows microbial biomass profiles related to the different strains. pH was also monitored during the tests and observed a progressive increase until 9.8÷10 for all strains except for *Viridiella fredericiana 035*, where pH was 7.5 and for

Chlorella saccarophila 042, where pH was 8. Chlorophyll a was measured for all samples to evaluate photosynthetic capacity (data not shown).

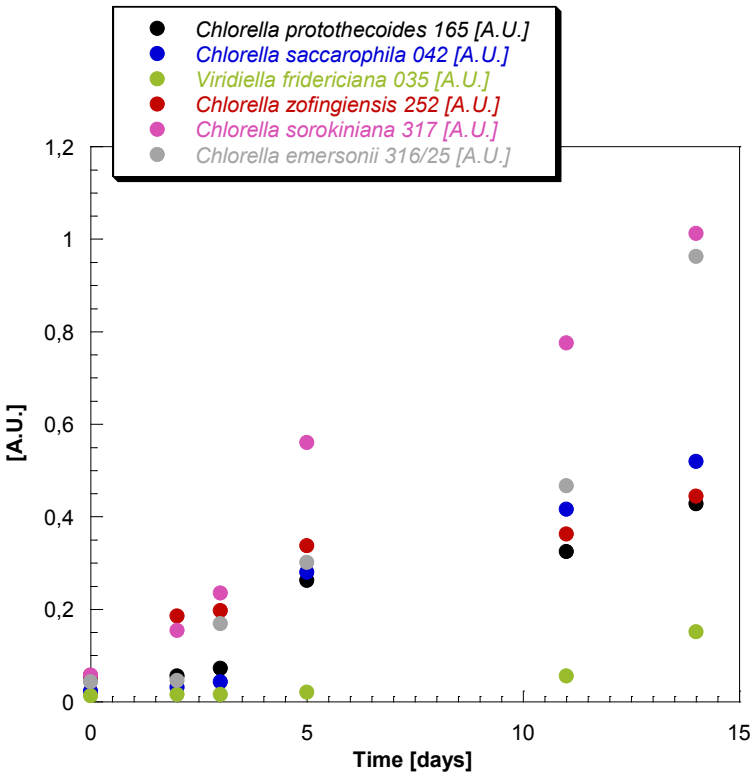

Fig. 3.20: Microbial biomass profiles in **BBM** medium

From a biorefinering perspective, lipid content is one of the most important performance indicators for evaluating the commercialization potential of lipid-producing microalgae. *Chlorella emersonii 316/25* was chosen for its capacity to accumulate lipids that is the aim of the experimental activity (*Gouveia and Oliveira, 2009*).

Figure 3.21 shows the profiles related to the microalga photosynthetic activity in a BBM medium. Microbial biomass was 2.3 g/L, pH monitoring was performed to evaluate possible fluctuations. pH reached values equal to almost 8. Chlorophyll a profile confirmed the photosynthetic activity associated to the cell proliferation.

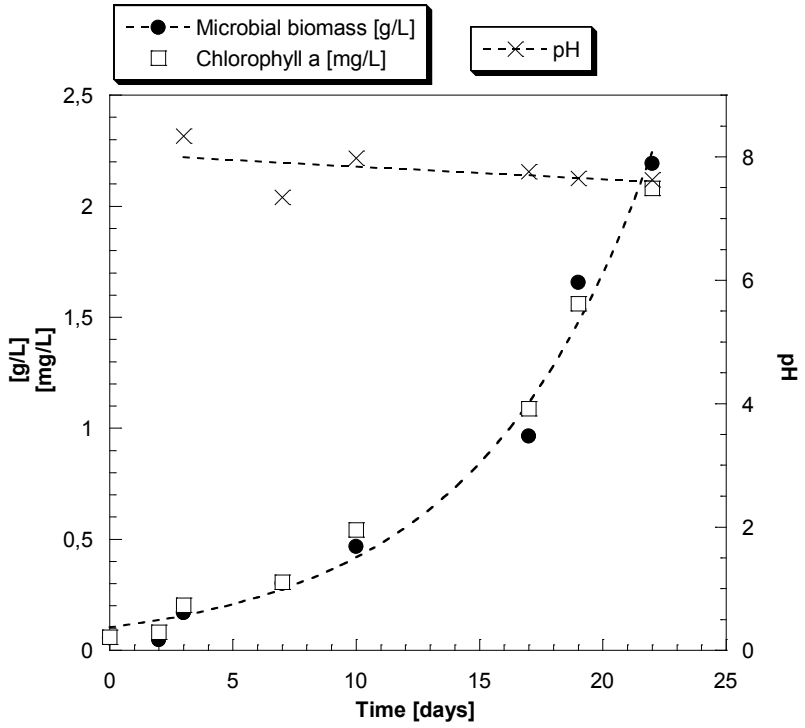

Fig. 3.21: Microbial biomass, Chlorophyll a and pH profiles in **BBM Chl**

The specific growth rate (μ_x) was calculated according to Equations 3.8 and 3.9:

$$\frac{dX}{dt} = \mu X \tag{3.8}$$

$$\mu_x = [\ln(X_t/X_0)]/(t - t_0) \tag{3.9}$$

where X is the concentration of biomass at time t, and μ_x is the specific growth rate. Exponential growth, related to microalga proliferation, was halted after the end of test. The value of CO_2 fixation rate (P_{CO2}) was obtained according to Equations 3.10 and 3.11 (*Ho et al., 2015*):

$$P_{CO_2}\left[mg\ L^{-1}\ d^{-1}\right] = 1.88 \times P_x \tag{3.10}$$

$$P_x = \frac{\Delta x}{\Delta t} \tag{3.11}$$

P_x is biomass productivity [mg L^{-1} d^{-1}], it was obtained according to Equation 3.11 where Δx represents the microbial biomass variation and Δt the relative time. The latter value was monitored to evaluate possible effects of photoinhibition strictly related to biomass growth. In **BBM Chl**, μ_x and P_{CO2} were 0.168 d^{-1} and 35 mg L^{-1} d^{-1}, respectively. To evaluate the ability of *Chlorella emersonii* to grow in a culture medium containing an organic source, BBM

medium with added glucose (10 g/L) (**BBM+G**) was chosen. Wide variety of organic carbon sources can be assimilated by microalgae for growth (*Liang et al., 2009*). The aim was to approach culture medium composition to hydrolysate composition, chosen as real feedstock for its low cost and, at the same time, to evaluate the versatility of *Chlorella emersonii* to grow in heterotrophic and mixotrophic conditions (*Chojnacka and Marquez-Rocha, 2004*). Figure 3.22 reports the profiles of microbial biomass concentration and the pH values. In this regard, it was observed that the progressive increase of microbial biomass, up to 3.57 g/L, did not match the profile of glucose consumption, that stopped after one day at about 4 g/L, but it was coherent with the increase of Chlorophyll a. These results attested that *Chlorella emersonii* was able to grow using organic substrate, such as glucose. The glucose consumption, during the first day of culture, not comparable to the growth of microbial biomass, probably, was related to an experimental error during the turbidometric analysis. In fact, the cells adopted down regulation of chlorophyll synthesis and conserved energy, since Chlorophyll a was almost constant, but it was simultaneously observed a remarkable glucose consumption. In the following cultivation time, after the first day [Figure 3.22], the increase of biomass growth was tied to Chlorophyll a increase, not to glucose conversion. This trend was confirmed by the change of metabolic activity.

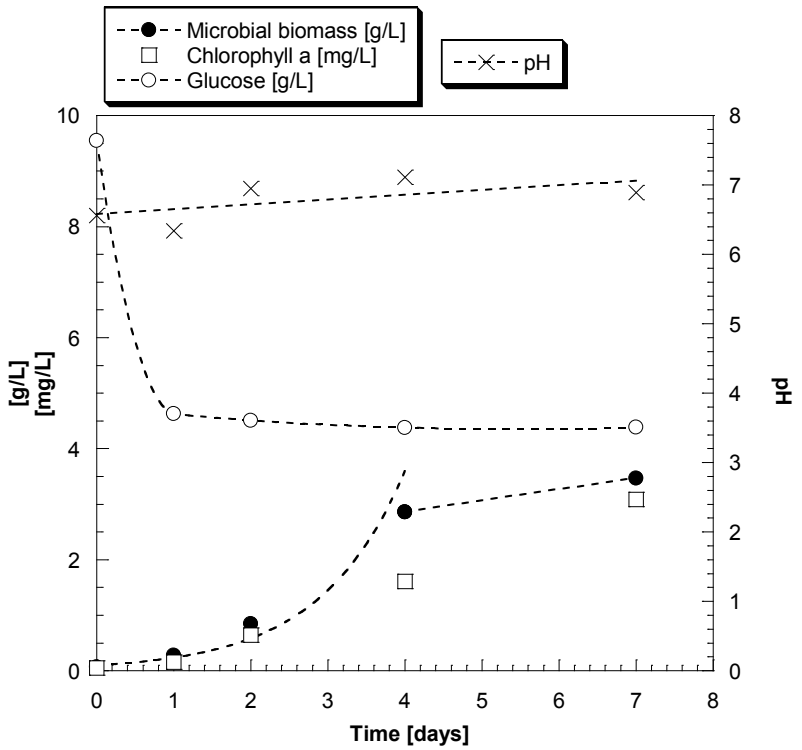

Fig. 3.22: Microbial biomass, Chlorophyll a, glucose and pH profiles in **BBM+G Chl**

Table 3.8 shows specific growth rate (μ_x) in **BBM+G** significantly higher than μ_x in **BBM**. Kinetic parameters were calculated according to Equations 3.8 and 3.9 and reported in Table 3.8. The yield factor ($Y_{x/s}$) was estimated according to Equation 3.12. P_{CO2} values were calculated using Equations 3.10 and 3.11 to evaluate phenomenon commonly known as photoinhibition.

$$Y_{x/s} = \frac{x - x_0}{s_0 - s} \tag{3.12}$$

Tab. 3.8: Kinetic parameters for **BBM Chl** and **BBM+G Chl**

	μ_x [d^{-1}]	$Y_{x/s}$ [g g^{-1}]	P_{CO_2} [mg L^{-1} d^{-1}]
BBM	0.168	-	35
BBM+G	0.910	0.176	130

Figure 3.23 shows microbial biomass, glucose concentration and pH values in BBM medium plus glucose, using *Lipomyces starkeyi DBVPG 6193* as inoculum (**BBM+G Lip**). Low metabolic activity of *Lipomyces starkeyi* in BBM+G medium suggested that this medium was not suitable for yeast growth. In fact microbial biomass was 0.3 g/L.

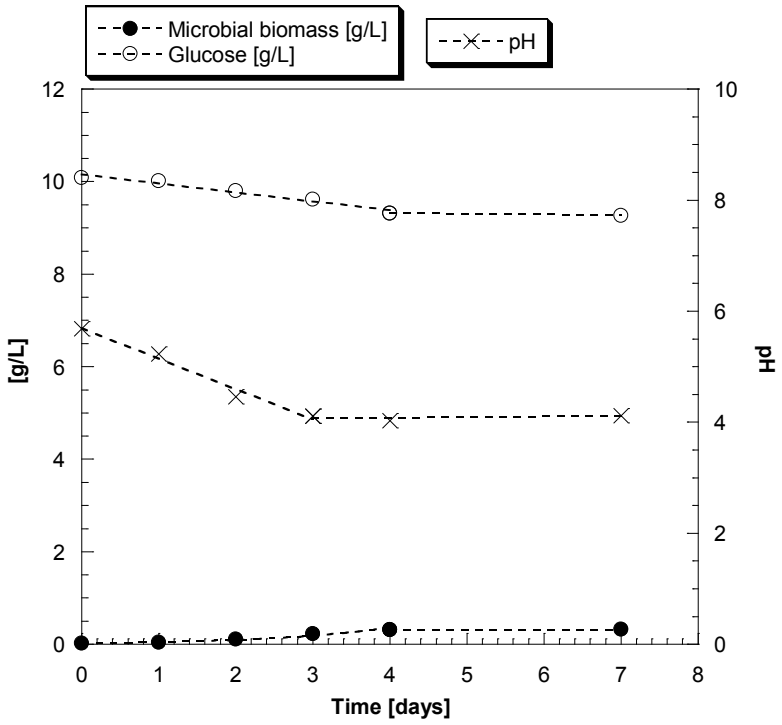

Fig. 3.23: Microbial biomass, glucose and pH profiles in **BBM+G Lip**

The next step was carried out using a culture medium for oleaginous yeasts containing in addition glucose and yeast extract (**YEG**) and reduced contribution in terms of external and supplementary elements that sometimes represent an increase of cost not negligible. Metabolic activity of two strains, *Lipomyces starkeyi* (**Lip**) and *Chlorella emersonii* (**Chl**), was investigated to evaluate the synergistic effects in comparison to the behaviour of individual strains. The cultures were marked as **YEG Chl**, **YEG Lip** and **YEG Chl+Lip**. In each case, in addition to the daily monitoring of substrate concentration, microbial biomass determination and by-products; cell count (Cells/mL) was carried out to evaluate the different growth of yeast and microalga in the same medium during the test. Figure 3.24 shows, in **YEG Chl**, an initial exponential growth of the microbial biomass, which corresponded to the symmetrical glucose consumption until the third day. After, the profiles related to microbial biomass growth and glucose consumption followed a kinetic reaction of apparent zero order, attributable to a maintenance metabolism, whose specific rate was calculated according to Equation 3.13:

$$s = s_0 - mx_0t \tag{3.13}$$

The other parameter that could significantly affect metabolic activity of *Chlorella emersonii* was represented by pH, which undergoes a drastic reduction, inspiring contextually an arrest of exponential growth rate.

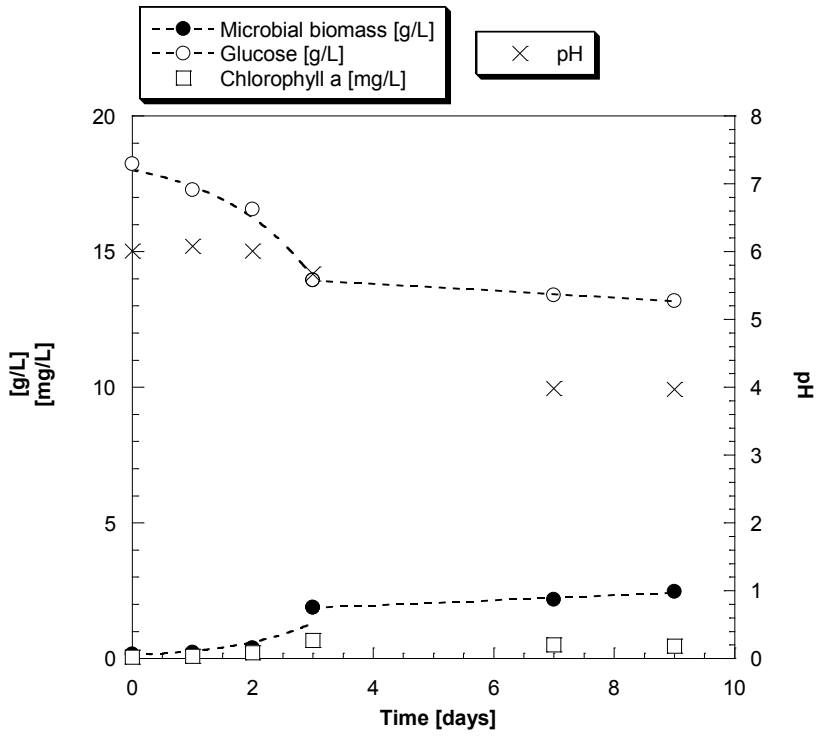

Fig. 3.24: Microbial biomass, Chlorophyll a, glucose and pH profiles in **YEG Chl**

Figure 3.25 allows to observe the correlation between the trend of microbial biomass growth and the cell count. The final value was equal to $1.4 \cdot 10^7$ Cells/mL. The profiles confirmed, after an initial phase of exponential growth, the arrest of cell proliferation attributable to the change in culture conditions, probably related to the pH decrease and a reduction of buffering ability, as above mentioned.

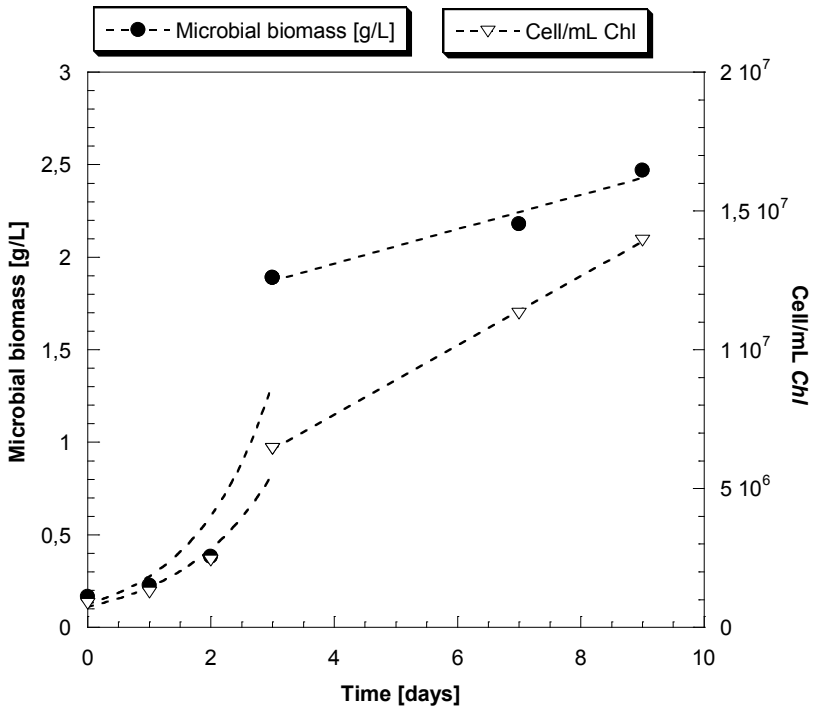

Fig. 3.25: Microbial biomass and Cell/mL profiles in **YEG Chl**

Figure 3.26 shows microbial biomass growth and glucose consumption profiles in **YEG Lip**. The difference that was observed in comparison to **YEG Chl** [Figure 3.24] was related to the microbial biomass value, that reached, 5 g/L, higher than the same value found in **YEG Chl**, where it was 2.5 g/L. These values are known also as dry cell weight and the last one obtained for *Chlorella emersonii* in YEG medium was considered promising if compared to the literature values (*Kula et al., 2017*). After 3 days, an evident acidification of the medium was observed, which would assume a loss of buffering capacity. Therefore, *Lipomyces starkeyi* is able to grow also in those changing conditions occurred during the test (*Calvey et al., 2016*).

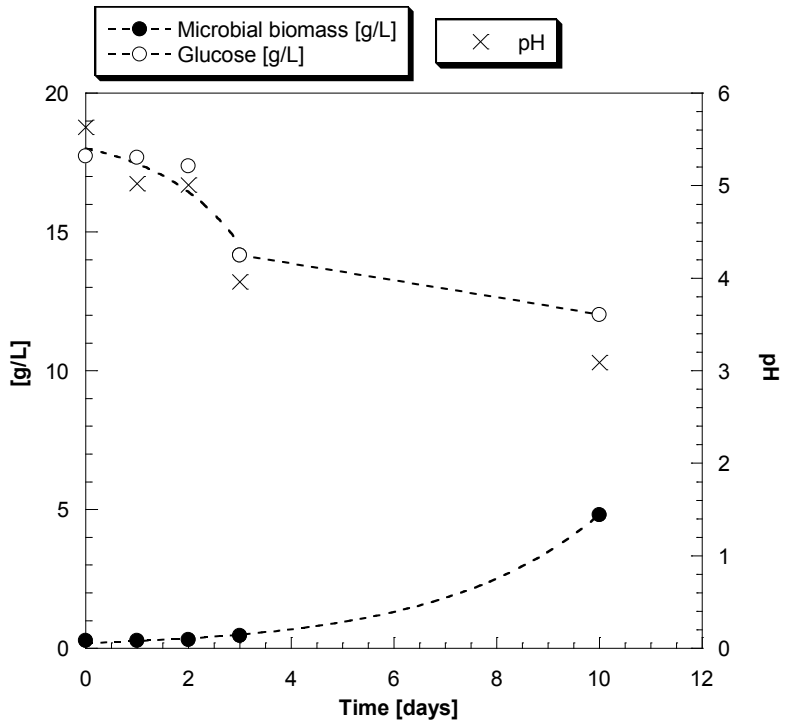

Fig. 3.26: Microbial biomass, glucose and pH profiles in **YEG Lip**

Figure 3.27 shows the profiles in terms of microbial biomass and cell count. The final value in **YEG Lip** was $3.0 \cdot 10^7$ Cells/mL instead of $1.4 \cdot 10^7$ Cells/mL for **YEG Chl** [Figure 3.25]. The inoculum was equal to $5.5 \cdot 10^5$ Cells/mL for both microorganisms. This result confirmed the ability of *Chlorella emersonii* to grow in YEG medium.

