Conversion of Agro-industrial Wastes into Lipids Suitable for Biodiesel Production

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Abstract

Microorganisms that can accumulate lipids at more than 20% of their dry mass are defined as oleaginous species. The majority of these lipids are triacylglycerol containing long-chain fatty acids, which are comparable to conventional vegetable oils. The recent, increasing interest towards the oleaginous microorganisms is due to the potential use of microbial triglycerides as feedstock for biodiesel production.

The oleaginous yeasts used in this thesis work appear to be very promising, due to their versatility, as they allow the use of different kinds of residues as nutrients. In particular, *Lipomyces starkeyi* is so far one of the best used, as it has been proved to store large amounts of lipids.

Lipomyces starkeyi were first grown in the presence of olive oil mill wastewaters (OMW), a medium difficult to process by biological treatments, due to the antimicrobial activities of their phenolic components. We demonstrated that *Lipomyces* can produce, without external organic supplements, a significant reduction of both the total organic carbon (TOC) and the total phenols content, leading to a significant increase of the germination index. The fatty acid distribution showed a prevalence of oleic acid, demonstrating the potential of *L. starkeyi* as a source of lipids to be used as a feedstock for the synthesis of II generation biodiesel. The performance of *Lipomyces was* improved by a preliminary dilution of OMW.

Lipomyces were able to grow also in the presence of wastewaters from cheese factory, leading to a satisfactory growth and to a significant reduction of the TOC levels.

Cellulosic agricultural residues were also evaluated as feedstock for oleaginous yeasts. *Lipomyces starkeyi* were first grown in the hydrolysate of tomato wastes, containing mainly peel

and seeds, at different nitrogen contents. The yeasts showed a favorable growth, with no need of addition of external nutrients.

Hydrolysates of Sorghum and Giant Reed were also studied as nutrients for the *Lipomyces starkeyi*. The conditions to maximize the lipid yield and the efficiency of the biomass conversion were found in terms of H_2SO_4 concentration (for the preliminary hydrolysis) and of medium composition (for the yeasts growth). Detoxification of hydrolysate with overlime and activated charcoal was carried out to reduce the concentration of microbial growth inhibitors, improving the growth of the yeasts in the undiluted hydrolysate.

In conclusion, the potential of oleaginous yeasts was demonstrated by the satisfactory microbial growth in the presence of different waste materials, and by the favorable composition of the triglycerides. Further studies are ongoing to optimize the preliminary hydrolysis of lignocellulosic materials and the lipid fraction of the yeasts.

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List of Abbreviations

BOD	Biological oxygen demand
C	Carbon
C/N	Carbon/Nitrogen
CFU	Colony forming Unit
COD	Chemical oxygen demand
dwb	Dry weight basis
et al.	et al.ii (and others)
EU	European Union
g	Gram
GC	Gas chromatography
GI	Germination index
GRS	Giant reed stem
hr	Hour
HMF	Hydroxymethylfurfural
IC	Inorganic carbon
IMF	International Monetary Fund
l./lt	Liter
M	Molar
mg	Milligram
min	Minute
ml	Milliliter

N	Nitrogen
NA	Not available
nm	Nanometer
OD	Optical density
OMW	Olive mill waste water
rpm	Rotation per minute
SCO	Single Cell Oil
SGM	Sorghum
T	Temperature
TC	Total carbon
TG	Triglycerides
TOC	Total organic carbon
TWH	Tomato waste hydrolysate
µg	Micro gram

CHAPTER-I: INTRODUCTION

1.1 Biorefineries

1.1.1 Biorefinery concept

The biorefinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass, using a variety of different technologies concept. Consequently, the concept of biorefinery, now widely accepted, is analogous to that of petroleum refinery (see Figure 1.1), except that it makes use of renewable plant-derived materials (derived from photosynthesizing plants), whereas an oil refinery uses non-renewable fossil-derived petroleum.

The technologies based upon the concept of biorefinery can provide a wide range of bio-based products that include: bio-fuels (bioethanol and biomethane), bio-materials (fibers, pulp for paper manufacture), and a host of bio-chemicals through downstream fermentation and refining processes, therefore maximizing the value derived from the biomass feedstock.