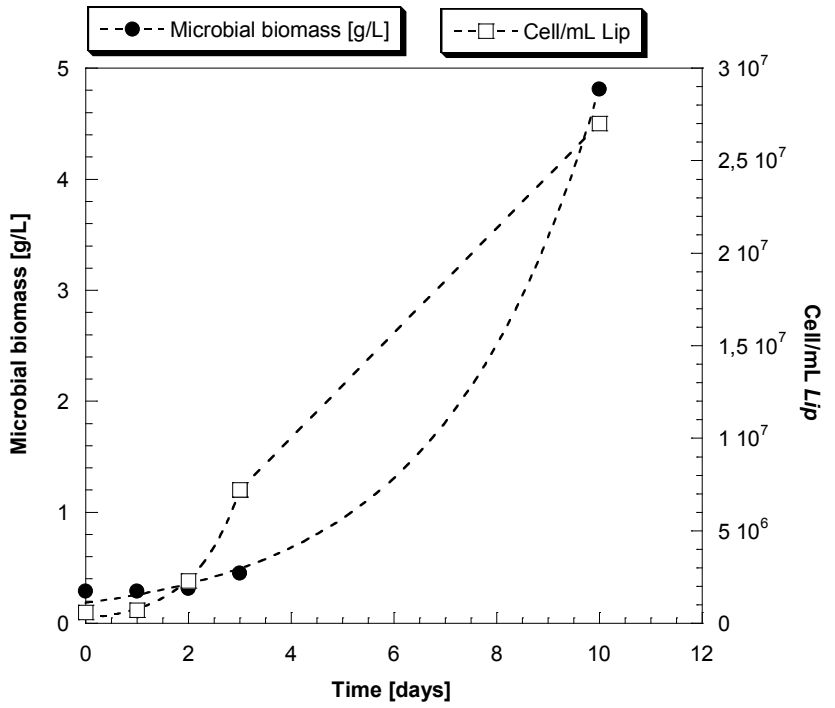

Fig. 3.27: Microbial biomass and Cell/mL profiles in **YEG Lip**

Synergistic effects of *Lipomyces starkeyi* and *Chlorella emersonii* mixed cultures and the presence of inhibition problems related to the photoinhibtion due to the cell density variation during the test were evaluated.

Figure 3.28 shows the profile of microbial biomass growth that was exponential in the first three days and then linear until the end of the test. The glucose consumption profile had a symmetrical trend. Probably, the trends were associated to the increase of osmotic pressure. The rapid changes caused by osmotic shock (dehydration, rehydration) might lead to modifications of the phospholipid structure of the cell membrane and even cell death. The value of microbial biomass was almost 10 g/L at the end of the experiment.

Figure 3.28 shows a progressive pH decrease that influenced cell proliferation of *Chlorella emersonii* more than *Lipomyces starkeyi* such as also confirmes Figure 3.29, where Cells/mL were $1.0 \cdot 10^8$ and $6.0 \cdot 10^6$, respectively. The cell proliferation of *Chlorella emersonii* in mixed cultures [Figure 3.29] was lower than observed in the individual culture (**YEG Chl**) [Figure 3.25] and stopped after the third day. In subsequent days, the cell death was observed, probably related to the decrease of pH or to the change of osmotic pressure.

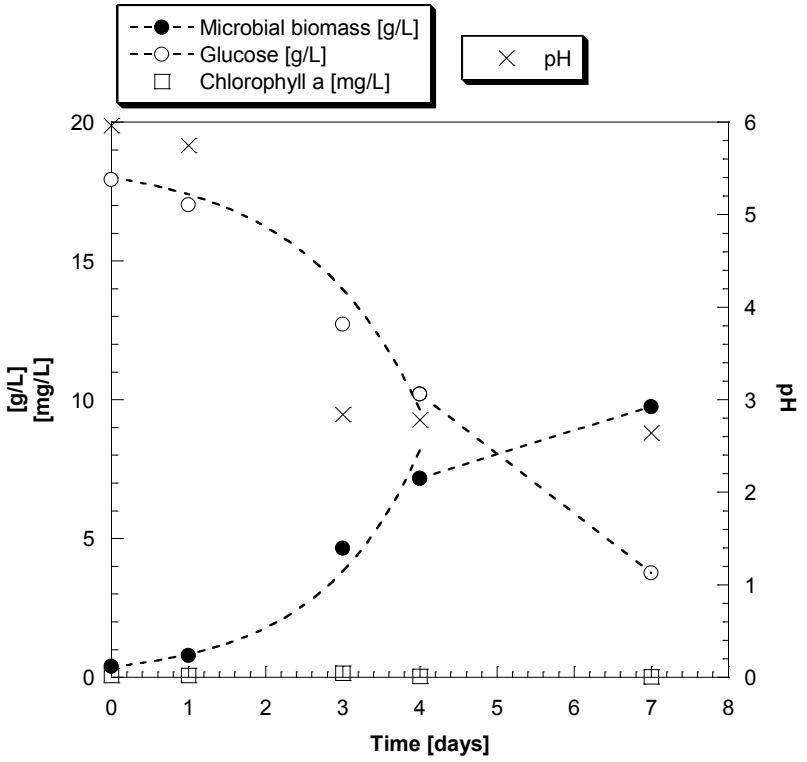

Fig. 3.28: Microbial biomass, Chlorophyll a, glucose and pH profiles in **YEG Chl+Lip**

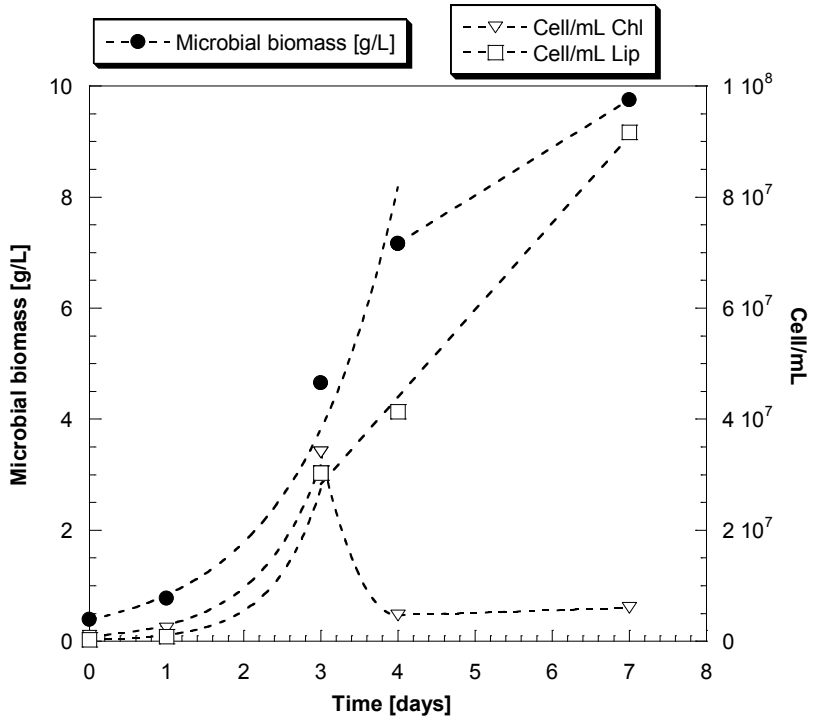

Fig. 3.29: Microbial biomass and Cell/mL profiles in **YEG Chl+Lip**

Table 3.9 shows kinetic values in terms of specific growth rate (μ_x) and glucose consumption rate (μ_s) were calculated according to Equations 3.8, 3.9 and 3.5, respectively. CO_2 fixation rate (P_{CO2}) according to Equations 3.10 and 3.11. Therefore, a discrepancy between the values related to the growth rate (μ_x) and glucose consumption rate (μ_s) was found. This problem could be associated to an experimental error of turbidometric analysis. CO_2 fixation rate (P_{CO2}) in mixed cultures (**YEG Chl+Lip**) was lower than in the individual culture (**YEG Chl**), probably related to the different cell proliferation of *Chlorella emersonii* in individual and mixed culture, up to $1.5 \cdot 10^7$ and $6.0 \cdot 10^6$ Cells/mL, respectively.

Tab. 3.9: Kinetic parameters related to **YEG**

	μ_x [d^{-1}]	μ_s [d^{-1}]	$Y_{x/s}$ [$g\ g^{-1}$]	P_{CO_2} [$mg\ L^{-1}\ d^{-1}$]
YEG Chl	0.784	0.181	0.233	168
YEG Lip	0.373	0.625	0.859	-
YEG Chl+Lip	0.763	0.655	0.647	44

Lipid extraction from the oleaginous biomass was performed according to the techniques already described in materials and methods [Chapter 2, § 2.6]. Figure 3.30 shows relative mass percentages. The resulting composition was relatively poor in fatty acids with mono or poly unsaturations which gives to the mix a low stability to oxidative degradation (*Cheirsilp et al., 2011*). It has been well established that the ease of formation of fatty acid radicals increases with increasing unsaturation, e.g., oleic acid (C18:1) has been estimated to be 10～40 times less susceptible to oxidation than linoleic acid (C18:2) (*McClements and Decker, 2008*). Among the fatty acids, n-3 PUFAs have been of particular interest since epidemiological and clinical researches have revealed possible effects of the n-3 PUFAs on brain development and curative and preventive effects on cardiovascular disease (*Simopoulos, 1989*). However, n-3 PUFAs might adversely affect human health because of their susceptibility to lipid oxidation. Therefore, Single Cell Oils represent intermediates for biodiesel production, polymers, biosurfactants and the control of unsaturation degree in their chain, for example by hydrogenation, could be critical to ensure selectivity and stability.

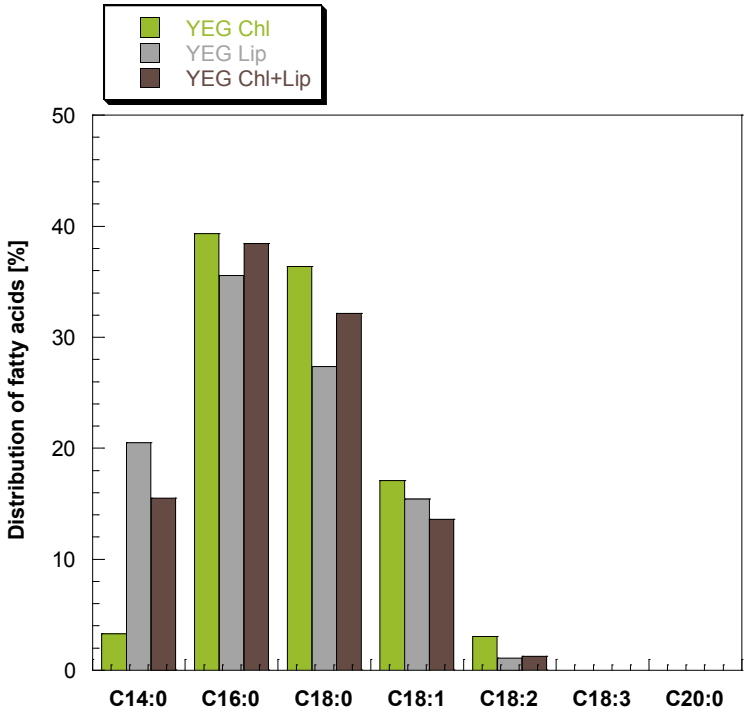

Fig. 3.30: Fatty acid distribution (% w/w) in **YEG**

Lipid yield (Y_{lipid}) and lipid productivity (ν_{lipid}) were also evaluated. Table 3.10 reports the values for **YEG Chl** lower than in **YEG Lip** and **YEG Chl+Lip**. The results partially confirmed the achieved values in terms of growth rate and substrate consumption. Therefore, the synergistic effect was able to produce a little increase in productivity and lipid yield. Leesing et al. (*2012*) studied microbial oil production in mixed culture using *Chlorella* sp. and *Torulospora maleeae* and they calculated kinetic parameters. Cheirsilp et al., (*2011*) studied the behaviour of *Chlorella vulgaris* and *Rhodotorula glutinis* mixed cultures for lipid production from industrial wastes. Both manuscripts confirmed symbiotic relationship and mutualism effect.

Tab. 3.10: Yields and productivity in **YEG**

	Y_{lipid} [g g^{-1}]	ν_{lipid} [g L^{-1} d^{-1}]
YEG Chl	0.008	0.168
YEG Lip	0.022	0.701
YEG Chl+Lip	0.027	0.705

Experiments were also carried out using a real system represented by *Arundo donax* hydrolysate (**ADH**) using methodologies already mentioned in materials and methods [Chapter 2, § 2.2], and simultaneously inoculated with *Chlorella emersonii* 316/25 (**Chl**) and *Lipomyces starkeyi DBVPG 6193* (**Lip**). The sample was labeled as **ADH Chl+Lip**. The aim was compare the operating conditions of synthetic cultures with real system, providing for the use of lignocellulosic biomass at low cost and with low environmental impact, and to evaluate the presence of synergism in yeast-microalga mixed cultures using *Arundo donax* hydrolysate as substrate (**ADH Chl+Lip**).

Figure 3.31 shows the relative profiles in terms of microbial biomass and different metabolites. The exponential growth of microbial biomass according to the adopted model [Equations 3.8, 3.9] was observed. The glucose consumption rate was symmetrical and calculated according to Equation 3.5. The exponential phase was followed by a kinetic reaction of zero order in which the increase of microbial biomass could be associated to the maintenance reaction. Figure 3.31 also reports pH value, that was constant and not able to decrease metabolic activity of oleaginous microorganisms. Phenol and furan concentrations were also measured during the test, with the aim to evaluate possible inhibition phenomena, already excluded using only *Lipomyces starkeyi* and only *Chlorella emersonii* as inoculum in steam exploded *Arundo donax* hydrolysate (data not shown). The remarkable biomass concentration, about 9 g/L, revealed that mixed culture of algae and yeast could achieve the symbiotic effects, which was mutually beneficial to the growth of both microorganisms. One of the possible reasons was the gas exchange in the medium.

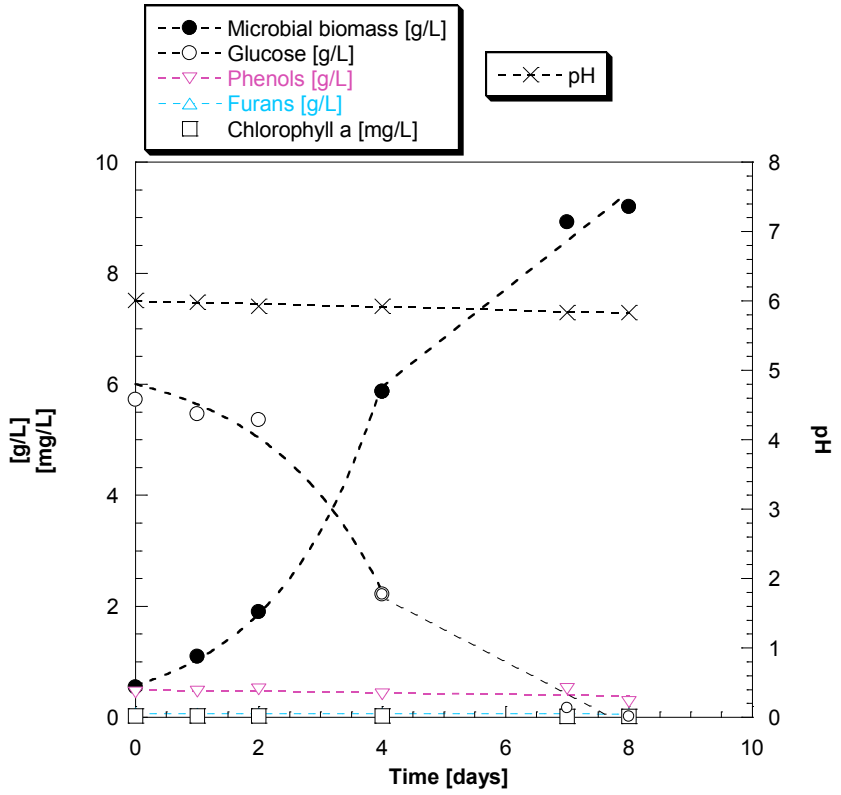

Fig. 3.31: Concentration and pH profiles in **ADH Chl+Lip**

Figure 3.32 shows microbial biomass and cell count profiles. Significant difference between yeast and microalga proliferation was observed, probably associated to a different adaptation in hydrolysate represented by a composition not able to ensure the normal metabolic activity of *Chlorella emersonii*. In fact the value in terms of Cell/mL was almost $2.0 \cdot 10^8$ for *Lipomyces starkeyi* and $1.0 \cdot 10^7$ for *Chlorella emersonii*. Microalga cell proliferation increased until the third day and then it was followed by a death phase.

This result probably confirmed an inhibition of *Chlorella emersonii* due to the cell proliferation of *Lipomyces starkeyi*, that inhibited microalga proliferation due to a reduced capacity of glucose assimilation and, simultaneously, a reduced light intensity caused by cell proliferation of yeast.

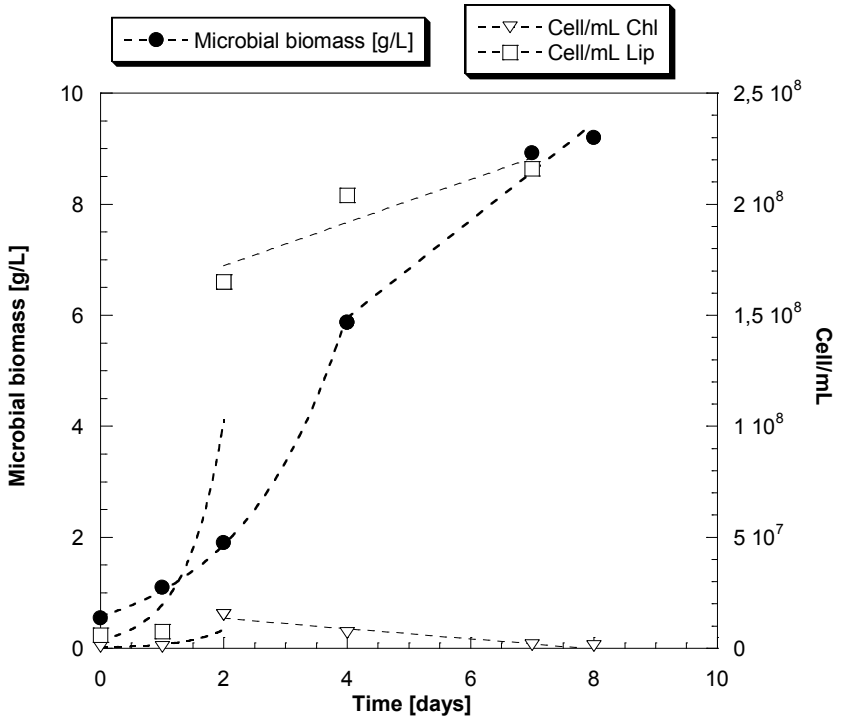

Fig. 3.32: Microbial biomass and Cell/mL profiles in **ADH Chl+Lip**

Kinetic parameters of **ADH Chl+Lip** in mixed culture were calculated. A correspondence between specific growth (μ_x), 0.587 d^{-1}, and consumption rate (μ_s), 0.523 d^{-1}, was observed. CO_2 fixation rate (P_{CO2}) was 103 mg L^{-1} d^{-1} [Equations 3.10, 3.11]. Lipid yield (Y_{lipid}) (calculated according to Equation 3.7, considering only glucose as measured substrate) and productivity (ν_{lipid}) were 0.122 g g^{-1} and 0.088 g L^{-1} d^{-1}, respectively. The values showed the ability to assimilate fermentable sugars (hexose and pentose sugars) and to contribute positively to a relatively high lipid yield (Y_{lipid}), suggesting a feasible scaling up of lipid production via mixed culture in batch mode. Papone et al., (*2012*) calculated kinetic parameters in mixed cultures using sugarcane juice as substrate that that allows to look at experimental obtained results as promising for further study.

The samples were subjected to lipid extraction and alkaline transesterification, in order to evaluate the composition in terms of the fatty acids. Figure 3.33 shows the data of main fatty acids that were long-chain with 16 and 18 carbon atoms including palmitic (32.7%), oleic (52.1%), stearic (12.1%) and linoleic (2.5%). The fatty acid compositional profile, similar to that of plant oil which contains mainly palmitic and oleic acid (*Gui et al., 2008*), indicated that Single Cell Oil in this study could be used as biodiesel feedstock. However, oleic acid being a typical ingredient of edible oil (*Simopoulos, 2008*). The high content of unsaturated

compound (oleic acid) would suggest lower oxidative stability, but excellent fuel properties at low temperatures, which is an advantage in winter operation (*Cheirsilp et al., 2011*).

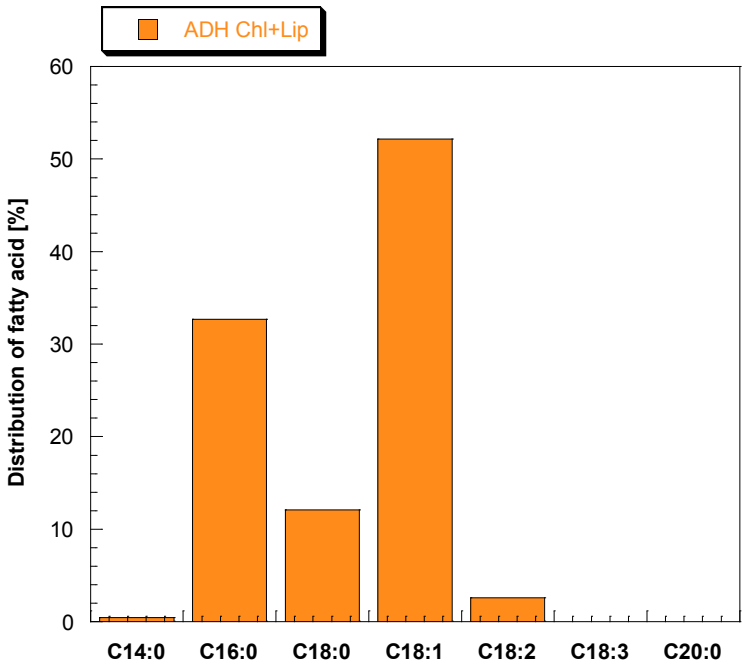

Fig. 3.33: Fatty acid distribution (% w/w) in **ADH Chl+Lip**

In an attempt to deepen and better understand the effects of gas exchange in oleaginous yeast-microalga mixed cultures, experimental tests were perfomed using as microalga *Chlamydomonas* sp., from culture collection of INRA-LBE (Laboratoire de Biotechnologie de l'Environnement), Narbonne, France and *Lipomyces starkeyi DBVPG 6193* as oleaginous yeast. *Chlamydomonas* sp. grows in heterotrophic and mixotrophic conditions, and it represents a suitable source for microbial oil production (*Ho et al., 2014*; *Nakanishi et al., 2014*; *Moon et al., 2013*). In particular, different organic sources, i.e. acetate and glucose, were examined on algal growth and lipid production. Furthermore, the variation in terms of gas composition was monitored. The final aim was to examine the behavior of selected oleaginous microorganisms in mixed cultures using a low cost feedstock, e.g., wheat straw, and, in the same way, to solve the problem that represent about 80% of the total cost of the growth medium (*Li et al., 2007b*). Figure 3.34 shows microbial biomass, glucose conversion and pH profiles for *Chlamydomonas* sp. (**Chla**) in a medium with MES buffer (**MP100**), with the addition of glucose (**G**) and Yeast extract (**YE**) [Chapter 2, § 2.4]. The final value for

microbial biomass was 1.22 g/L and final glucose concentration 9.8 g/L. This result confirmed what it has been achieved in preliminary tests (data not shown). Figure 3.35 shows cell proliferation in tems of Cells/mL. The final value was equal to $1.3 \cdot 10^6$ Cells/mL, attesting a reduced metabolic activity of microalga in a medium containing glucose as carbon source.

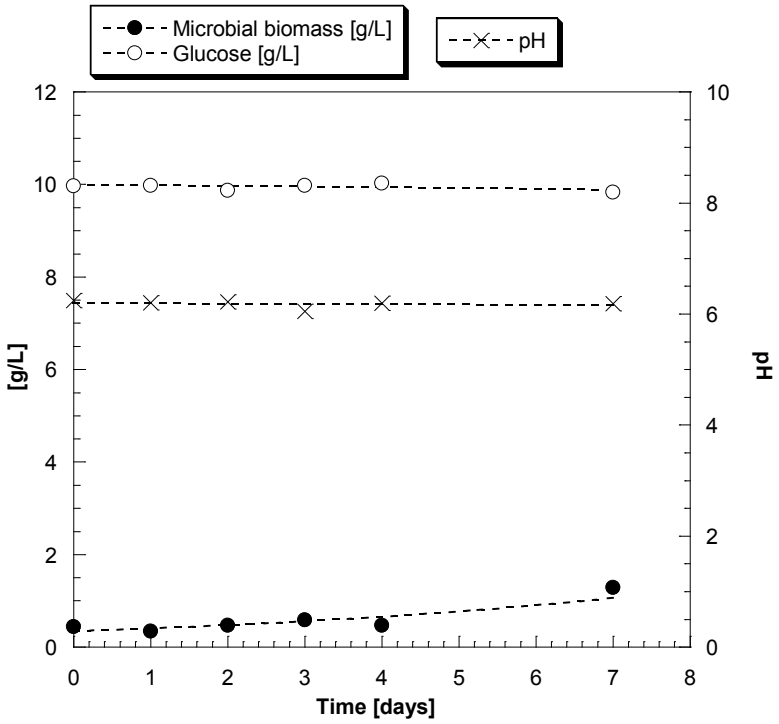

Fig. 3.34: Concentration and pH profiles in **MP100+G+YE Chla**

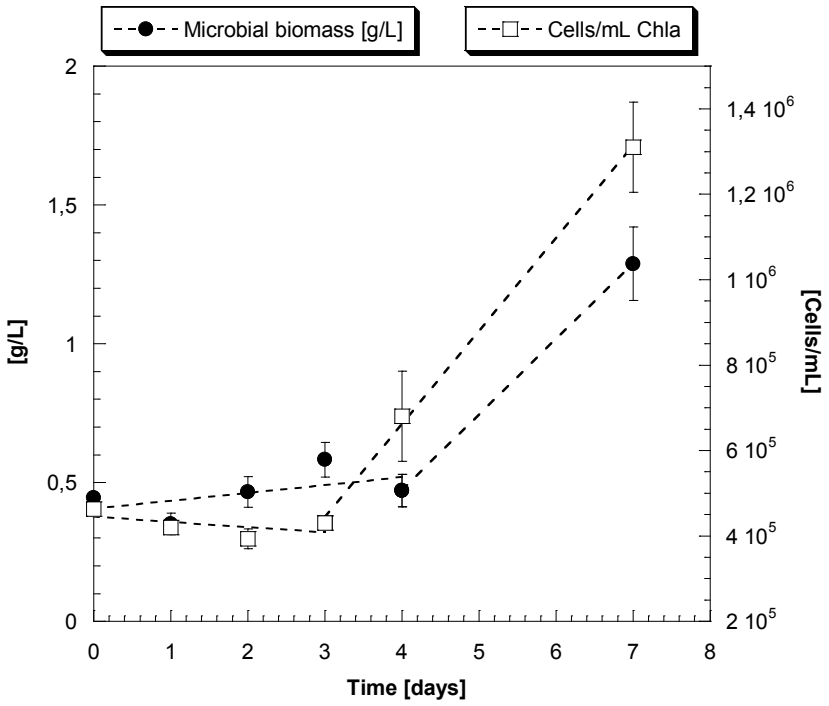

Fig. 3.35: Microbial biomass and cell/mL values in **MP100+G+YE Chla**

The time course data of different gas concentrations (% v/v) and dissolved oxygen (D.O.) value in **MP100+G+YE Chla** showed in Figure 3.36. The concentrations of O_2, CO_2 and N_2 were constant. The dissolved oxygen (D.O.) value, obtained using D.O. probe [Chapter 2, § 2.6], was also compared with the value obtained according to Equation 3.14:

$$[O_2]_{eq} = \frac{P\, y_{O_2}}{H_{O_2}(24\,°C)} \tag{3.14}$$

$$H_{O_2}(24\,°C) = 769.2\ atm\ L\ mol^{-1}$$

Saturated conditions of O_2/CO_2 were chosen for the experimental tests. The reason was to avoid external contributions in terms of gas, for the accuracy CO_2, and to study the behaviour of microalgae in operating conditions, that should be similar to the real system in microalga-yeast mixed cultures, where yeast should grow faster than microalga and simultaneously supply CO_2 during the growth.

Under high irradiance, high O_2 and reduced CO_2 concentrations, the equilibrium in the reaction was shifted towards the photorespiration, activating Rubisco's catalytic site, decreasing O_2 competition and enhancing carbon fixation. Figure 3.36 shows only a slight

decrease of dissolved oxygen (D.O.) during the test from 9 to about 8 mg/L. The other monitored values were almost constant.

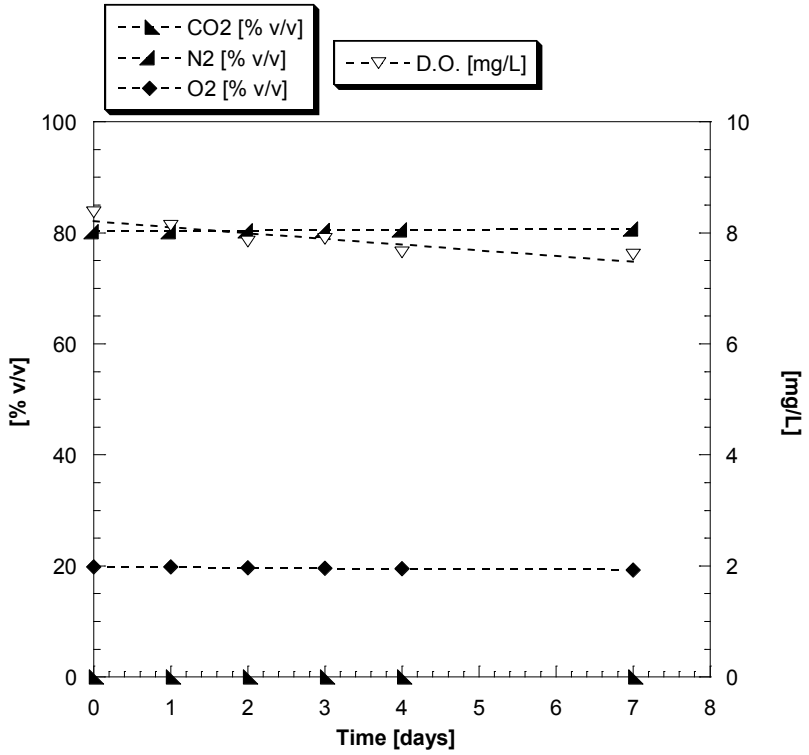

Fig. 3.36: Gas phase and dissolved oxygen (D.O.) values in **MP100+G+YE Chla**

Figure 3.37 shows the profiles in terms of microbial biomass, glucose and pH related to *Lipomyces starkeyi* in MP100+G+YE medium (**MP100+G+YE Lip**). The experimental test was conducted to evaluate the ability of yeast to grow in a culture medium containing glucose as carbon source and a buffering solution able to preserve the pH stability. In fact, fixed pH at 6.25 didn't change during the test.

Figure 3.37 illustrates an exponential increase of microbial biomass and a linear next phase due to a maintenance metabolism. The final microbial biomass was 3.23 g/L, instead of glucose consumption that stopped at 6.96 g/L. The change in metabolism from exponential to linear stretch was not yet clear. Probably this phenomenon is associated to the osmosis or to the pH value, which may be higher than required by the yeast for its better proliferation.

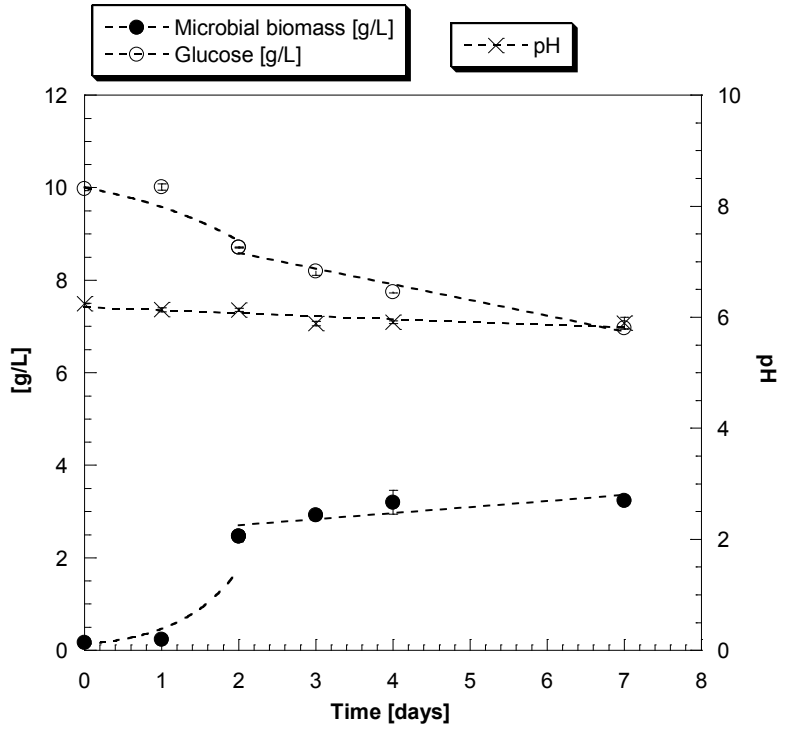

Fig. 3.37: Concentration and pH profiles in **MP100+G+YE Lip**

Figure 3.38 shows the cell proliferation profiles in terms of microbial biomass and Cells/mL. The latter was equal to $2.6 \cdot 10^7$ Cells/mL. **MP100+G+YE** medium was considered able to promote the activity of the oleaginous yeast, but limited nitrogen content (data not shown) for these cultures probably could lead to a cessation of cell growth while still allowing fatty acid production.

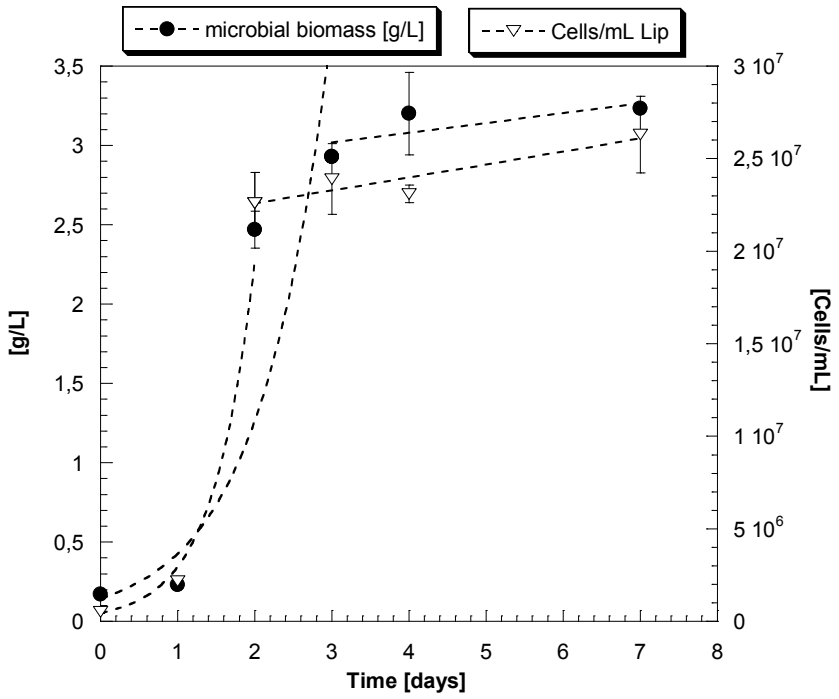

Fig. 3.38: Microbial biomass and Cell/mL values in **MP100+G+YE Lip**

Figure 3.39 shows the variations of the gas-phase concentrations. O_2 concentration decreased from 19.83% to 5.62% and CO_2 increased from 0.14% to 16.29%. These results suggested that *Lipomyces starkeyi* had a great growth rate in aerobic conditions wherein large amount of O_2 must be required and CO_2 emitted. D.O. declined from 8.5 mg/L to 2 mg/L. Probably, the agitation system was not able to ensure the sufficient aeration and when the dissolved oxygen decreased also the exponential biomass growth ended. The highly oxygenated conditions may contribute to a rapid cell division. Oxygen limitation reduced the growth rate, leads to incomplete glucose oxidation, and may enhance lipid production (*Calvey et al., 2016*).

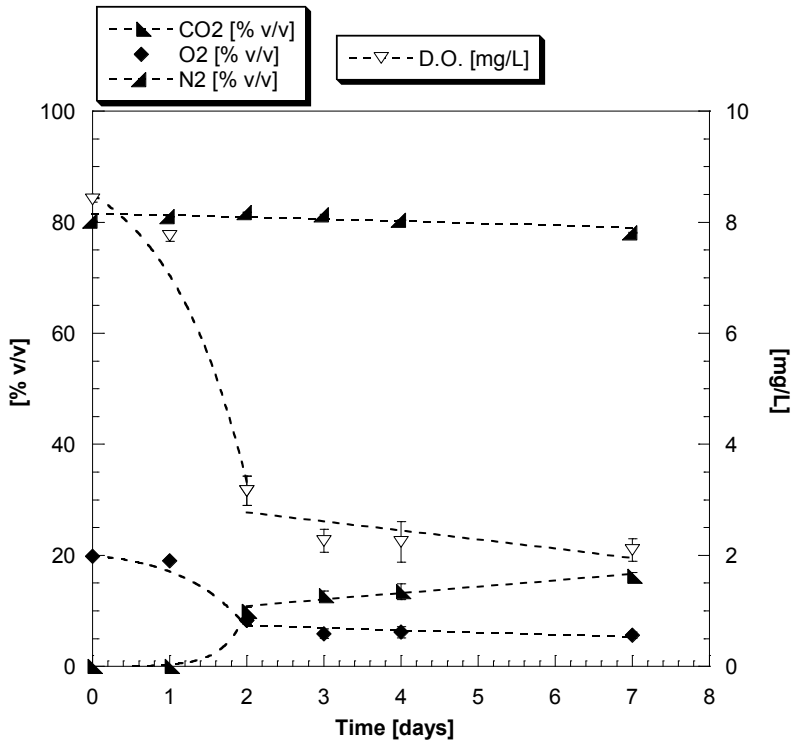

Fig. 3.39: Gas phase and dissolved oxygen (D.O.) values in **MP100+G+YE Lip**

The next step provided for the preliminary selection of microalga-yeast ratio to use as inoculum in mixed cultures, and, simultaneously, to verify the increase of metabolic activity related to the individual microorganisms. Since the microalga usually grows slower than the yeast, an increase in the initial amount of microalga was attempted. The effect of the amount of microalga was investigated by increasing the amount of microalga of two and three times compared oleaginous yeast concentration. Figure 3.40 shows the results in terms of Cells/mL for three different ratio of **Chla:Lip** equal to **1:1**, **2:1**, **3:1** and two cultures represented by **Chla** and **Lip,** that were chosen as control. **MP100+G+YE** was chosen such as medium. It was possible to observe an increase of the *Chlamydomonas* cell proliferation in mixed cultures in comparison to the individual culture. In fact, the value of Cells/mL in **Chla:Lip 1:1** was equal to $6.7 \cdot 10^6$, while in **Chla** was $2.4 \cdot 10^6$ [Figure 3.41a].

The metabolic activity of yeast in mixed cultures (**Chla:Lip**) suffered, however, a reduction compared to single culture (**Lip**). In fact Cell/mL values were $7 \cdot 10^7$ for **Chla:Lip 1:1** and $1.1 \cdot 10^8$ for **Lip**, respectively [Figure 3.41b]. Probably, in mixed culture *Chamydomonas sp.* benefited from the synergy with *Lipomyces starkeyi* to increase its cell proliferation due to the increase of CO_2 availability during yeast fermentation, while the yeast suffered a reduction of

its metabolic activity, related to pH value (data not shown) and to the lack of microelements that were also metabolised by the microalga.

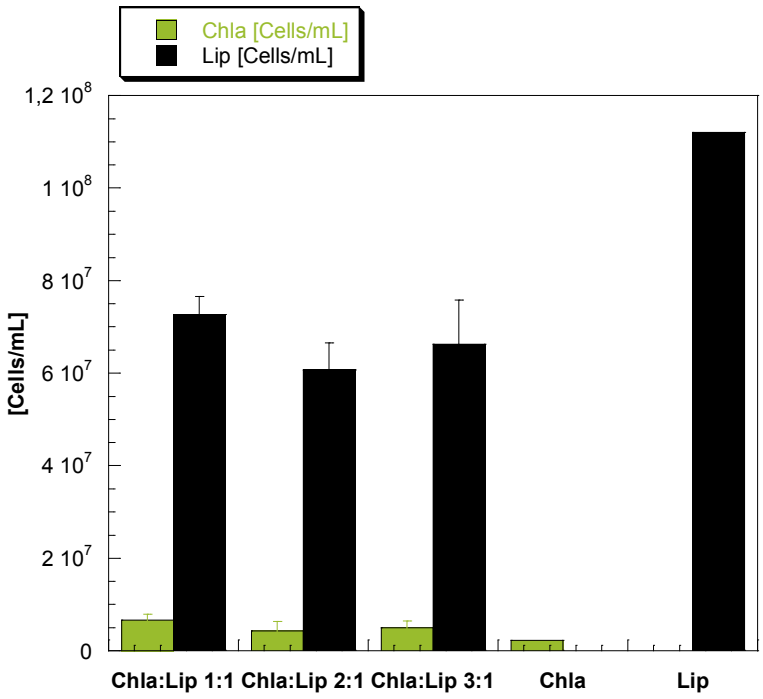

Fig. 3.40: Cell proliferation (Cells/mL) in **MP100+G+YE**

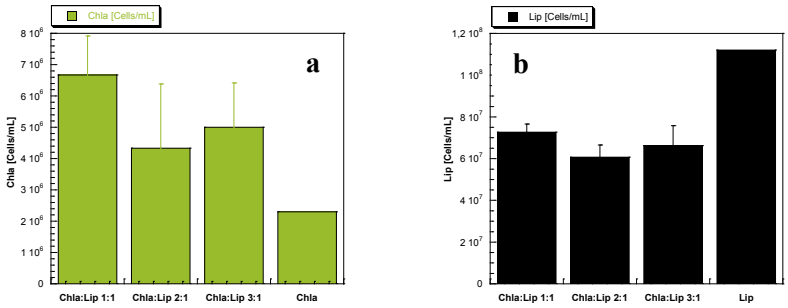

Fig. 3.41 a, b: Cell proliferation (Cells/mL) in **MP100+G+YE**
(Chla:Lip and Chla, **a**; Chla:Lip and Lip, **b**)

Figure 3.42 shows the lipid content calculated as ratio between mass of lipid extracted divided by mass of treated dry biomass multiplied by 100 [% w/w]. The results showed an improvement in lipid content, in **MP100+G+YE** medium, from **Chla** (2.5 %w/w) to **Chla:Lip 1:1** (4 %w/w). The values of **Chla:Lip 2:1** and **Chla:Lip 3:1** were almost comparable to **Chla**.