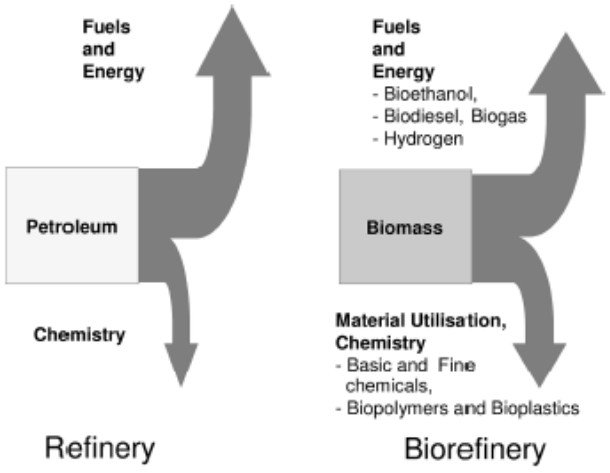

Figure 1.1 Comparison of the basic-principles of the petroleum refinery and the biorefinery

Among the several definition of biorefinery, the most exhaustive was recently performed by the IEA Bioenergy Task 42 ‘‘Biorefineries” (IEA, 2007): ‘‘Biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy”.

1.1.2 Development of Biorefineries as an alternative to Petroleum refineries

There are at least three distinct advantages of a biorefinery using renewable feedstocks for production of bioenergy, biofuels and biochemicals, compared to chemical refining of petrochemical feedstocks: **energy security, climate change and rural development** (Cherubini, 2010).

Plant biomass (agricultural and forestry residues) constitute a **renewable** resource, whereas crude fossil oil has a limited and finite supply, that could run dry during this century depending upon its increasing consumption as an energy source. Plant biomass will therefore increase our **energy security**, reducing the dependency on crude oil (non-renewable).

It should also reduce waste streams and minimize pollution, assisting against **climate change** by reducing the amount of products of fossil fuel combustion released to the atmosphere (Cherubini, 2010; IPCC, 2007).

In addition, a Biorefinery-based economy will promote the **rural development**, creating new businesses that will lead to new jobs, and generate wealth for the agro-based countries.

If compared to petroleum, biomass generally has too little hydrogen, too much oxygen, and a lower fraction of carbon. The compositional variety in biomass feedstocks is both an advantage and a disadvantage. An advantage is that biorefineries can make more classes of products that can petroleum refineries and can rely on a wider range of raw materials. A disadvantage is that a relatively larger range of processing technologies is needed, and most of these technologies are still at a pre-commercial stage, though they are being rapidly developed.

1.1.3 Biorefineries versus alternative energies

While the energy production can be based on various alternative resources, such as wind, sun, water, biomass, as well as nuclear fission and fusion, the economy of substances is fundamentally depending on biomass, in particular biomass from plants.

It can be said that “the development of biorefineries represents the key for the access to an integrated production of food, feed, chemicals, materials, goods, and fuels of the future” (National Research Council U.S.A. 1, 2).

1.1.4 Non-food agriculture

The recent evolution of the concept of Biorefinery has been also affected by the concept of "non-food agriculture". In a world hungry for food (and fuel), it is a questionable practice to displace a foodstock resource (e.g., starch, sugar, seed oils) derived from agricultural crops such as cereals (wheat, rice), maize (corn), sugarcane, soybean, oil palm, vegetables (potato, cassava), and fruits (dates) for the production of energy and chemicals.

Instead, a non-food material such as **cellulose** (the most abundant organic chemical on Earth) offers an alternative feedstock from which different products can be derived: chemicals (bio-based products) and biofuels. The industrial utilization of raw materials from agriculture, forestry and green landscape care for the energetic, biotechnological and chemical industry is still in the beginnings. Currently, only 6 billion tons of the annual produced biomass (1.7 -2.0 10^{11} tons), are used, and only 3.0 to 3.5 percent of this amount is used in the non-food area, such as chemistry (Zoebelin, 2001).

1.1.5 Biorefining as a new science

Biorefining is still largely unexplored territory and presents many research and business opportunities for the production of bio-based products from agricultural and forest residues. Currently, petrol-chemistry is based on the principle to generate from hydrocarbons simply to handle and well defined chemically pure elements in refineries. In efficient product lines, a system based on family trees has been built, in which basic chemicals, intermediate products and sophisticated products are produced.

This principle of petroleum refineries must be transferred to Biorefineries. Biomass contains the synthesis performance of the nature and has another C:H:O:N-ratio than petroleum. The process of biomass genesis should be modified to adapt the biomass produced to the purpose of the subsequent processing and particular target products already have been formed.

Plant biomass always consists of the basic products carbohydrates, lignin, proteins and fats, beside various substances such as vitamins, dyes, flavors, aromatic essences of most different chemical structure. Biorefineries combine the essential technologies between biological raw materials and the industrial intermediates and final products.

A technically feasible separation operation, which would allow a separate use or subsequent processing of all these basic compounds, exists up to now only in form of an initial attempt. Assuming that out of the estimated annual production of biomass by biosynthesis of 170 billion tons 75 percent are carbohydrates, mainly in form of cellulose, starch and saccharose, 20 percent lignin and only 5 percent other nature compounds such as fats (oils), proteins and various substances, the main attention firstly should be focused on an efficient **access to carbohydrates,**

their subsequent conversion to chemical bulk products and corresponding final products. Glucose, accessible by microbial or chemical methods from starch, sugar or cellulose, predestined for a key position as basic chemical, because a broad palette of biotechnological or chemical products is accessible from Glucose.

In the case of **starch** the advantage of enzymatic compared to chemical hydrolysis is today already realized.

In the case of **cellulose** this is not yet realized. Cellulose-hydrolyzing enzymes can only act effectively after pre-treatment to break up the very stable lignin/cellulose/hemicellulose composites. These treatments are still mostly thermal, thermo-mechanical or thermo-chemical and require a considerable input of energy. The arsenal for microbial conversion of substances out of glucose is large, the reactions are energetically profitable. It is necessary to combine the degradation processes via glucose to bulk chemicals with the building processes to their subsequent products and materials (Kamm, 2006).

Biobased products are prepared for an economic use by a reasonable combination of different methods and processes (physical, chemical, biological and thermal). Consequently, Biorefining is an interdisciplinary science involving the interaction between Biology (microbiology), Chemistry (biochemistry), Botany (forestry), Engineering (bio/chemical & process), and Mathematics (physics). It is therefore much like Biotechnology, and in this respect applies the principles of the basic sciences and is heavily reliant upon Enzyme and Fermentation Technology. Thus it appears to be reasonable to refer to the term “biorefinery design” that means bringing together smart scientific and technologic basics with practical technologies, products and product lines inside the biorefineries.

Special attention must be given to the combination of biotechnological and chemical substance-converting and the required energy input for the conversion. The basic conversions of each biorefinery can be summarized as follows:

Currently four complex biorefinery systems are forced in research and development:

(1) the 'Green Biorefineries' using ‘nature-wet’ biomasses such as green grass, alfalfa, clover, or immature cereal (Kromus et al., 2006).

(2) the 'Lignocellulosic Feedstock Biorefinery' using 'nature-dry' raw material such as cellulose-containing biomass and wastes (Koutinas et al., 2006).

(3) the 'Whole Crop Biorefinery' uses raw material such as cereals or maize (Kamm et al., 2006).

(4) the ‘Biorefinery Two Platforms Concept’ includes the sugar platform and the syngas platform (Werpy et al., 2004).

1.2 Biofuels

From the 19th century, technological visionaries dreamed that internal-combustion engines would run on plant-based fuels. Subsequently, both Henry Ford and Rudolf Diesel supported the notion. Unfortunately, the interest towards this subject was reduced owing to the diffusion of oil-based fuels. In the last years, the strategic and economic matters associated to the oil economy have promoted new interest for the so-called biofuels, which include bioethanol, biodiesel and biogas.