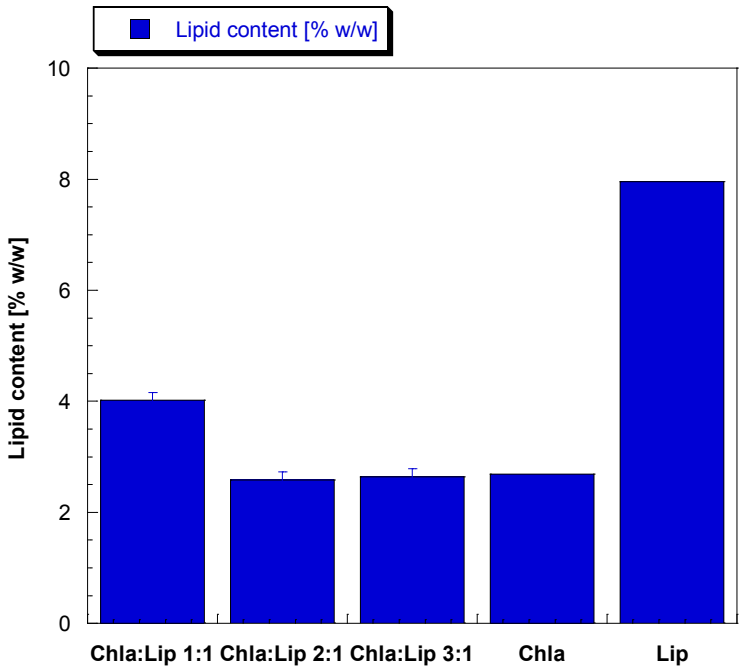

Fig. 3.42: Lipid content in **MP100+G+YE**

The **MP100+G+YE Chla:Lip** mixed culture, with **1:1** as inoculum ratio, showed the better growth in terms of cell proliferation [Figure 3.40] and it was selected for the next tests.

Figure 3.43 shows the growth of microbial biomass. It was exponential in the first part, as well as glucose consumption rate. The value in terms of microbial biomass reached 5.96 g/L higher than obtained in the single cultures of **Chla** and **Lip**, where it was equal to 1.22 g/L and 3.23 g/L, respectively. The growth changed its behaviour from exponential to linear after three days probably due to osmotic phenomena that influenced glucose metabolism. The pH was stable during the test and not represented an inhibition factor for yeast growth.

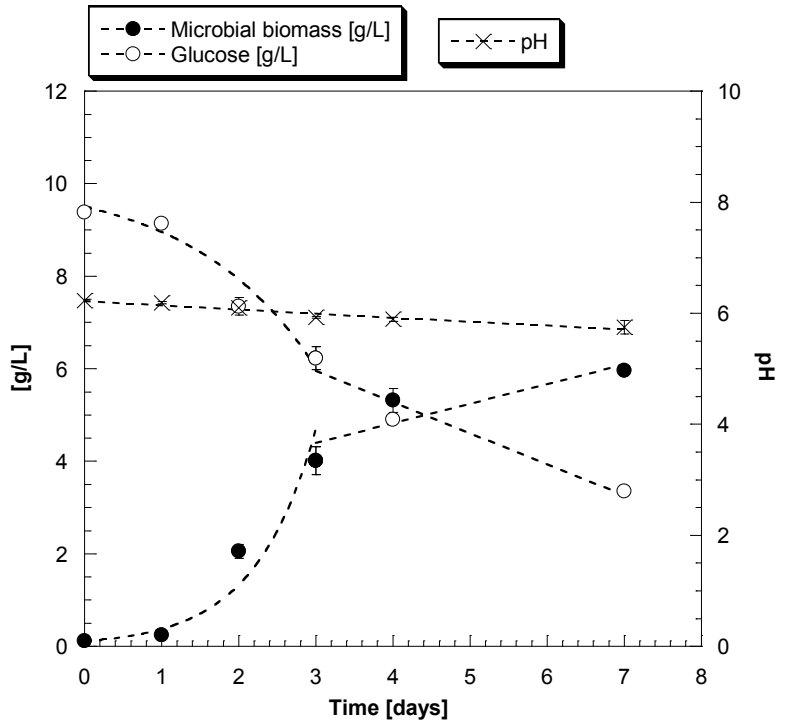

Fig. 3.43: Concentration and pH profiles in **MP100+G+YE Chla:Lip 1:1**

Figure 3.44 reports cell proliferation of *Lipomyces starkeyi* and *Chlamydomonas* sp., in terms of Cells/mL. The yeast and microalga growths stopped after three days. The growth of both microorganisms suffered the consequences of inhibition factor that, probably, was related to osmosis effect or to nitrogen source uptake. Nitrogen removal by algae provides the potential benefit of produced lipids that is also related to a high C/N ratio that promotes lipid accumulation for several microalgae under heterotrophic conditions (*Eriksen, 2008*).

The synthesis of nitrogen-containing compounds such as protein and nucleic acids is reduced, and cells enter into the stationary phase and begin to accumulate storage lipids (*Jiang and Chen, 2000*).

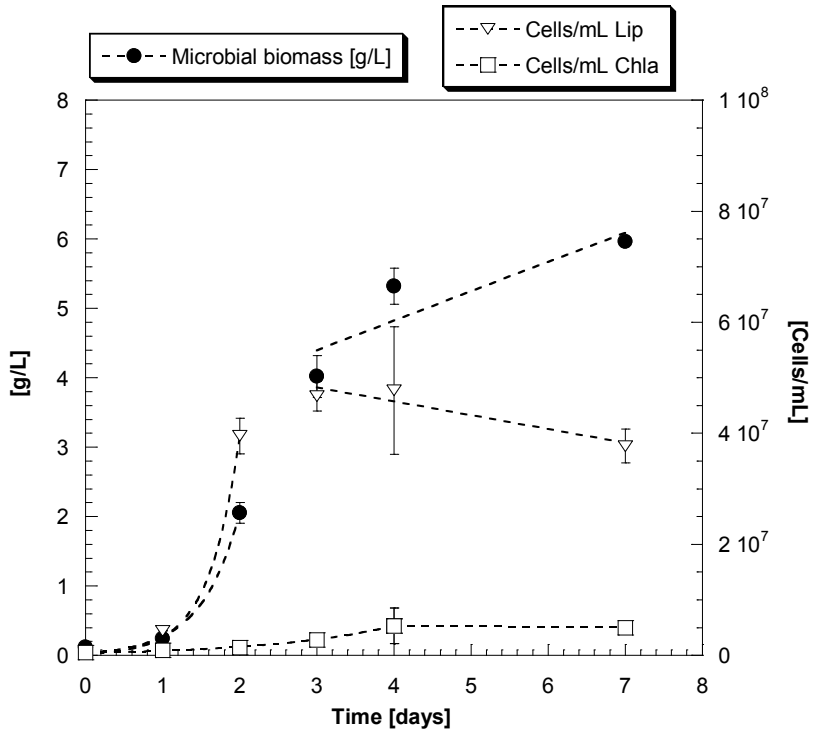

Fig. 3.44: Microbial biomass and cell/mL values in **MP100+G+YE Chla:Lip 1:1**

The gas concentrations reached a dynamic equilibrium during 48–72 h and a O_2/CO_2 balance for enhancing of both yeast and alga growths [Figure 3.45]. O_2 concentration decreased rapidly due to the faster growth of yeast. This result confirmed the trends of Cell/mL profiles [Figure 3.44]. In the same time, microalga grew using part of CO_2 that was produced during yeast fermentation. After three days, it was observed a decrease of D.O. value from saturated conditions (almost 8.5 mg/L), to 3 mg/L, also calculated according to Equation 3.14, that influenced the change of metabolism and also the production of some metabolites such as ethanol (0.358 g/L) under partial anaerobic conditions strictly related to D.O. decrease.

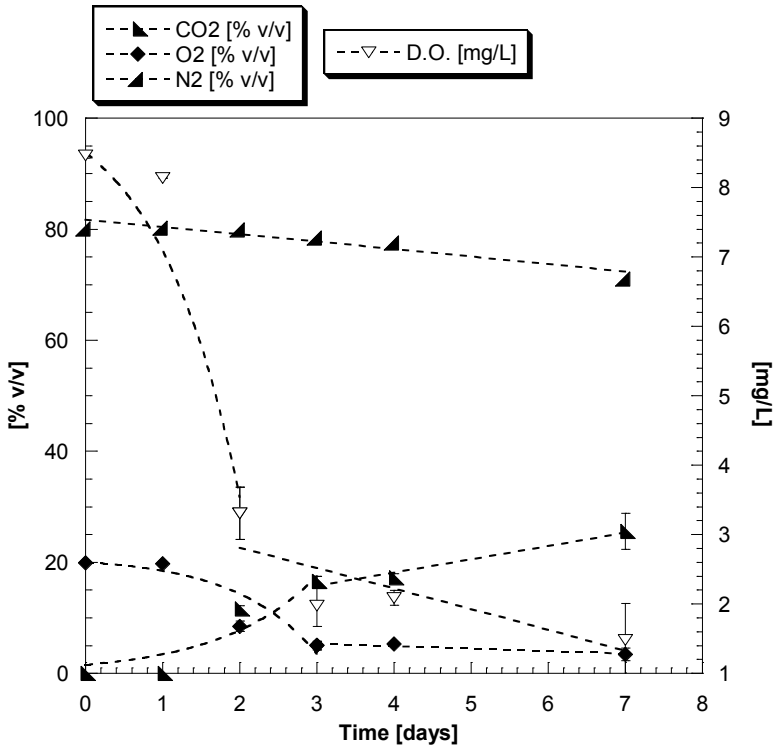

Fig. 3.45: Gas phase and dissolved oxygen (D.O.) values in **MP100+G+YE Chla:Lip 1:1**

A further test was performed to validate the presence of the gas exchange between the oleaginous microorganisms and, at the same time, verify the presence of a symbiotic effect. For this purpose, a batch reactor with the same ratio of yeast-microalga in terms of Cells/mL (**Chla:Lip 1:1**), was inoculated. The selected medium was **MP100+G+YE**, as in the previous tests and it was previously removed the air ($\mathbf{N_2}$).

Figure 3.46 shows the same concentration profiles reported in Figure 3.43 (in **MP100+G+YE Chla:Lip 1:1** $\mathbf{N_2}$ was observed the same behavior of **MP100+G+YE Chla:Lip 1:1**). This result also certified that in the mixed culture in absence of initial saturated conditions (**MP100+G+YE Chla:Lip 1:1** $\mathbf{N_2}$) metabolic activity was comparable to the mixed culture in presence of oxygen saturated conditions (**MP100+G+YE Chla:Lip 1:1**), without additional gas intake. To check the growth of both microorganisms, separated cell count was carried out. Figure 3.47 shows an exponential increase of *Chlamydomonas* (**Cells/mL Chla**) and *Lipomyces strakeyi* (**Cells/mL Lip**) until the fourth day. This result confirmed the beneficial balance of mutualism that was further confirmed, monitoring the gas phase in terms of CO_2, O_2, N_2 concentrations such as showed in Figure 3.48. CO_2 increased from 0.11% to 7.67%, strictly related to the faster growth of yeast, that was not able to inhibit cell proliferation and microalga growth, also in non-aerated conditions. The value of D.O., equal to 1.2÷1.7 mg/L

[Figure 3.48] according to Equation 3.14 and experimental analysis using D.O. probe [Chapter 2, § 2.6], confirmed, for its part, that the yeast was able to grow because microalga was able to supply O_2, and it was used by yeast for its growth.

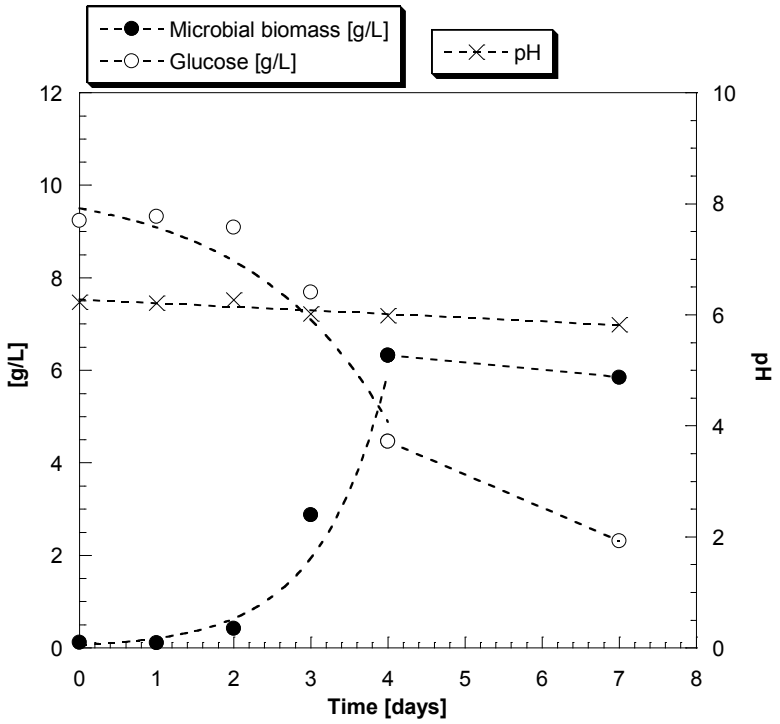

Fig. 3.46: Concentration and pH profiles in **MP100+G+YE Chla:Lip 1:1 N_2**

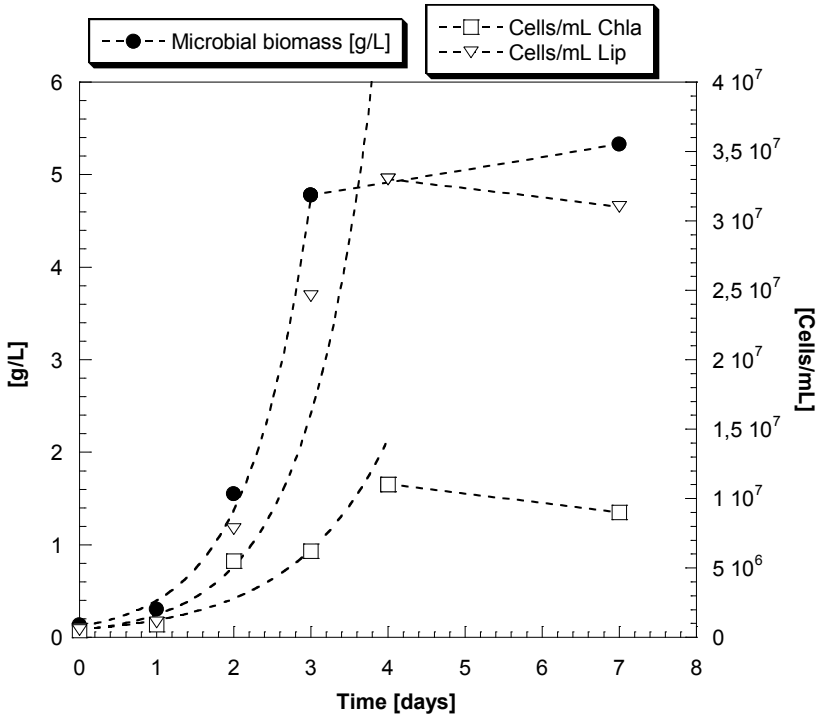

Fig. 3.47: Microbial biomass and cell/mL values in **MP100+G+YE Chla:Lip 1:1 N_2**

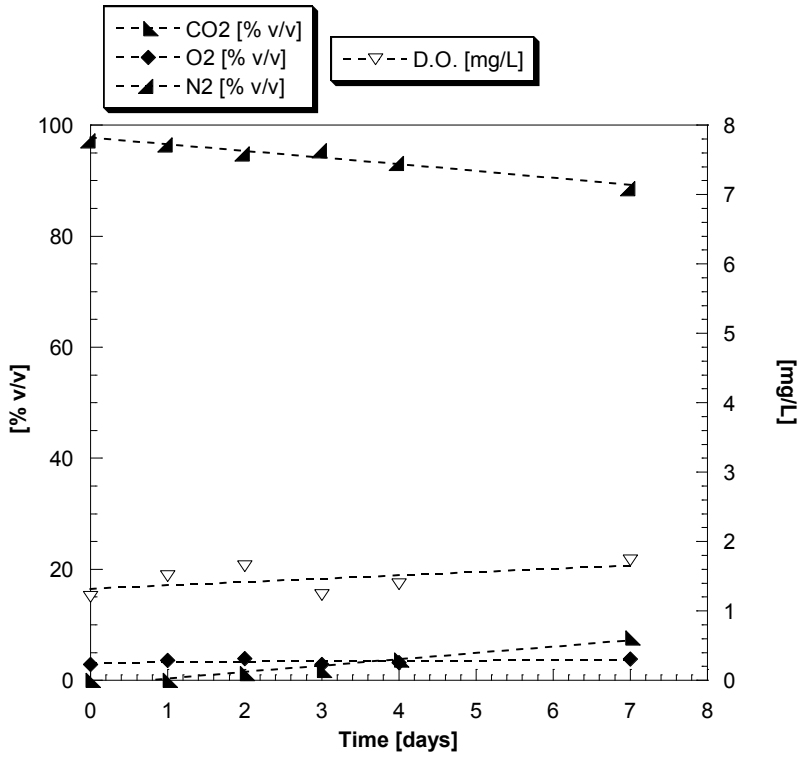

Fig. 3.48: Gas phase and dissolved oxygen (D.O.) values in **MP100+G+YE Chla:Lip 1:1 N_2**

Table 3.11 shows the values of specific growth rate (μ_x) according to Equations 3.8 and 3.9, that increased from 0.144 d^{-1} in **MP100+G+YE Chla** to 1.266 d^{-1} in **MP100+G+YE Chla:Lip 1:1**. This result was associated to glucose consumption rate (μ_s) according to Equation 3.5. mx_0 was also calculated according to Equation 3.13 and lipid productivity (ν_{lipid}) as a value of biomass accumulation at the end of culture time.

The glucose consumption rate certified that *Chlamydomonas* didn't grow as a result of glucose intake (**MP100+G+YE Chla)**. μ_s was low (0.144 d^{-1}), while in **MP100+G+YE Lip** was high and equal to 1.320 d^{-1}. In this case, however, the growth was due to glucose metabolism which was almost the same in the case of mixed cultures (**MP100+G+YE Chla:Lip 1:1** and **MP100+G+YE Chla:Lip 1:1 N_2**). The increase of the metabolic activity of *Chlamydomonas* and *Lipomyces starkeyi*, already discussed, could be attributed to the presence of a synergistic effect, in which the microalga has benefited by CO_2 production during the yeast fermentation for its growth, and the yeast of the partial CO_2 removal, and, probably, the availability of O_2 by the metabolic activity of the microalga. The result of the increase in metabolic activity due to the presence of a mutualism, increased lipid productivity (ν_{lipid}) [Table 3.11] and lipid yield (Y_{lipid}). A different tendency was observed for cell yield coefficient ($Y_{x/s}$) calculated according to Equation 3.12, probably, due to different way to metabolise glucose.

Tab. 3.11: Kinetic parameters related to **MP100+G+YE**

	μ_x [d^{-1}]	μ_s [d^{-1}]	mx_o [g g^{-1} d^{-1}]	$Y_{x/s}$ [g g^{-1}]	Y_{lipid} [g g^{-1}]	ν_{lipid} [g L^{-1} d^{-1}]
MP100+G+YE Chla	0.144	0.014	0.014	0.078	$7.0 \cdot 10^{-3}$	0.014
MP100+G+YE Lip	1.320	1.718	0.298	0.967	0.039	0.032
MP100+G+YE Chla+Lip 1:1	1.266	1.165	0.776	0.908	0.115	0.053
MP100+G+YE Chla+Lip 1:1 N_2	1.121	1.224	0.715	0.843	0.128	0.056

Figure 3.49 shows the lipid content vs time in **MP100+G+YE** for all samples, where different inoculum were used. In **MP100+G+YE Chla** was not possible to observe an increase in terms of lipid content. In the mixed cultures (**MP100+YE Chla:Lip 1:1**, **MP100+G+YE Chla:Lip 1:1N_2**) and in the individual culture (**MP100+G+YE Lip**), the increase of lipid content was found until the fourth day, strictly related to biomass growth profiles. The subsequent

decrease was probably related to the cell death that was also observed during cell proliferation monitoring in mixed cultures [Figures 3.44, 3.47]. Lipid content descrease for **MP100+G+YE Lip** was, instead, attributed to dissolved oxygen consumption and, probably, to the change of metabolic pathway from lipid accumulation to fermentation, confirmed by final accumulation of ethanol (0.299 g/L) [Figure 3.49].