In principle, biofuels offer a huge advantage over fossil fuels. The source plants absorb carbon dioxide from the air as they are growing, and consequently, the carbon dioxide that is released when biofuels are burned does not represent a net addition of that greenhouse gas to the atmosphere (Naik et al. 2010; Cherubini, 2010).

Though, a disadvantage associated with biofuels is a lower energy density than diesel and petrol. More than a liter of biodiesel or bioethanol is necessary to substitute a liter of diesel or petrol. However, both biofuels are also reported to have higher combustion efficiency, which partially makes up for the lower energy density (International Energy Agency- IEA, 2004).

Furthermore, there is considerable attention on the environmental impacts from biofuels. In terms of tailpipe emissions, both biodiesel and bioethanol are generally considered to be less polluting than petrol and diesel (Biofuels Research Advisory Council-BIOFRAC, 2006). The well to wheels (WTW) greenhouse gas balance of biofuels is also attracting interest. A range of studies indicate that it depends on the way feedstocks are produced, processed into biofuels, and distributed (IEA, 2004).

The **First generation biofuels** are produced from raw materials in competition with food and feed industries, such as ethanol made from corn or sugar cane, or biodiesel made from vegetable oil.

Because of this competition, these biofuels give rise to ethical, political and environmental concerns (Cherubini, 2010). They have been blamed for causing unintended environmental damage and for displacing production of food crops, which may have helped raise world food prices (Sims et al., 2010; Cherubini, 2010). Biofuels are estimated by the IMF to have been responsible for 20-30% of the global food price spike in 2008 when 125 m tonnes of cereals were diverted into biofuel production. The amount of biofuels in Europe's car fuels is expected to quadruple in the next decade. Amid these attacks, the political momentum of biofuels has slowed in the last couple of years. In addition, it should be noted that some fossil fuels, especially natural gas, are consumed in refining today's biofuels, another source of controversy about them.

The production of 1st generation biofuels is commercial today, with almost 50 billion liters produced annually. There are also other niche biofuels, such as biogas which have been derived by anaerobic treatment of manure and other biomass materials. However, the volumes of biogas used for transportation are relatively small today.

In order to overcome the limitations of 1st generation biofuels, the production of **second generation biofuels** gained an increasing worldwide interest in the last few years as a possible ‘‘greener” alternative to fossil fuels and conventional biofuels. The second-generation biofuels are obtained using waste residues, or making use of land not suitable for food production, culturing crops specially grown.

Low-cost crop and forest residues, wood process wastes, and the organic fraction of municipal solid wastes can all be used as lignocellulosic feedstocks. Where these biomass materials are available, it should be possible to produce biofuels from them with virtually no additional land requirements or impacts on food and fiber crop production (Sims et al., 2010).

Contrarily to first generation biofuels, where the utilized fraction (grains and seeds), represents only a small portion of the above-ground biomass, second generation biofuels can rely on the whole plant for bioenergy production. Biofuels are considered to be the best way to reduce green house gas emissions and alternate to the pollutant fossil fuels. The main advantages offered by second-generation biofuels are (Searcy and Flynn, 2008; Fleming et al., 2006; Sims et al., 2010):

- Not directly affecting the human food chain
- Not using fertile soils (grown in places that are not suitable for agriculture)
- Enhanced efficiencies or reduction in cost
- Environmental performance

Typical examples of second-generation biofuel are those produced from the microbial fermentation, using sugars obtained from the hydrolysis of cellulosic biomass:

- bioethanol (primary metabolite synthesized during the alcoholic fermentation of yeasts)

- biodiesel from microalgae (using as feedstock the triglyceride fraction of the microalgae biomass)

- biodiesel from oleaginous microorganisms (using as feedstock the triglyceride fraction of the oleaginous yeasts or bacteria)

As a development of 2nd generation biofuel production, the use of biomass in biorefinery complexes is expected to ensure additional environmental benefits and implement national energy security, thanks to the coproduction of both bioenergy and high value chemicals.