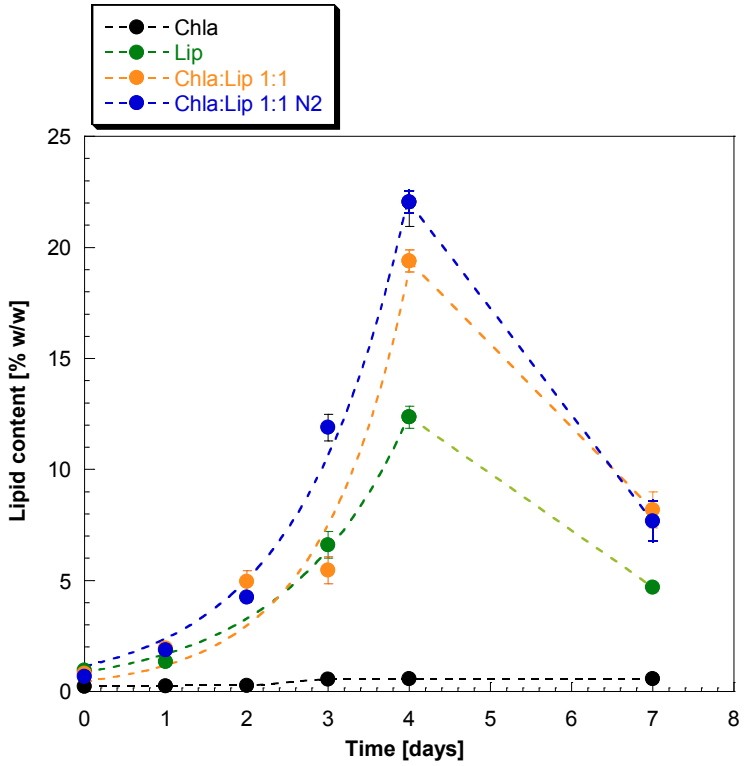

Fig. 3.49: Lipid content in **MP100+G+YE**

Fatty acid profiles of total lipids for **MP100+G+YE** media were summarized in Figure 3.50. The high mass percentage of oleic (C18:1) for **MP100+G+YE Lip** was in agreement with previous results for *Lipomyces starkeyi* (*Wild et al., 2010*). The main lipid components were mid and long-chain saturated fatty acids (C14:0, C16:0, C18:0), monounsaturated fatty acids (MUFAs, C18:1) in all samples which together represented almost 89.8% w/w in **MP100+G+YE Chla**, 89.4% w/w in **MP100+G+YE Lip**, 96.1% w/w in **MP100+G+YE Chla:Lip 1:1** and 99.7% w/w in **MP100+G+YE Chla:Lip 1:1 N_2**.

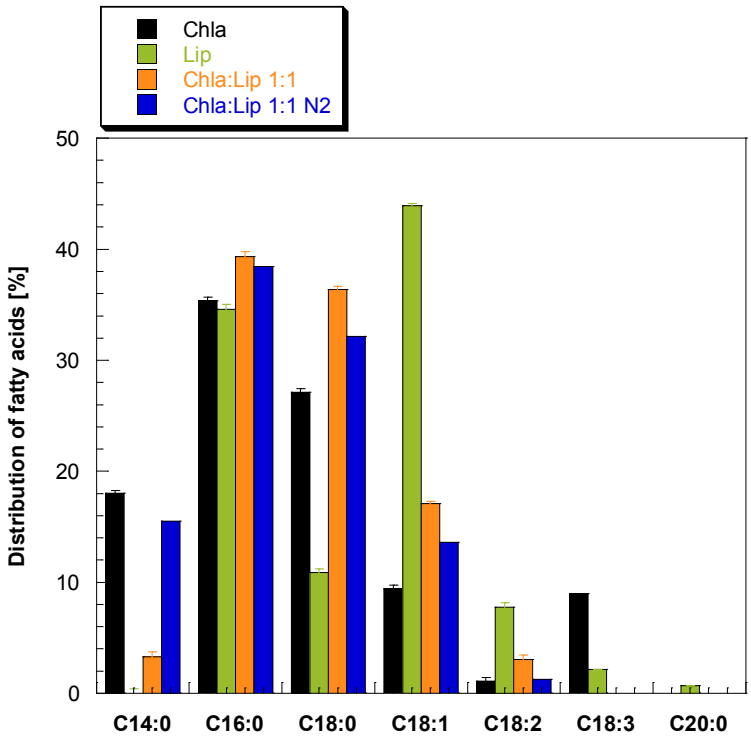

Fig. 3.50: Fatty acid distribution (% w/w) in **MP100+G+YE**

In an attempt to justify the choice of *Chlamydomonas*, able to metabolize organic substrates such as acetic acid, and, at the same time, with the aim to approach the composition of culture medium to the real system represented by a hydrolysates of lignocellulosic materials containing sugars but also volatile organic acids; the research activity was focused on the study of the behavior of the two selected strains, *Lipomyces starkeyi* (**Lip**) and *Chlamydomonas* (**Chla**) in culture media (**MP100**) containing glucose (**G**), acetic acid (**Ac**) and yeast extract (**YE**) at the same operating conditions just discussed.

Figure 3.51 shows an exponential behavior of microbial biomass in **MP100+G+YE+Ac Chla** until the third day. The value in terms of g/L was equal to 4.11. In this case, the metabolic activity was also associated to the acetic acid consumption, from 1.6 g/L to 0.00 g/L in 48 hours, while the glucose concentration was almost unchanged. The value of microbial biomass was higher than observed in the **MP100+G+YE Chla**, in absence of acetic acid, where it was 1.22 g/L. This result confirmed those reported in literature (*Moon et al., 2013*) that supplementation of the growth medium with appropriate amounts of organic carbon, particularly acetate, under mixotrophic conditions increases biomass production, as just discussed, and lipid production. The pH value was particularly stable, probably related to

buffering solution that was chosen to guarantee process conditions suitable for the yeast growth.

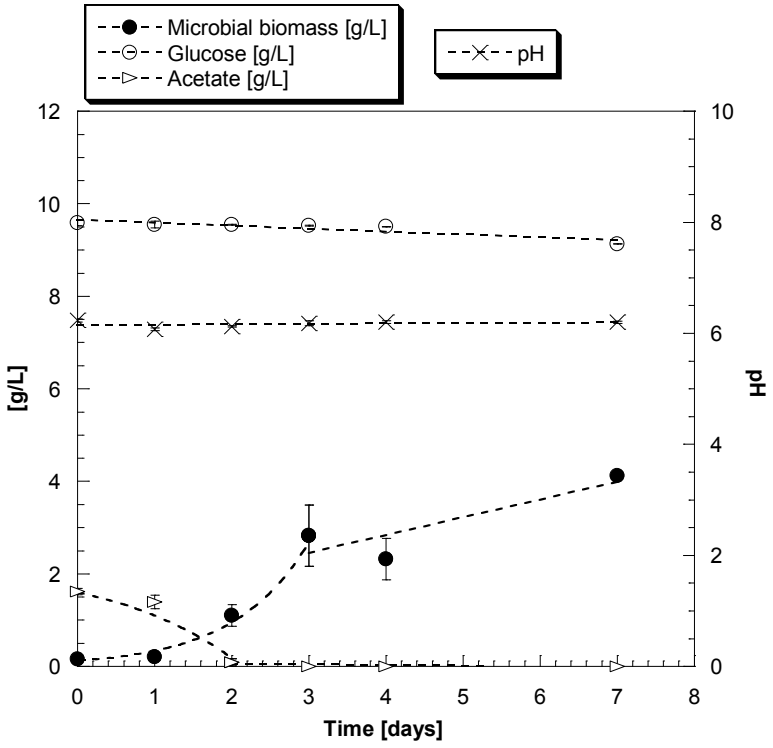

Fig. 3.51: Concentration and pH profiles in **MP100+G+YE+Ac Chla**

Figure 3.52 shows cell proliferation in terms of Cells/mL. The final value was $6.0 \cdot 10^6$, higher if compared to $1.3 \cdot 10^6$ for **MP100+G+YE Chla** [Figure 3.35], that attested an increase of metabolic activity of microalga in a medium containing also acetate as carbon source instead of only glucose, not metabolized by *Chlamydomonas* sp.

The concentrations in the gas phase of O_2, CO_2 and N_2 dind't change [Figure 3.53]. The low CO_2 concentration of atmospheric air was sufficient for cell growth if compared these values to dynamic models observed in Figures 3.51 and 3.52.

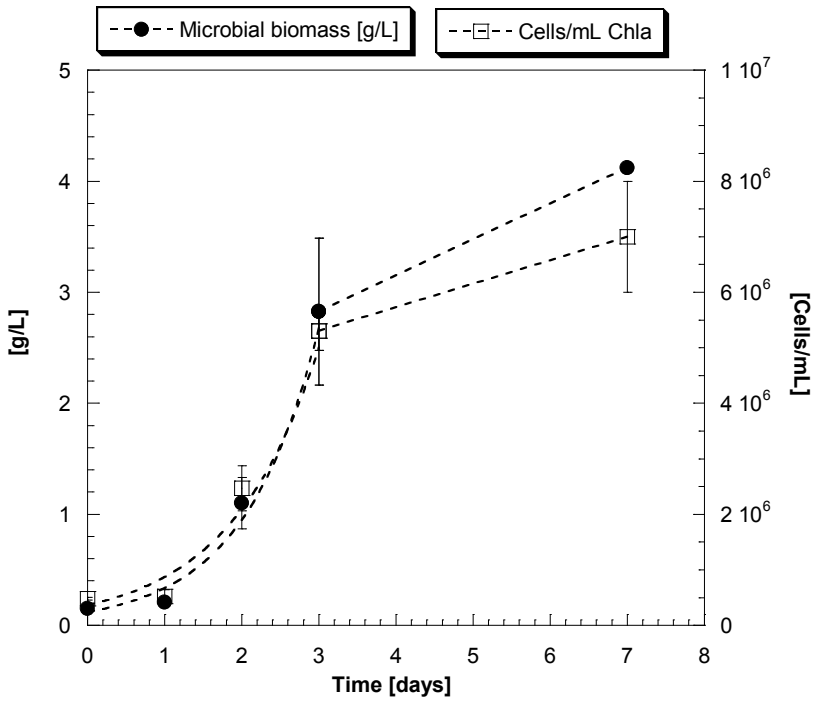

Fig. 3.52: Microbial biomass and cell/mL values in **MP100+G+YE+Ac Chla**

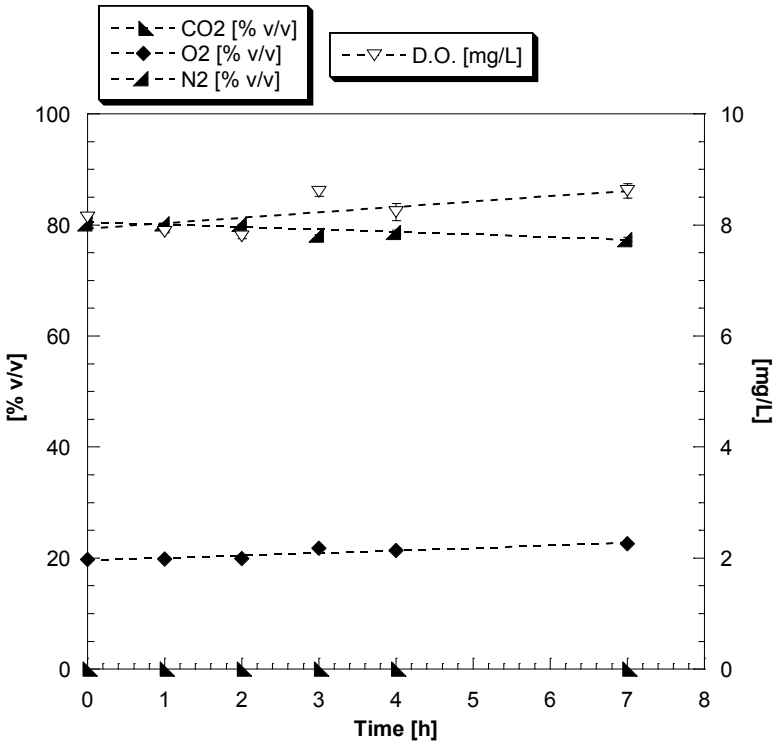

Fig. 3.53: Gas phase and dissolved oxygen (D.O.) values in **MP100+G+YE+Ac Chla**

Subsequent tests were conducted to evaluate the behavior of *Lipomyces starkeyi* in synthetic medium with an organic substrate not only represented by glucose (10 g/L), but also by acetic acid with an initial concentration of 1.6 g/L (**MP100+G+YE+Ac**). Figure 3.54 summarizes the profiles of substrates and microbial biomass. It was observed a trend with an exponential growth rate until the third day and, after, a linear trend that was not strictly related to glucose consumption profile. Acetate was not used during the fermentation. This results suggested the possibility to diversify carbon source metabolism. *Chlamydomonas* and *Lipomyces starkeyi* could be used to metabolize acetate and glucose, respectively.

In Figure 3.55, Cells/mL and microbial biomass have been reported. The maximum value in terms of Cells/mL was $2.5 \cdot 10^7$ that was comparable to the result in **MP100+G+YE** without acetate, where it was $2.6 \cdot 10^7$ Cells/mL. It confirmed that acetate dind't contribute to increase cell proliferation or microbial biomass that was almost the same if compared to the culture with only glucose as substrate (**MP100+G+YE Lip**) [Figure 3.38].

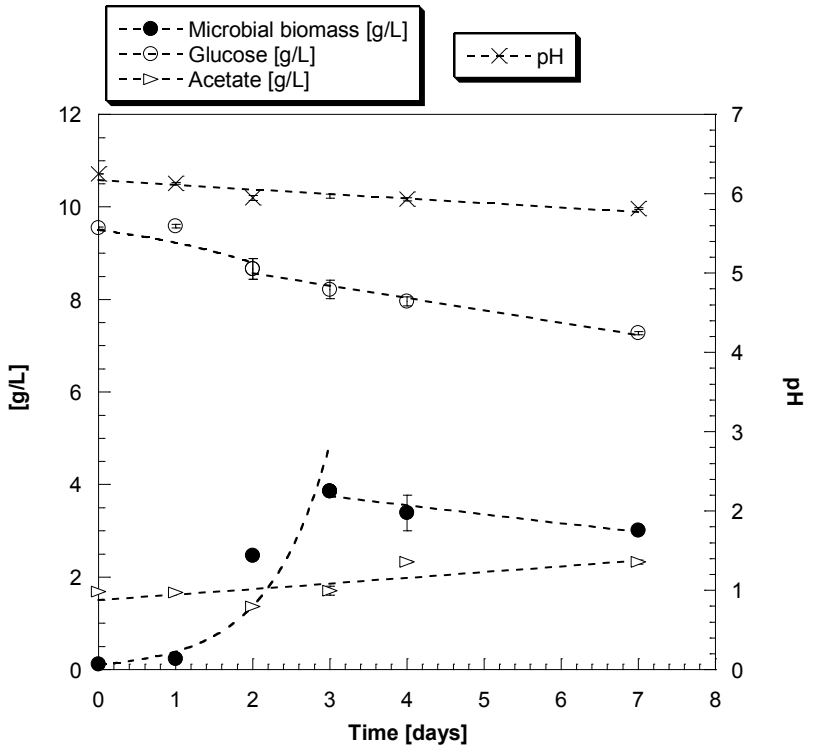

Fig. 3.54: Concentration and pH profiles in **MP100+G+YE+Ac Lip**

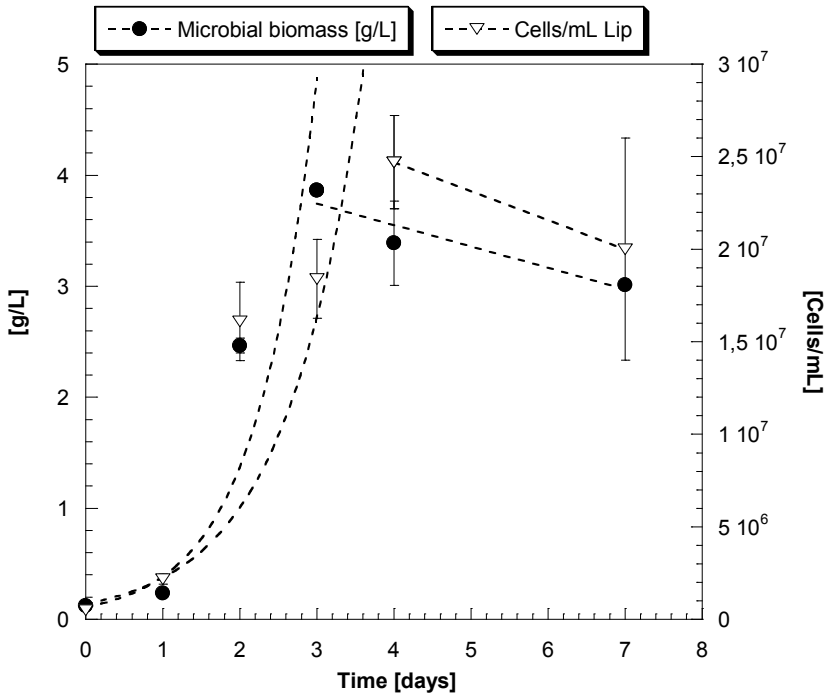

Fig. 3.55: Microbial biomass and cell/mL values in **MP100+G+YE+Ac Lip**

Figure 3.56 shows D.O. value calculated according to Equation 3.14. It decreased from 8.5 to 2 mg/L. The trends of dissolved oxygen (D.O.) and O_2 concentration in liquid and gas phase were compared to microbial biomass profile [Figure 3.54]. After three day, microbial biomass growth stopped due to a low oxygen concentration from 20% to 6.4% v/v. In the same time CO_2 concentration increased until 16% v/v. This results demonstated that, after three days, a different metabolic pathway was occurred due to a partial anaerobic conditions.

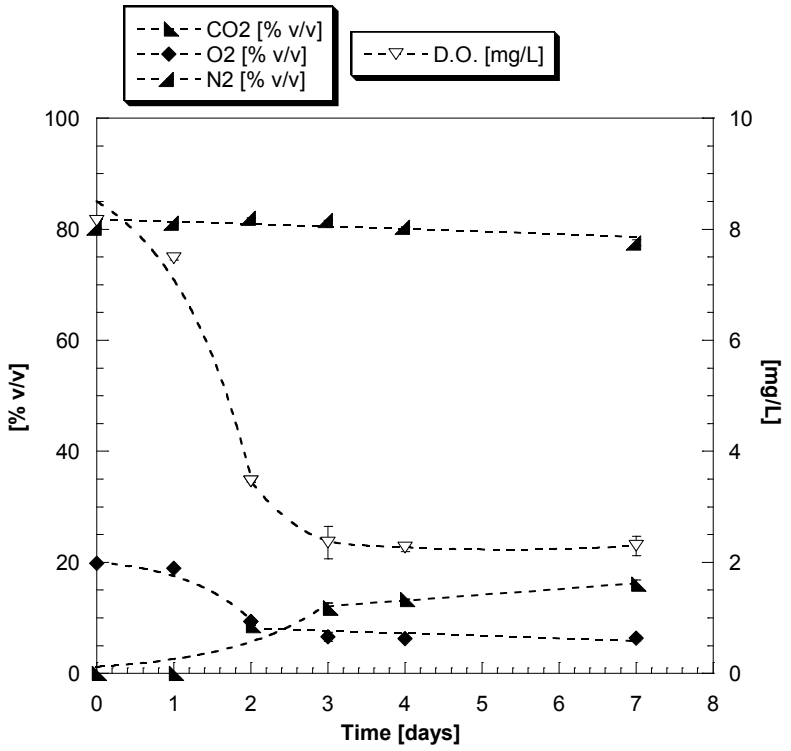

Fig. 3.56: Gas phase and dissolved oxygen (D.O.) values in **MP100+G+YE+Ac Lip**

Before starting next experimental tests for microalga-yeast mixed cultures, a preliminary phase was carried out with synthetic culture media containing two organic substrates, such as glucose and acetic acid, as previous tests, just discussed, with different inoculum ratio of *Chlamydomonas* (**Chla**) and *Lipomyces starkeyi* (**Lip**) equal to 1:1, 2:1 and 3:1. Figures 3.57 and 3.58 report higher cell proliferation of *Chlamydomonas* in mixed cultures (**Chla:Lip 1:1, 2:1, 3:1**) if compared to the culture using only *Chlamydomonas* (**Chla**) as inoculum. Cells/mL of *Chlamydomonas* were $9.67 \cdot 10^6$ in **MP100+G+YE+Ac Chla:Lip 1:1**, $8.33 \cdot 10^6$ in **MP100+G+YE+Ac Chla:Lip 2:1**, $1.50 \cdot 10^7$ in **MP100+G+YE+Ac Chla:Lip 3:1** and $2.80 \cdot 10^6$ in **MP100+G+YE+Ac Chla**, respectively. Figure 3.59 shows the values of lipid content, defined as g lipid/g of treated dry cell weight multiplied by 100 (% w/w), for each sample in single and mixed culture. The results confirmed the presence of synergistic effect of mixed cultures and a slight increase in lipid content from Chla:Lip 1:1 to Chla:Lip 3:1, already observed during cell proliferation monitoring. The ratio **Chla:Lip 1:1** was chosen for the next tests in mixed culture to compare the results with the previous tests in absence of acetate as additional carbon source.

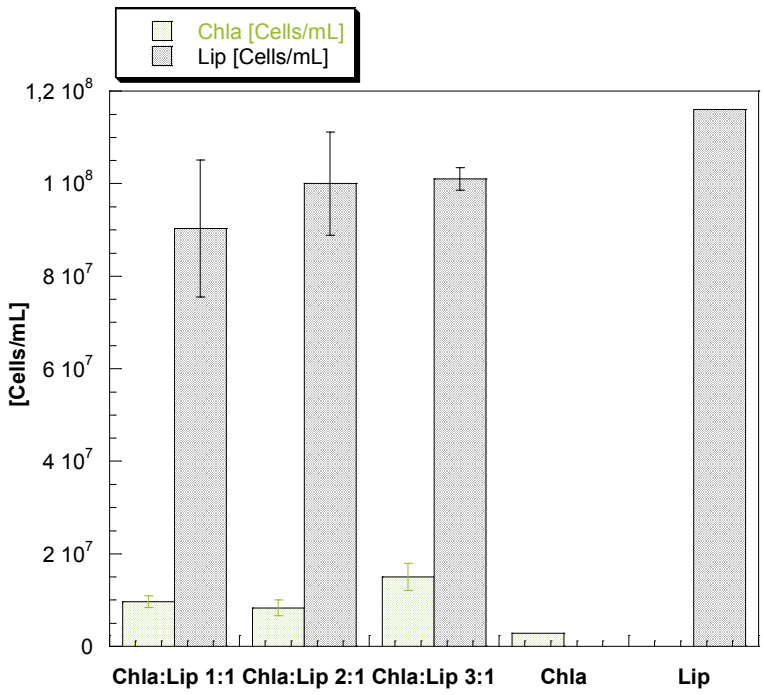

Fig. 3.57: Cell proliferation (Cells/mL) in **MP100+G+YE+Ac**

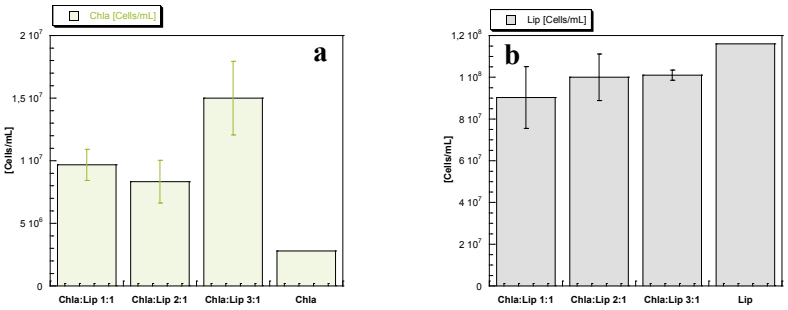

Fig. 3.58 a, b: Cell proliferation (Cells/mL) in **MP100+G+YE+Ac**
(Chla:Lip and Chla, **a**; Chla:Lip and Lip, **b**)

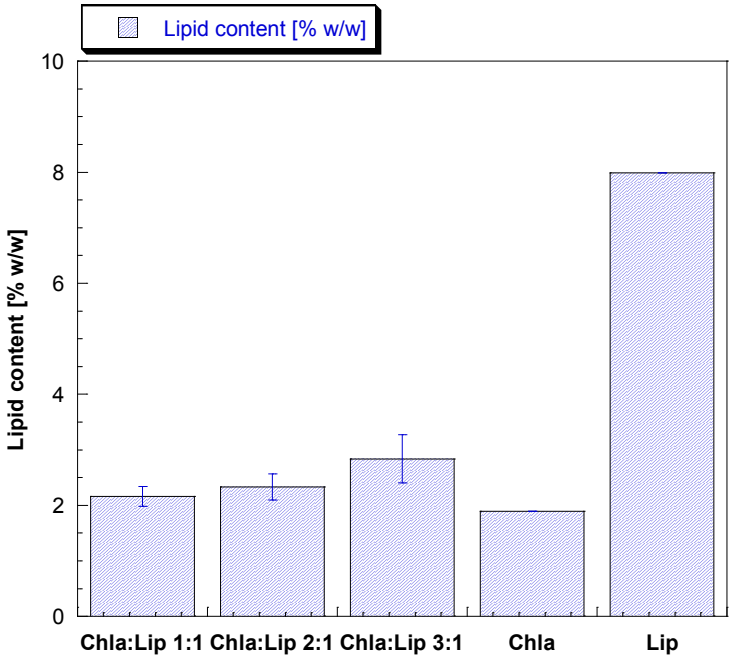

Fig. 3.59: Lipid content (% w/w) in **MP100+G+YE+Ac**

An extra test was performed on medium, removing the air in head space. The samples were marked as **MP100+G+YE+Ac Chla:Lip 1:1** and **MP100+G+YE+Ac Chla:Lip 1:1 N_2**, respectively. Figure 3.60 describes the dynamic model associated to the conversion of organic substrates and associated to the metabolic activity of both microorganisms in mixed cultures. Glucose and acetate were metabolized, as already described, respectively by *Lipomyces starkeyi* and by *Chlamydomonas*. Firstly, glucose was consumed for the growth and cell proliferation and subsequently for maintenance reaction, as it was possible to understand, observing the microbial biomass profile. At the end of the test, ethanol (0.329 g/L) was produced. Probably, this results was related to the reduction of dissolved oxygen [Figure 3.62] and the additional glucose was metabolized to pyruvate after which it is discarded in the form of ethanol. Alternatively, in respiration, pyruvate is completely metabolized to CO_2, which generates an additional ATP.

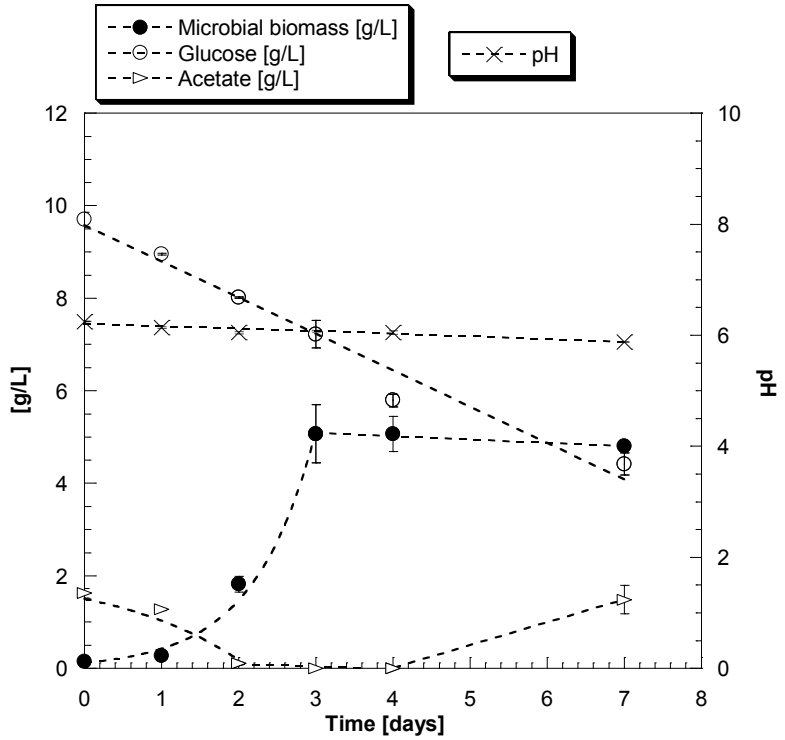

Fig. 3.60: Concentration and pH profiles in **MP100+G+YE+Ac Chla:Lip 1:1**

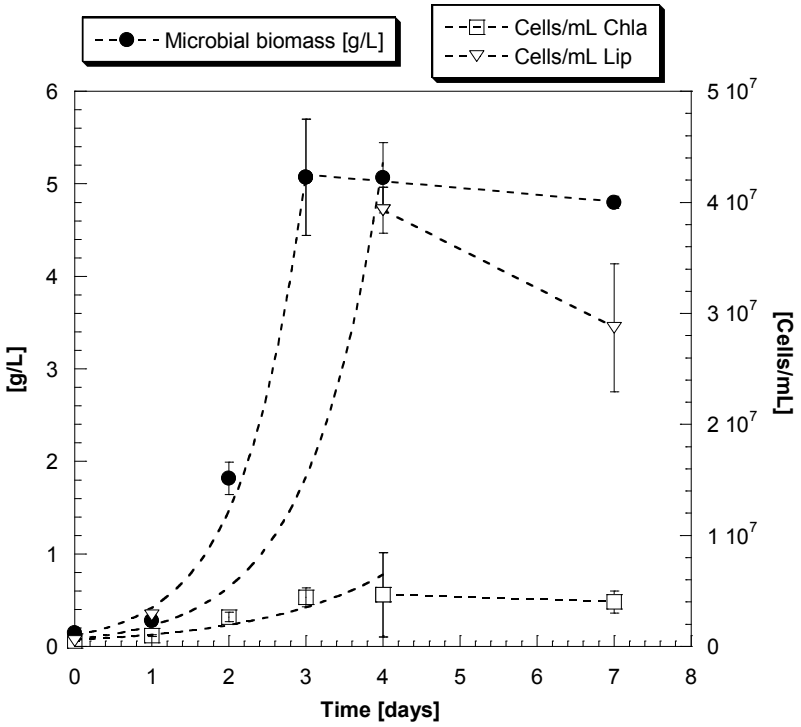

Fig. 3.61: Microbial biomass and cell/mL values in **MP100+G+YE+Ac Chla:Lip 1:1**

Figure 3.62 shows that during yeast and microalga growth, CO_2 concentration increased until 22% v/v at the end of the test. This result confirmed that yeast grew faster than microalga, that was not completely inhibited by yeast growth.

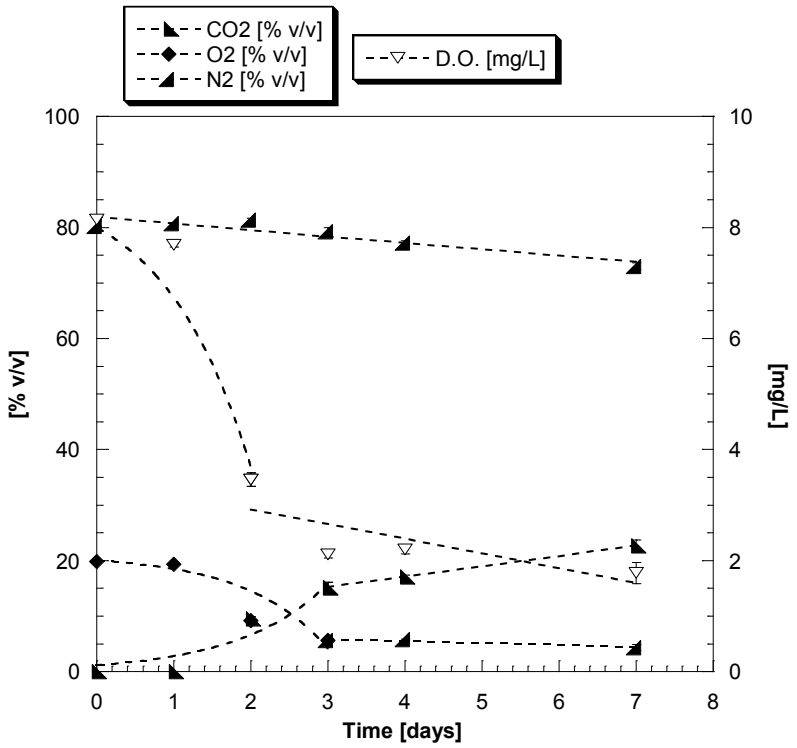

Fig. 3.62: Gas phase and dissolved oxygen (D.O.) values in **MP100+G+YE+Ac Chla:Lip 1:1**

The next experiment was performed to confirm mutualism effect in yeast-microalga mixed culture (**MP100+G+YE+Ac Chla:Lip 1:1 N_2**). Operating conditions already used for the previous test were used also in the next one, plus air removal in order to evaluate gas exchange and substrate conversion.

Figure 3.63 shows microbial biomass growth, equal to 5.32 g/L at the end of the test. Glucose and acetate were metabolized, probably by *Lipomyces starkeyi* and *Chlamydomonas*, respectively. Part of glucose not metabolized by *Lipomyces starkeyi*, was used for the maintenance metabolism when the change of profile from exponential to linear was observed. The same trends were observed also in Figure 3.64, where cell proliferation in terms of Cells/mL for *Lipomyces starkeyi* and *Chlamydomonas* grew following the metabolic pathway, already described.

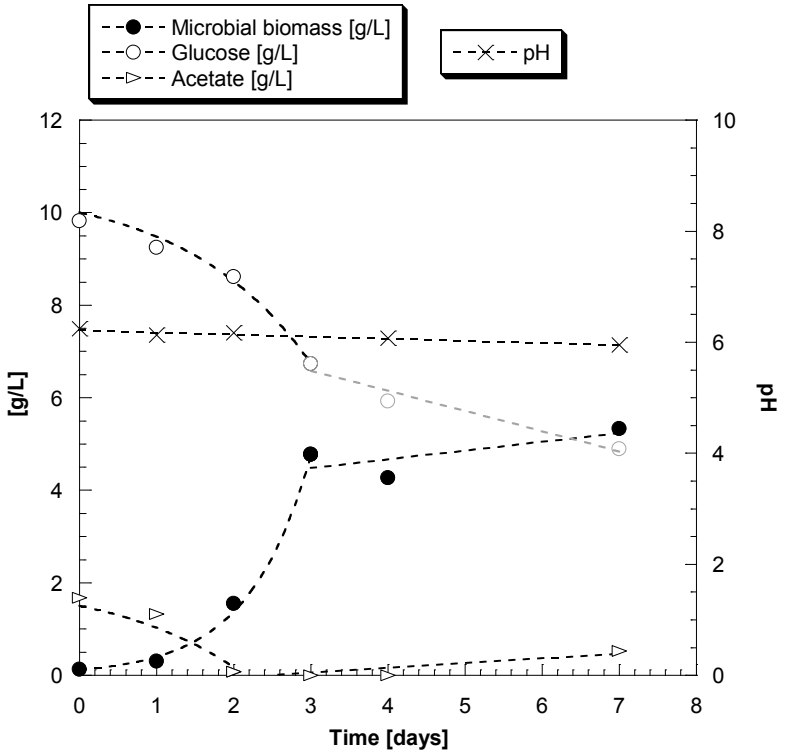

Fig. 3.63: Concentration and pH profiles in **MP100+G+YE+Ac Chla:Lip 1:1 N_2**

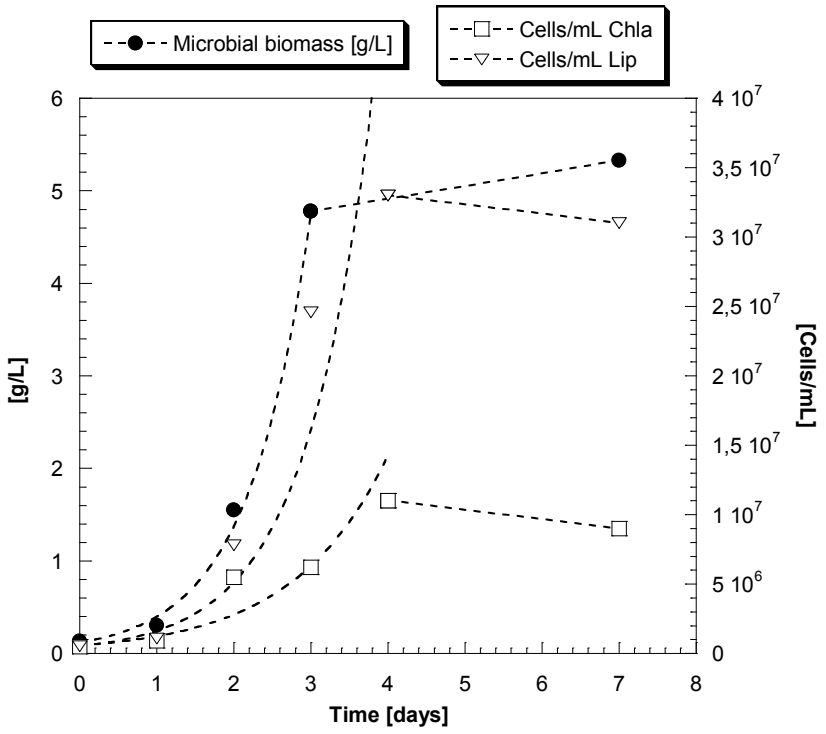

Fig. 3.64: Microbial biomass and cell/mL values in **MP100+G+YE+Ac Chla:Lip 1:1 N_2**

Figure 3.65 reports the value of gas concentrations (CO_2, O_2, N_2) in % v/v. It was observed CO_2 increase from 0.04% to 7.67% v/v, while the value of O_2 was almost constant. The absence of CO_2 and O_2 in gas phase (in the initial stage) and the observed microbial growth [Figure 3.63], suggested the presence of synergistic effect of yeast and microalga strains used for the mixed cultures.

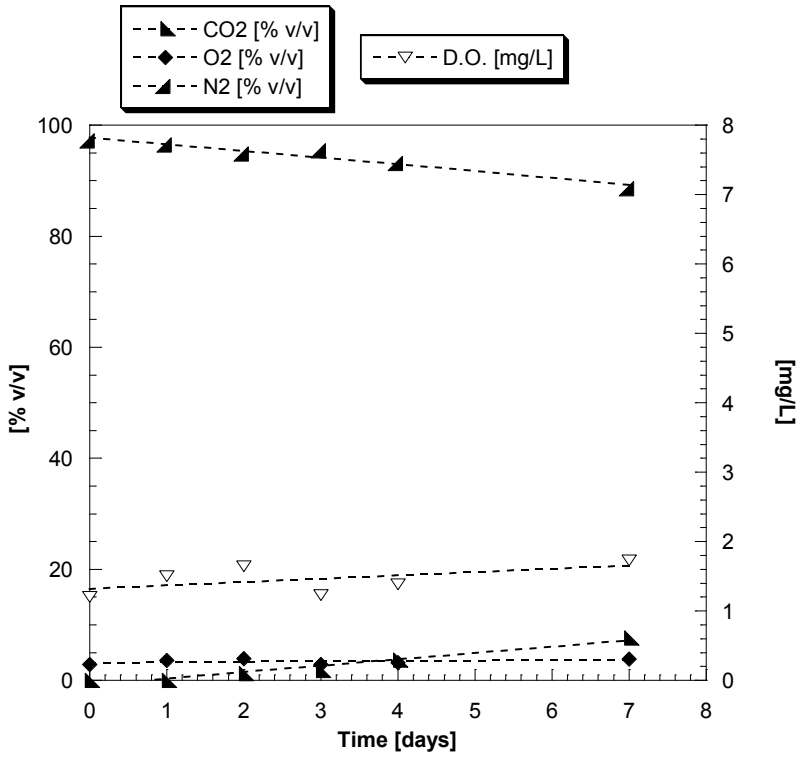

Fig. 3.65: Gas phase and dissolved oxygen (D.O.) values in **MP100+G+YE+Ac Chla:Lip 1:1 N_2**

The presence of a mutualism increased lipid productivity (ν_{lipid}) and lipid yield (Y_{lipid}) [Table 3.12]. The latter values reported in Table 3.12 were slightly lower than observed in Table 3.11 for MP100+G+YE media, expect for **MP100+G+YE Chla**, that confirmed the inability of *Chlamydomonas* to grow on glucose. The presence of an additional organic substrate positively influenced specific growth rate (μ_x), but, probably, part of organic substrate was able to favorite different metabolic pathways not directly linked to the lipid accumulation.