Unfortunately, the technology to make these newer fuels is in its infancy and the claims of its advocates have yet to be proved. Innovative technologies are needed to produce biofuels in an energy efficient way, from a wider range of biomass resources and to reduce costs. The options, which will be developed, need to be sustainable in economic, environmental and social terms. This means that apart from purely economic factors, e.g. investment, operating cost, and productive capacity, other factors have to be taken into account such as the greenhouse gas and energy balances, the potential competition with food production and the impact of biomass production on the environment.

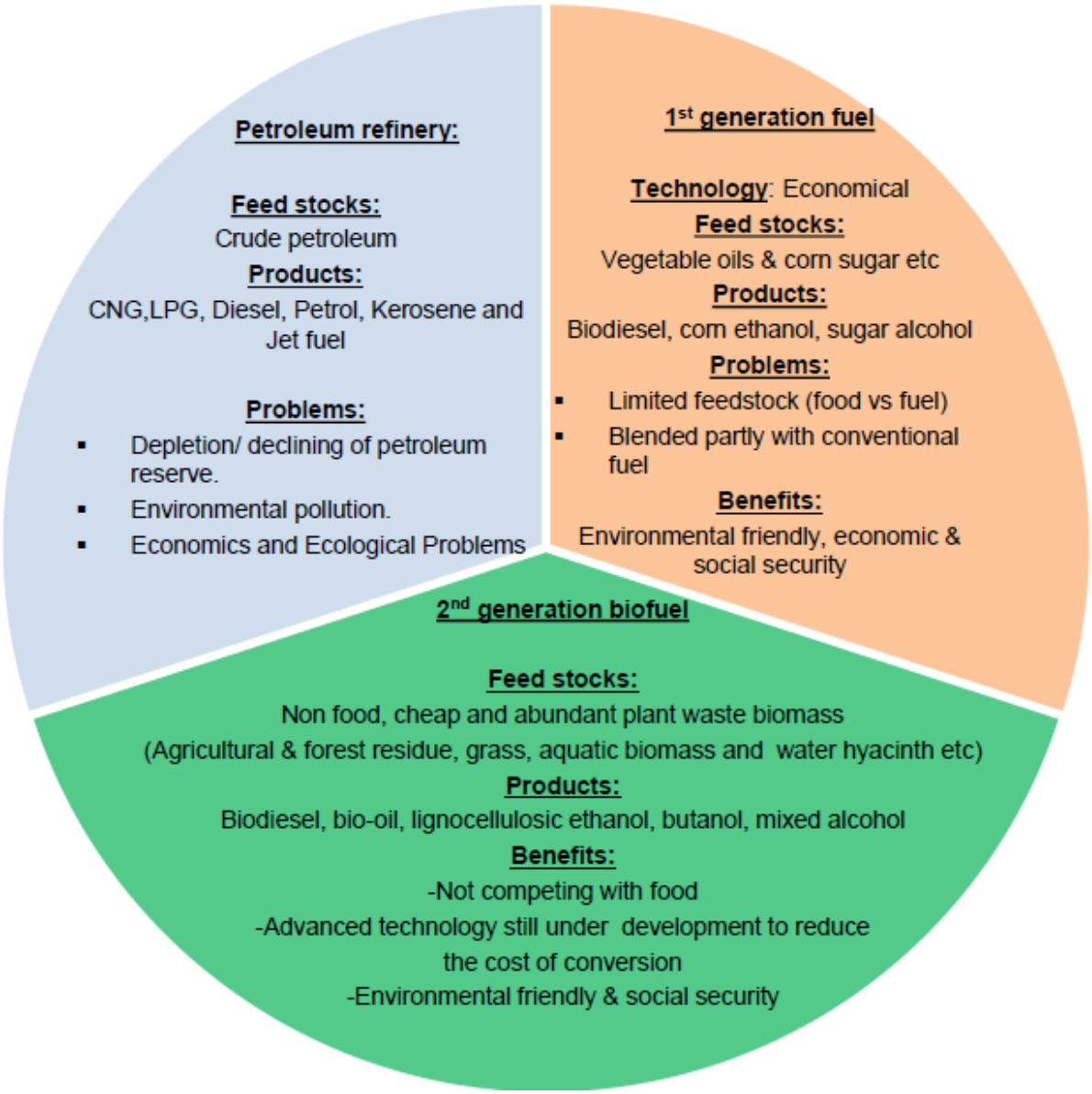

Figure 1.2 Comparison of first, second generation biofuel and petroleum fuel (Naik et al., 2010)