Tab. 3.12: Kinetic parameters related to **MP100+G+YE+Ac**

	μ_x [d^{-1}]	μ_s [d^{-1}]	mx_o [$g\ g^{-1}\ d^{-1}$]	$Y_{x/s}$ [$g\ g^{-1}$]	Y_{lipid} [$g\ g^{-1}$]	v_{lipid} [$g\ L^{-1}\ d^{-1}$]
MP100+G+YE+Ac Chla	1.049	0.062	0.124	0.388	0.068	0.019
MP100+G+YE+Ac Lip	1.271	0.352	0.228	1.003	0.036	0.025
MP100+G+YE+Ac Chla+Lip 1:1	1.252	0.785	0.457	0.799	0.083	0.029
MP100+G+YE+Ac Chla+Lip 1:1 N_2	1.241	0.764	0.346	0.961	0.089	0.047

Figure 3.66 reports lipid content in **MP100+G+YE+Ac** media. The trends showed a decrease in the final side of the graph that was related to the observed cell proliferation of *Lipomyces starkeyi* in single and mixed cultures and *Chlamydomonas* only in mixed cultures [Figures 3.53, 3.61, 3.64]. Different behaviour was shown by *Chlamydomonas* in single culture, where an increase in terms of cell proliferation was also observed in the final part of the cultivation time [Figure 3.52], but the lipid content didn't reflected the same trend [Figure 3.66].

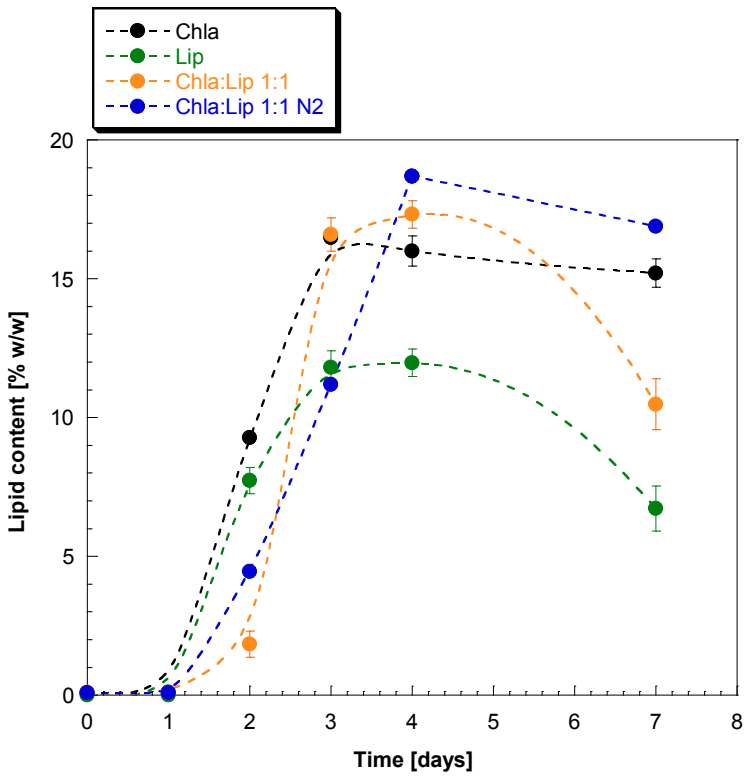

Fig. 3.66: Lipid content in **MP100+G+YE+Ac**

Figure 3.67 illustrates fatty acid distribution. The mass percentage of palmitic acid (C16:0) was almost the same for all samples, about 35% w/w. **MP100+G+YE+Ac Lip** showed a higher concentration in C18:1, almost 42% w/w, than the other samples that confirmed those reported in scientific literature (*Wild et al., 2010*). In **MP100+G+YE+Ac Chla**, **Chla:Lip 1:1** and **Chla:Lip 1:1 N_2** was observed a high value in terms of saturated fatty acids (C14:0, C16:0 and C18:0) that grants an high stability to oxidative degradation.

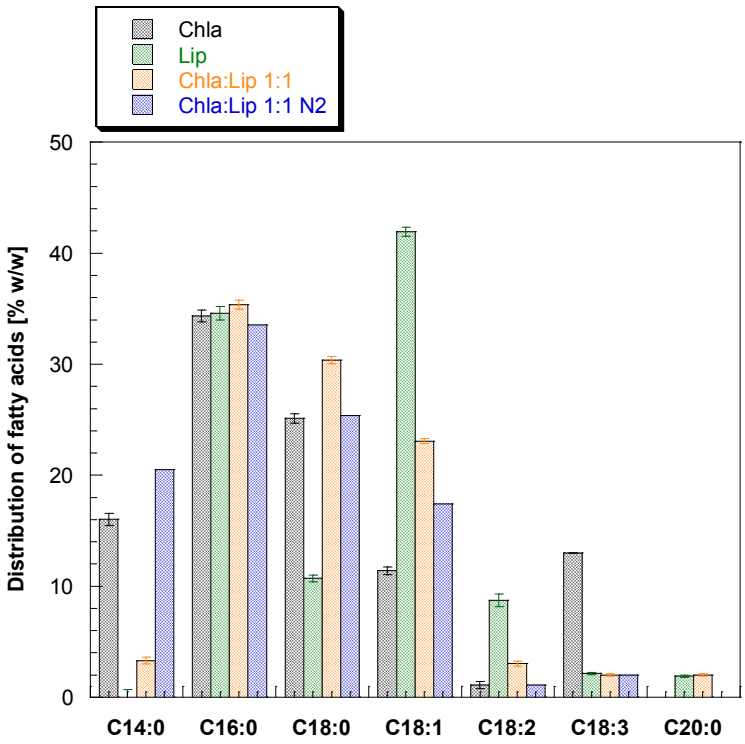

Fig. 3.67: Distribution of fatty acids in **MP100+G+YE+Ac**

The experimental activity just discussed and focused on the evaluation of the correct process parameters to achieve *Chlamydomonas* and *Lipomyces starkeyi* mixed cultures gave promising results.

The choice of the two strains was positive for the ability to set up mutualism phenomena and, simultaneously, for the ability of co-metabolize organic substrates, which represented an important achievement in view of subsequent applications. The next step involved the use of wheat straw which was previously subjected to pretreatment processes able to hydrolyze lignocellulosic fraction to obtain fermentable sugars but also fractions of volatile organic acids [Chapter 2, § 2.2].

The samples, were marked as:

- **WSAH Chla:Lip 1:1** (Wheat Straw subjected to Acid Hydrolysis, using *Chlamydomonas*:*Lipomyces starkeyi* ratio of 1:1);
- **WSAELH Chla:Lip 1:1** (Wheat Straw subjected to Acid Hydrolysis plus Enzymatic hydrolysis on the Liquid fraction, using *Chlamydomonas*:*Lipomyces starkeyi* ratio of 1:1);
- **WSAESH Chla:Lip 1:1** (Wheat Straw subjected to Acid Hydrolysis plus Enzymatic hydrolysis on the Solid fraction, using *Chlamydomonas*:*Lipomyces starkeyi* ratio of 1:1).

Figures 3.68 a, b, c show the concentration profiles in terms of microbial biomass, glucose and acetate concentration in **WSAH Chla:Lip 1:1** with three different dilution factors (**dil 0**, **2**, **4**). The aim was to assess, as the dilution affected concentration reduction of inhibitory compounds such as phenols and furans (data not shown), but at the same time, the loss of glucose. In all the samples, it was not observed any form of metabolic activity since all values were almost constant.

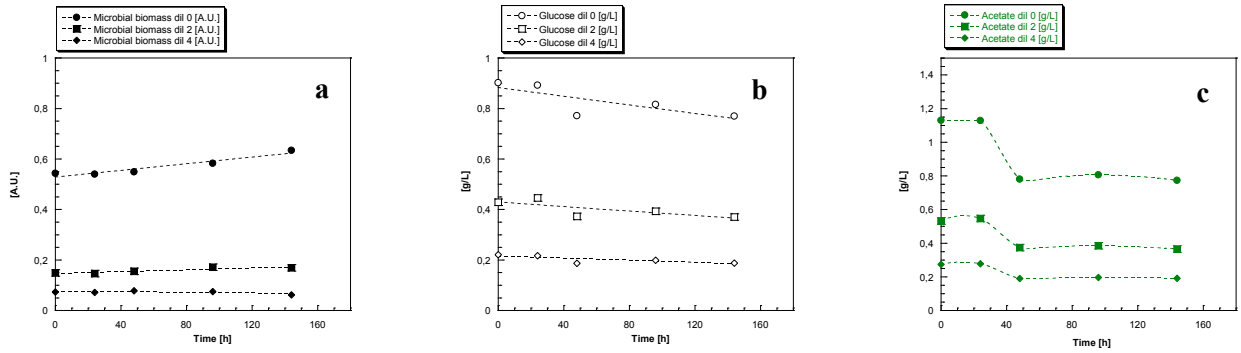

Fig. 3.68 a, b, c: Concentration profiles in **WSAH Chla:Lip 1:1 dil 0, 2, 4**
(**a** = microbial biomass dil 0, 2, 4; **b** = glucose dil 0, 2, 4; **c** = acetate dil 0, 2, 4)

The next step was performed to evaluate the behavior of *Chlamydomonas* and *Lipomyces starkeyi* in a culture medium represented by an acid hydrolysate, then subjected to enzymatic hydrolysis of its liquid fraction. The chosen pH for the tests was the same of synthetic samples already described and discussed (pH 6.25). Even in this case, dilution factors, equal to 0, 2 and 4, were chosen to mediate the decline in terms of fermentable sugars with the reduction of inhibitory compounds and the turbidity of the culture media able to decrease the growth phase of the microalga.

Figures 3.69 a, b, c show exponential growth for microbial biomass (a) related to the samples with dilution factor equal to 2 and 4. The same exponential trends were observed for glucose (b) and acetate (c) consumptions. Even for these samples, the dilution factor 0 inhibited

microorganism growth, probably due to furan concentration (data not shown). In the other samples with dilution 2 and 4, the increase of lag phase was related to inhibitory compounds.

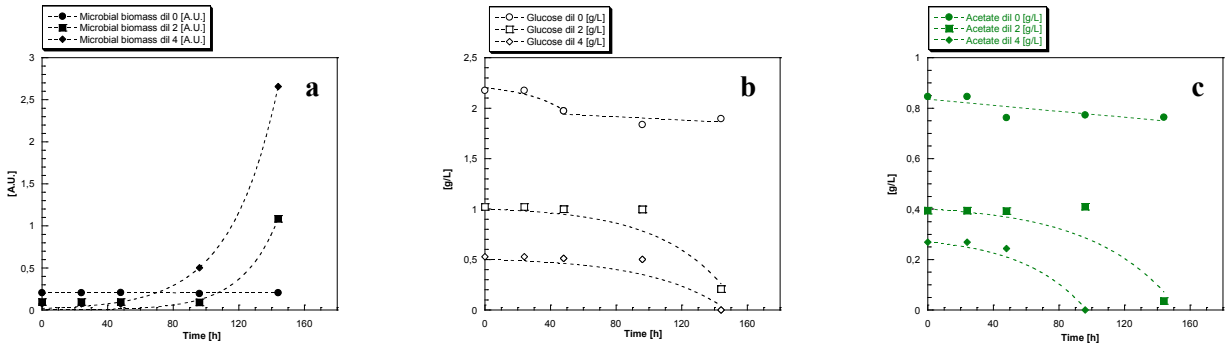

Fig. 3.69 a, b, c: Concentration profiles in **WSAELH Chla:Lip 1:1 dil 0, 2, 4**
(**a** = microbial biomass dil 0, 2, 4; **b** = glucose dil 0, 2, 4; **c** = acetate dil 0, 2, 4)

Figure 3.70 reports the values of Cells/mL for *Chlamydomonas* and *Lipomyces starkeyi* in mixed cultures with different dilution factor. These results confirmed that in **WSAELH Chla:Lip 1:1 dil 0**, yeast and microalga didn't grow. In **WSAELH Chla:Lip 1:1 dil 2** and **4**, an increase of lag phase was observed and related to inhibitory compounds, as above discussed. The distribution of fatty acids for **WSAELH Chla:Lip 1:1 dil 2** and **4** was evaluated [Figure 3.71]. The main compounds were oleic acid (C18:1) and palmitic acid (C16:0). High cell proliferation of *Lipomyce starkeyi* in the sample with dilution 4, probably, induced the increase in oleic acid.

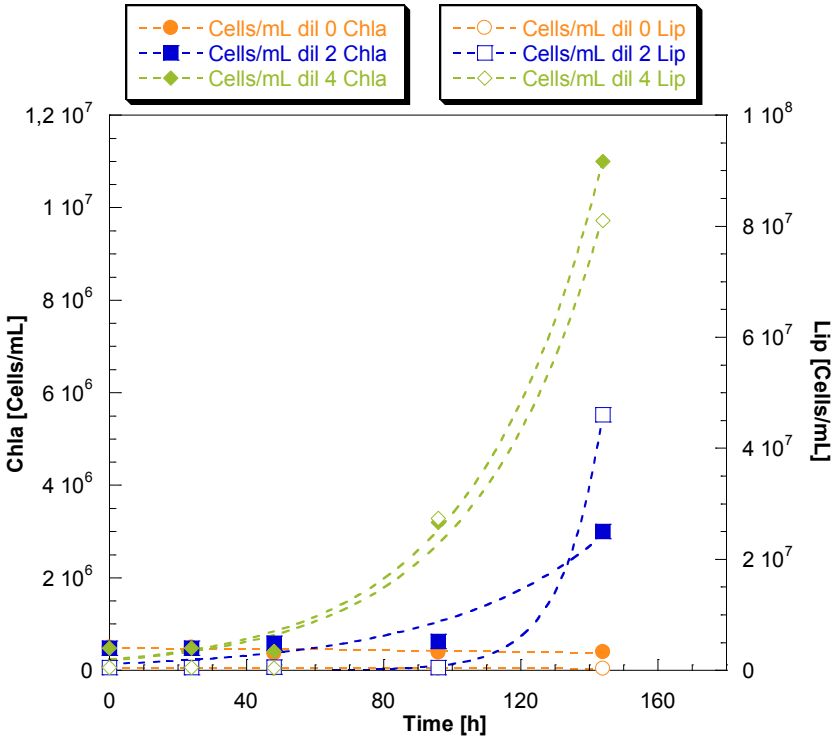

Fig. 3.70: Cell/mL values in **WSAELH Chla:Lip 1:1 dil 0, 2, 4**

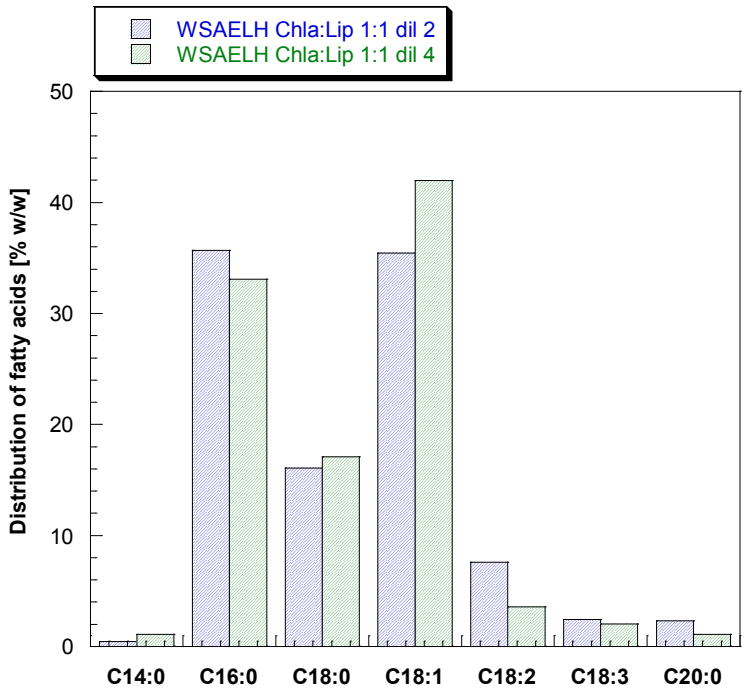

Fig. 3.71: Distribution of fatty acids in **WSAELH Chla:Lip 1:1 dil 2, 4**

The next step was carried out to compare the results just discussed with the results in **WSAESH Chla:Lip 1:1 dil 0, 2, 4** [Figures 3.72 a, b, c]. In these samples, the same increase of lag phase was observed, as shown in **WSAELH Chla:Lip 1:1 dil 2** and **dil 4**, and the absence of metabolic activity for **dil 0** [Figures 3.69 a, b, c]. The increase in terms of microbial biomass compared to the previous tests (**WSAELH Chla:Lip 1:1 dil 0, 2, 4**) was found and it was directly related to the initial glucose concentration equal to 4 and 2 g/L for dil 2 and dil 4, respectively; higher than 1 and 0.5 g/L for dilution factor equal to 2 and 4 in **WSAELH Chla:Lip 1:1**. The increase of microbial biomass was not attributed to acetate that was lower than observed in **WSAELH Chla:Lip 1:1 dil 0, 2, 4** [Figures 3.69 a, b, c].

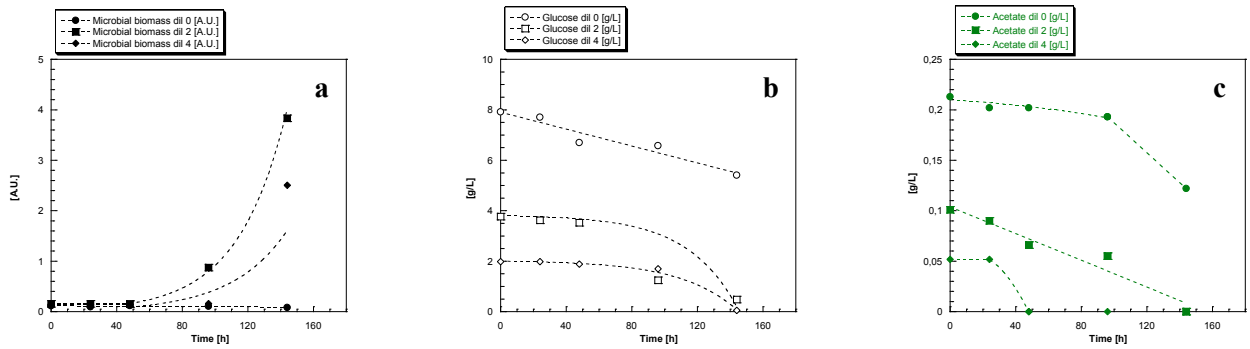

Fig. 3.72 a, b, c: Concentration profiles in **WSAESH Chla:Lip 1:1 dil 0, 2, 4**
(**a** = microbial biomass dil 0, 2, 4; **b** = glucose dil 0, 2, 4; **c** = acetate dil 0, 2, 4)

Figure 3.73 shows that *Lipomyces starkeyi* and *Chlamydomonas* grew exploiting synergistic effects, in fact, a cell proliferation of yeast and microalga was observed in mixed cultures with dil 2 and 4, while with dil 0, it was not found. In **WSAESH Chla:Lip 1:1 dil 2**, Cells/mL of *Chlamydomonas* (**Chla**) and *Lipomyces starkeyi* (**Lip**) were $5.00 \cdot 10^6$ and $1.16 \cdot 10^8$, respectively. Figure 3.74 reports the distribution of fatty acids, that confirmed as above discussed about the correlation between the increase in oleic acid (C18:1) and cell proliferation of *Lipomyces starkeyi* (*Wild et al., 2010*; *Calvey et al., 2016*).