Currently, the only biofuels that can be supplied in considerable amounts are the first generation biofuels of bioethanol (from sugar and starch) and biodiesel (Bomb et al., 2007). Bioethanol and biodiesel have some important advantages over many alternative fuels in that they can be used in conventional vehicles. Biodiesel consists of fatty acid methyl esters (FAME) and it is agreed that it can be used in pure form or any blends in conventional diesel vehicles

with only minor engine alterations (International Energy Agency -IEA, 2004). For bioethanol, it is generally accepted that all recently produced conventional petrol vehicles are compatible with blends up to 10% bioethanol and 90% petrol or E10 (International Energy Agency -IEA, 2004). Flexi-fuel vehicles can use both bioethanol and petrol. They are often designed for blends of 85% bioethanol or E85. Furthermore, biodiesel can use the transport, storage and retail systems of diesel. Bioethanol transport faces a few difficulties. To avoid some problems it can be converted to ethyl tertiary butyl ether (ETBE) and then blended with petrol (Biofuels Research Advisory Council -BIOFRAC, 2006).

1.3 Methods for biodiesel production

1.3.1 First-generation biodiesel

Presently, first-generation biodiesel is produced using triglycerides obtained from plant oils. The majority of reported studies on this subject implemented soybean oil (Wei et ai, 2004; Samukawa et al., 2000; Kaieda et al., 1999; Noureddini et al., 2005; Watanabe et al., 2000, Watanabe et al. 2002), rapeseed oil (Georgogianni et al., 2009; Shi and Bao,2008; Yuan er al., 2008), palm oil (Salamatinia et al., 2010; Pleanjai and Gheewala 2009) and sunflower oil (Hama et al.,2004; Orcaire et al., 2006; Soumanou and Bornscheuer, 2003). Some work also has been reported on the enzymatic production of biodiesel using olive oil (Sanchez and Vasudevan, 2006), rice bran oil (Lai et al., 2005), and canola oil (Chang et al.,2005).

1.3.2 Second-generation biodiesel

Second-generation biodiesel has been so far produced by different methods:

a) **from waste oils**

As an alternative to the plant oil, waste cooking oils (Zhang et al., 2003) have been used. However, the amount of waste cooking oil is limited and cannot meet up the vast demand of increasing need of biodiesel (Zhu et al., 2008).

b) **from animal oils**

Animal fats (Tashtoush et al., 2004) can be used as a triglyceride source, though they require addition of organic solvent to be dissolved, due to the high melting points. Consequently, a solvent recovery unit must be considered in the plant design (Al-Zuhair, 2007).

c) from use of non-fertile soils

Inedible oils like Jatropha oil (Shah and Gupta, 2006) are being used as a starting material for biodiesel synthesis, as well. They are produced from plants, like Jatropha, that can be cultured on drought and sandy lands.

d) from fermentation of waste, non-lignocellulosic materials

The use of agro-industrial residues has attracted the great attention in the last years. These residual materials are used as nutritional source for microorganisms which are able to accumulate intracellular lipid with in short time.

The microorganisms so far used for this process are:

- microalgae

- oleaginous microorganisms (yeasts or bacteria), which able to produce more than 20% of their weight in the form of triacylglycerols,

Different waste materials have been so far tested (e.g., kitchen waste, tomato waste, oil mill waste water, milk-industry waste water etc.), as nutrient for microorganisms. The agro-industrial residues which are used in the recent studies to produce microbial oils are shown in the Table 1.1.

e) from fermentation of lignocellulosic materials

The same microorganisms mentioned in the previous paragraph can be cultured in the presence of sugar mixtures obtained form hydrolysis of lignocellulosic materials.

Lignocellulosic biomass is the most abundant agricultural residue in the world, mainly they are used as fuel in rural area, biofertilizer and animal feed. From the economic point of view, it's a promising strain to produce microbial oil from agro-industrial residues, especially from lignocellulosic materials (Huang et al., 2009; Dai et al., 2007).

Huang et al., (2009) explored the possibility of lipid production from sulphuric acid treated rice straw hydrolysate (SARSH) by *T. fermentans*. They also reported that amongst various agricultural crop residues, rice straw, whose hydrolysate mainly contains glucose, xylose, and arabinose, which proved that *T. fermentans* can grow well and accumulate lipid efficiently not only on glucose but also on xylose. *T. fermentans* could also grow well in pretreated waste molasses and addition of various sugars (fructose, sucrose, xylose and lactose) to the pretreated molasses could efficiently enhance the accumulation of lipid (Zhu et al., 2008).

Table 1.1 The agro-industrial residues and their fermentation process to accumulate lipid by oleaginous microorganism

Residual Materials	Fermentation process	Pretreatment method	References
Wheat straw and wheat bran	Solid-state	Steam explosion	Peng and Chen, 2008
Pitch pine	Liquid	Organosolv	Park et al., 2010
Cane molasses	Liquid	Acid hydrolysis	Zhu et al., 2008
Pear pomace	Solid-state	Without treatment	Fakas et al., 2009
Sewage sludge	Liquid	Acid hydrolysis, alkaline hydrolysis, thermal treatment and ultrasonic treatment	Angerbauer et al., 2008
Rice straw	Liquid	Acid hydrolysis	Huang et al., 2009
Wheat straw	Liquid	Organosolv	Sun and Chen, 2008
Sweet Sorghum	Semi-solid state	Without treatment	Economou et al., 2010
Tomato waste	Liquid	Acid hydrolysis	Fakas et al., 2008
Olive mill waste water	Liquid	Without treatment	Yousuf et al., 2008
Corn stalk, Tree (*populus euramevicana)* leaves and rice straw	Liquid	Acid hydrolyis	Dai et al., 2007

Costly glucose was substituted by corn stalk and *P. euramevicana* leaves hydrolytes as alternative carbon sources and encouraging results were observed for lipid production(11.78% and 28.59% respectively) at the same time poor growth of *R. glutinis* was observed in rice straw

hydrolytes (lipid content, 5.74%), presumably because the presence of inhibitory compounds in this hydrolytes (Dai et al., 2007).

A novel method was described by Peng and Chen (2008) for the production of SCO in SSF (solid-state fermentation) from steam-exploded wheat straw mixed with wheat bran, using the endophytic fungus *Microsphaeropsis sp.* which was capable of accumulating SCO and of secreting cellulase. They showed, cellulase produced by *Microsphaeropsis sp*. itself was limited, leading to low SCO yield (42 mg/gds), which could however be increased by adding cellulase to the solid-state medium, leading to a maximal SCO yield of 80 mg/gds.

1.4 Oleaginous microorganisms

Oleaginous microorganisms, which able to produce more than 20% of their weight in the form of triacylglycerols, are ever-increasing attention for several reasons (Angerbauer et al., 2008; Dai et al., 2007; Li et al., 2007; Liu et al., 2007; Papanikolaou et al., 2007). An important advantage offered by the application of the oleaginous microorganisms is to produce aerobically lipids from residual organic matters. Consequently, in order to optimize the cost of the process, as well as to increase its environmental benefit, residual materials have been tested as possible nutrients for the oleaginous microorganisms, such as nutritional residues from agriculture and industry (Xue et al., 2006; Angerbauer et al., 2008), sewage sludge (Angerbauer et al., 2008), olive-mill wastewater (Papanikolaou et al., 2008) thus lowering the cost of oils. It is remarkable that compared with the production of vegetable oils, the culture of oleaginous microorganisms is affected neither by seasons nor by climates (Zhu et al., 2008).

Microbial lipids that also known as single cell oil (SCO) was not started to commercial production until 1995 and this was lasted only for 6 years before it was closed down as no longer being cost effective (Ratledge, 2004). By this time several oleaginous yeasts and microalgaes have been reported to grow and accumulate significant amount of lipids similar to vegetable oil (Aggelis and Sourdis, 1997; Meng et al., 2009). There is no unique microorganism found to use as lipid producer from agro-industrial residue either in liquid or solid state fermentation process. Table 1.2 shows some most usable and higher efficient in terms of lipid accumulation oleaginous microorganisms.