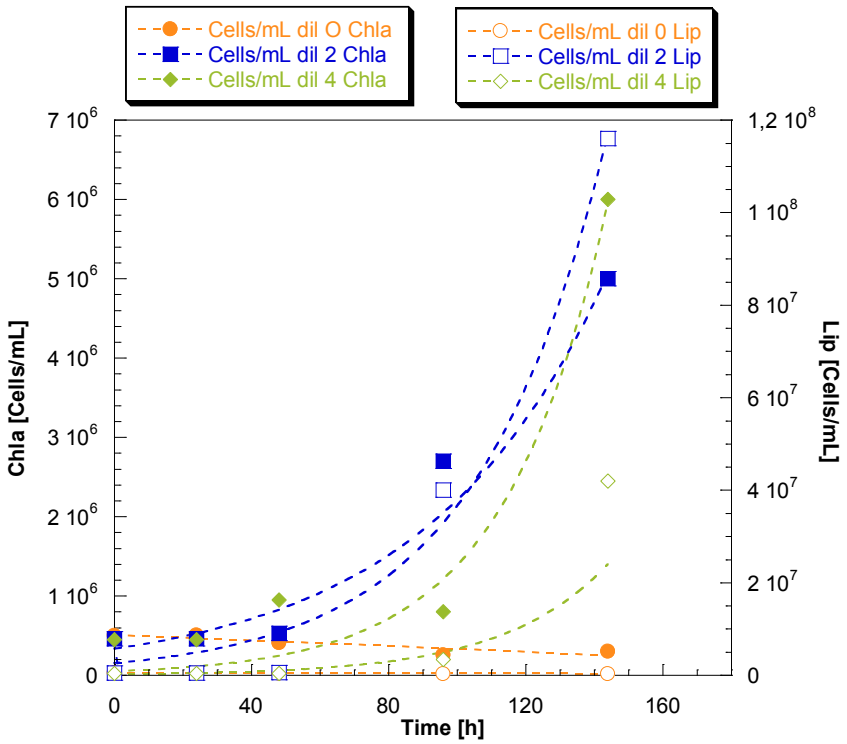

Fig. 3.73: Cell/mL values in **WSAESH Chla:Lip 1:1 dil 0, 2, 4**

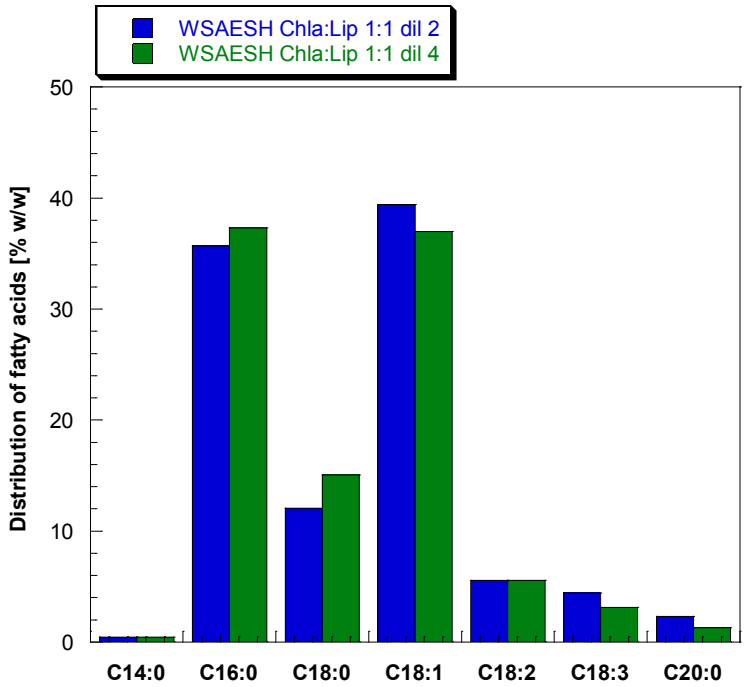

Fig. 3.74: Distribution of fatty acids in **WSAESH Chla:Lip 1:1 dil 2, 4**

The results of tests carried out on wheat straw hydrolysates showed the cell proliferation and metabolic activities of both microorganisms, in **WSAELH dil 2, 4 Chla:Lip 1:1** and in **WSAESH dil 2, 4 Chla:Lip 1:1**. However, metabolic activity wasn't observed in all samples with dilution factor equal to 0 and in case of acid hydrolysis (**WSAH dil 0, 2, 4 Chla:Lip 1:1**). The reason was associated to the presence of high concentration of inhibitory compounds related to the selected pretreatment.

The difference in terms of organic substrate concentrations in **WSAELH Chla:Lip 1:1 dil 2, 4** and in **WSAESH Chla:Lip 1:1 dil 2, 4** was observed [Figures 3.69, 3.72]. *Lipomyces starkeyi* was not able to metabolize acetic acid, whose conversion was ensured by *Chlamydomonas* sp. Because acetic acid has a pKa of 4.75, at acid pH, acetate appears largely in undissociated form, which imposes inhibitory effects on cell proliferation. Acetate exists largely in dissociated form at neutral pH, which is less toxic (*Gong et al., 2016*). The co-metabolism is of great interest to solve inhibition problems due to volatile organic acids which are formed during the pretreatments and it should be valuable towards conversion of acetate lignocellulosic biomass materials into Single Cell Oils. These results confirmed the presence of synergistic effect of mixed culture using wheat straw hydrolysate as low cost feedstock and they were validate also from the results shown in Figures 3.71 and 3.74 in terms of distribution of fatty acids. Further study should extend what has been studied in this research project, investigating new technology for Single Cell Oil production exploiting different low cost organic substrates, e.g., urban or industrial wastes.

3.4 Combined yeast and microalga consortium cultivation in pilot scale raceway pond for urban wastewater treatment and potential Single Cell Oil production

To test if urban wastewater can be used as solely feedstock for yeast-microalga culture, pre-cultured *Lipomyces starkeyi* and microalgal consortium were inoculated into primary wastewater without any nutrient supplementation. Non-sterile culture experiments were conducted (**UWRP Lip:m.c.**) [Chapter 2, § 2.5].

Figure 3.75 shows the increase in terms of microbial biomass, that was related to cell proliferation of microalga consortium, used as inoculum, and bacteria, mould and fungus colonies present in the starting substrate and isolated on Petri dish using selective media in collaboration with the Dipartimento di Medicina Sperimentale, Seconda Università di Napoli [Figures 3.77, 3.78]. The oleaginous yeast, *Lipomyces starkeyi*, did not show a significant metabolic activity except for the first day. The absence of yeast growth was attributed to the low concentration of easily assimilated organic substrates, such as fermentable sugars (data not shown). TOC (Total Organic Carbon) was equal to 48.32 mg/L at initial time. The value decreased up to 15 mg/L in the first two days and then stabilized at 25 mg/L. The consumable carbon source in the effluent was limited, namely not all of TOC could be consumed by yeast and microalgae. The exponential growth of the microbial biomass and specific growth rate equal to 0.101 d^{-1}, calculated according to Equations 3.8 and 3.9. Exponential growth, related to microalga proliferation, was halted after the end of test, except for initial lag phase. It was observed a cell proliferation of microalgal consortium until a value of $1.00 \cdot 10^7$ Cells/mL [Figure 3.75], mainly represented by *Scenedesmus* sp. [Figure 3.76], but surprisingly also the stop of bacterial, fungus and mould growth [Figure 3.78]. Probably, it was related to the stop of carbon substrate conversion measured in terms of TOC and to the high pH value equal to 10.9 at the end of the test run (data not shown). This result was very interesting due to the bactericidal and antifungal functions probably associated to the proliferation of microalgal consortium able to perform this task.

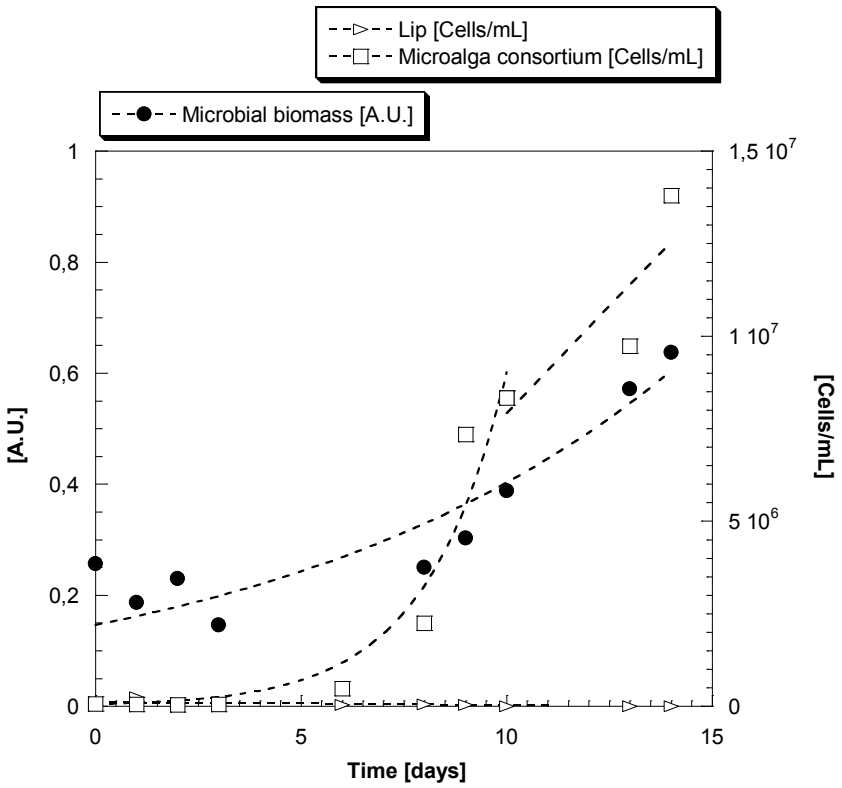

Fig. 3.75: Microbial biomass, pH and cell proliferation in **UWRP Lip:m.c.**

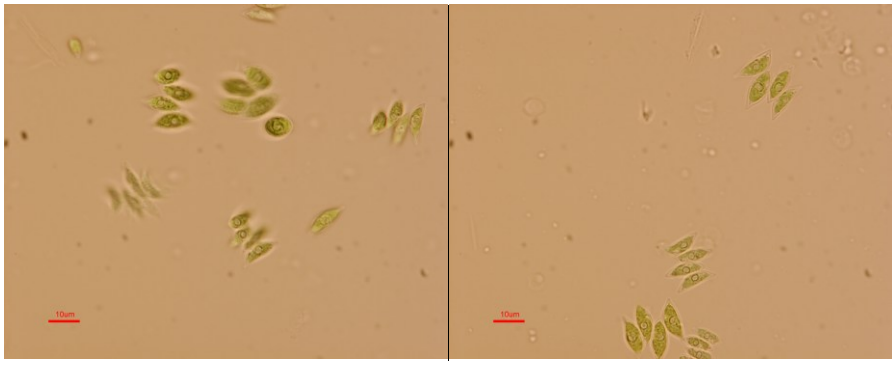

Fig. 3.76: Cells in **UWRP Lip:m.c.**

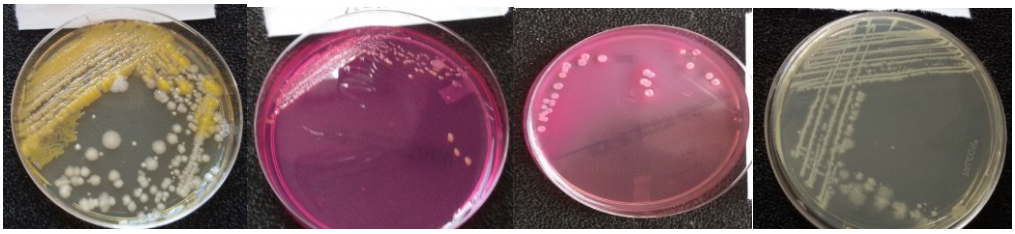

Fig. 3.77: Selective media to bacterial, mould and fungus evaluation in **UWRP Lip:m.c.**
(Time = 0 day)

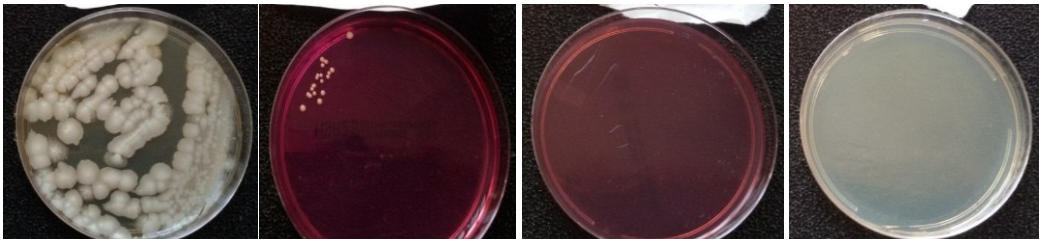

Fig. 3.78: Selective media to bacterial, mould and fungus evaluation evaluation in **UWRP Lip:m.c.**
(Time = 14th day)

Figure 3.79 shows lipid accumulation profile measured in terms of percentage in gram of lipids divided by gram of treated dry cell weight. The final value was 14% that was related mainly to microalgal growth. Only in the first part of test, lipid accumulation was related to oleaginous yeast. Probably, the low lipid content in this study could be caused by the low C/N ratio in urban wastewater, which was 17. Generally, oleaginous yeasts don't accumulate a high content of lipid in a medium with a C/N ratio less than 20, whereas a C/N ratio from 40 to 80 is preferred for lipid accumulation in most oleaginous microorganisms. The low C/N ratio in urban wastewater may be improved by supplementation with an extra carbon source. For example, crude glycerol or biomass hydrolysate contains no nitrogen, or very low nitrogen, and is thus good candidate to be used as supplemental carbon sources for this culture process.

The fatty acid composition of lipid extracted from mixed yeast/microalga culture was determined using GC. The data showed that the main fatty acids were long-chain fatty acids with 20 and 16 carbon atoms including palmitic acid (3%), and arachidic acid (97%).

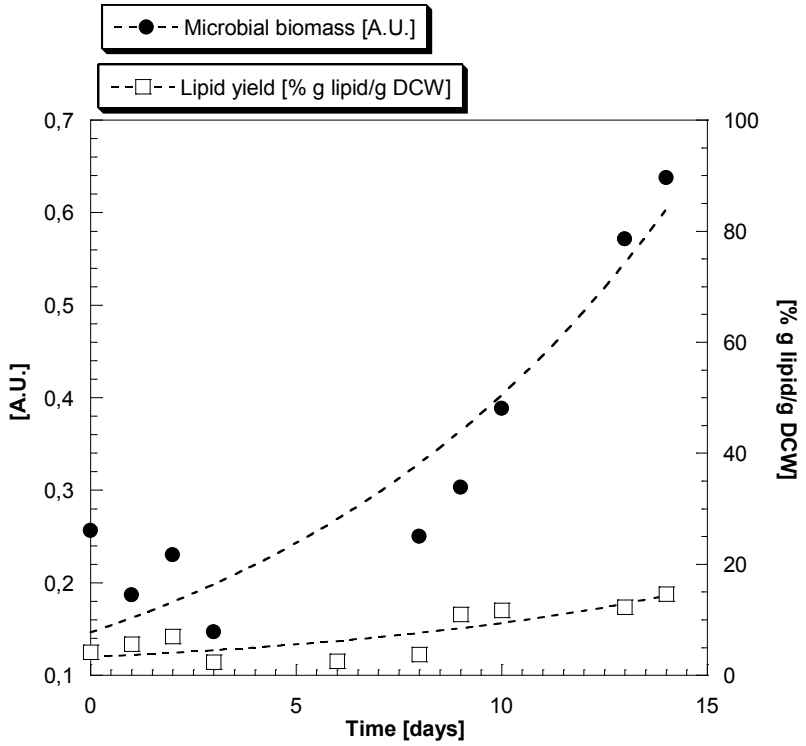

Fig. 3.79: Microbial biomass and lipid content in **UWRP Lip:m.c.**

Yeast-microalgal consortium mixed culture was monitored to evaluate also to the residue COD, nitrogen, and phosphorus in the effluent stream.

Cheirsilp et al. (*2011*) verified the improvement of COD and nitrate removal of industrial wastes by *Rhodotorula glutinis* and *Chlorella vulgaris* mixed cultures. It was found higher than that by the pure culture of yeast and microalga. The results showed that this process removed most residue nitrogen and phosphorus from the fermentation effluent [Figure 3.80] and that oleaginous yeast and algae biomass were produced at the same time, but converting wastewater into Single Cell Oils has to face two major challenges. First, the oleaginous microbial strains that can grow in wastewater environment have to be further screened. Second, the nutrients in the organic waste have to be completely removed during the cell culture processes. The wastewater stream from mixed culture process still contained very high residue carbon source concentrations, in terms of TOC (25 mg/L) and chemical oxygen demand COD, equal to 91 mg/L, which requires further treatment. Thus, a process is to be developed to completely remove the nutrients in food waste and wastewater, to convert them into microbial biomass and Single Cell Oils.

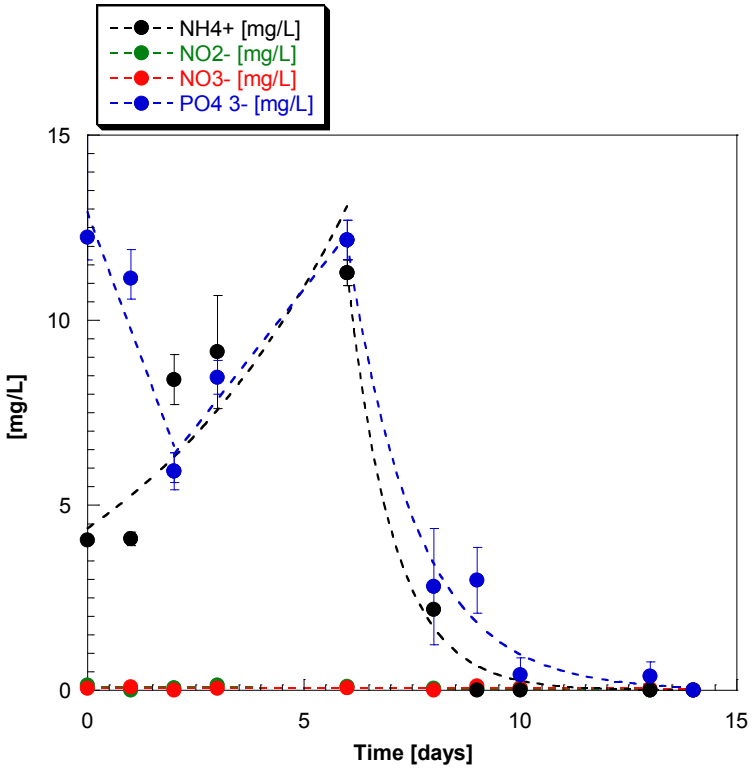

Fig. 3.80: Nitrogen and phosphorus concentrations in **UWRP Lip:m.c.**

4. SUMMARY AND CONCLUSIONS

Undoubtedly, the market for Single Cell Oils (SCOs) is attractive due to its important bi-function as a supplier of functional oils and feedstock for biodiesel production. However, high costs related to fermentation and hydrolysis processes should be mastered by specialists of SCOs for their industrialisation. Therefore, various low-cost, hydrophilic and hydrophobic substrates were used for SCO production. Of these substrates, lignocellulosic biomass, which is the most available and renewable source in nature, might be an ideal raw material for SCO production. Although many reviews on SCOs have been published, few have focused on SCO production from low-cost substrates or evaluated the possibility and potential of its industrialisation.

The purpose of this doctoral research was to evaluate the important steps for an integrated approach for efficient SCO production.

Optimization of operating parameters in the enzymatic hydrolysis of lignocellulosic biomass was performed. Experimental activity was focused on evaluation of synergistic effect of enzyme mix (cellulase from *Trichoderma reesei* and cellobiase from *Aspergillus niger*) to obtain fermentable sugars and the fair compromise between process condition of hydrolysis and fermentation in order to setting up the transition from hydrolysis and fermentation in two reactors to a single reactor process to reduce capital costs.

A simple kinetic model was also tested with the aim to justify experimental values and the presence of inhibition phenomena by products related to hydrolytic enzyme use.

Exploiting the optimum process conditions for the enzymatic hydrolysis, the subsequent experimental activity has concerned the identification of a choice able to replace classic separate hydrolysis and fermentation in two reactors with a choice able to reduce the costs but, at the same time, to balance the enzymatic hydrolysis process standards with those of oleaginous fermentation. In this regards, tests were performed on an enzymatic hydrolysis and oleaginous fermentation in a single reactor.

The experimental data have shown the ability of oleaginous microorganisms to grow and to accumulate lipids using a very complex culture medium composition, such as the hydrolysate of *Arundo donax*.

The results showed a remarkable ability in both processes SHF (Separate Hydrolysis and Fermentation) or, more hopefully, in SRF (enzymatic hydrolysis and oleaginous Fermentation in a Single Reactor), that represent the discovered innovative element, in terms of lipid yield (Y_{lipid}).

Finally, oleaginous yeast-microalga mixed cultures were explored. Utilizing a mixed culture of microalgae and yeasts could be a strategy to improve lipid production. In theory, microalgae could produce oxygen for yeasts while yeasts could provide carbon dioxide for microalgae.

In order to demonstrate the synergistic effect of the mixed cultures, different strains and different process parameters were investigated. Tests were performed on lignocellulosic hydrolysates and civil wastewater. In the first case, the aim was to verify the capacity to metabolize organic substrates, avoiding inhibition phenomena of both microoganisms. Promising results were achieved using *Chlamydomonas* sp., more than *Chlorella emersonii* in mixed cultures with *Lipomyces starkeyi*. In the latter case, very few studies have been done to produce Single Cell Oils from high strength real non-sterile wastewater without the addition of other nutrients for the mixed culture of yeast and microalga. The aim was to find a strategy to improve both Single Cell Oil production as well as to investigate the associated removal efficiency for organic matters (COD) and nutrients (nitrogen and phosphorus) present in wastewater under non-sterile conditions. Yeasts, in fact, mainly uptake organic matters and microalgae essentially require nitrogen and phosphorus from wastewater.

Single Cell Oil production from lignocellulosic biomass offers a new direction for bio-refinery approach, and it will have a great future if the above mentioned problems will be handled properly. In fact, Single Cell Oils represent intermediates for biodiesel production, polymers, biosurfactants and the control of unsaturation degree in their chain, for example by hydrogenation, could be critical to ensure selectivity and stability. The exploitation of Single Cell Oils for other purposes was studied by Simopoulos (*2008*) and Bharathiraja et al. (*2017*) and related to the possibility of ensuring mono or polyunsaturation, known as EFAs, essential fatty acids of ω-6 and ω-9 families; chemical mediators that, for example, allow to fight the emergence of diseases such as atherosclerosis and PUFAs as components of thrombocytes, neuronal and muscle cells, cerebral cortex as well as the immunocompetent cells.

A step toward better profitability might be the realization of high cell densities achieved in short time, product purity and the development of an universal and cost-efficient method for SCO recovery.

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