Table 1.2 Oleaginous microorganisms with their lipid accumulation efficiency from residual materials

Strain	Lipid content (%)	Lipid yield (g/l)	Biomass (g/l)	Carbon sources	References
Lipomyces starkeyi	68.0	6.4	9.4	Sewage sludge	Angerbauer et al., 2008
Mortierella isabellina	9-11	NA*	NA	Sweet Sorghum	Economou et al., 2010
Trichosporon fermentans	40.1	11.5	28.6	Rice straw	Huang et al., 2009
Rhodotorula glutinis	11.78	2.01	17.04	Corn stalk	Dai et al., 2007
Rhodotorula glutinis	28.59	4.73	16.56	Tree leaves	Dai et al., 2007
Rhodotorula glutinis	5.74	0.21	3.58	Rice straw	Dai et al., 2007
Trichosporon fermentans	35.3	12.8	36.4	Cane Molasses	Zhu et al., 2008
Cunninghamella echinulata	1.7	NA	NA	Orange peel	Gema et al., 2002

*NA= Not available

Lipids serve as storage materials in some lipid accumulating yeasts, e.g. *Rhodotorula graminis*. Guerzoni et al. (1985) reported that yeasts can store up to 70% of lipids in dry matter. First data in lipid accumulation and the conditions for the fermentation have been reported already more than 40 years ago (Mulder et al., 1962). They also monitored that the presence of a carbon-source in excess and under nitrogen limiting conditions organisms started to store lipids. Therefore a high carbon to nitrogen (C/N)-ratio, around 100, is a basic requirement for the accumulation of lipids. Almost similar result was found by Zhu et al. (2008) when they studied with *T. fermentans.* Lipid content was quite low at the C/N molar ratio of 108, then showed a sharp increase when C/N molar ratio increased from 108 to 140, and reached the maximum of

63.1% at 140. Further rise in C/N molar ratio beyond 140 resulted in a slight drop in lipid content but a continuous increase in biomass up to 163 and the highest lipid yield of 14.8 g/l was achieved at 163.

Compared to other lipid accumulating yeasts like *C. curvata* D, *T. cutaneum* and *R. toruloides*; *L. starkeyi* seemed to store the largest quantities of lipids (Holdsworth and Ratledge, 1988) and showed only a minimal reutilization of the stored lipids (Holdsworth et al., 1988). Physical factors such as the concentration of some ions like Zn^{2+} and Mn^{2+} affected lipid accumulation and to a lesser extent Fe^{3+}, Ca^{2+}, K^{+} and NH_4^{+} (Naganuma et al., 1985a,b). Natural habitat of *L. starkeyi* is soil and ensilage (Lodder, 1970), where the organism degrades carbohydrates using extracellular carbohydrolases. Both, α-amylase and dextranase from *L. starkeyi* (Kang et al., 2004; Park et al., 2003; Lee et al., 2003a,b) and the biodegradation of triazine herbicides (Nishimura et al., 2002) have been subject of recent studies.

1.5 Objectives

This study is aimed at demonstrating that the oleaginous yeast *Lipomyces starkeyi* can be grown using different waste materials as nutrients, offering an useful method to obtain microbial oils. These microbial oils can be used as feedstock for the production of II generation biodiesel.

The waste materials considered are: olive oil mill wastewaters (OMW), cheese factory wastewaters, lignocellulosic materials (tomato wastes, *Sorghum vulgaris*, *Arundo donax*), glycerol (co-produced in the synthesis of biodiesel by triglyceride alcoholysis).

The experimental work has been aimed at evaluating the potential of these residues as sources of nutrient for the yeasts. The growth of the microorganisms has been analysed as regards both the conventional growth parameters (biomass, CFU) and the yield of lipids (almost totally triglycerides) within the biomass, being these lipids a potential feedstock for the synthesis of biodiesel (see Figure 1.3). The composition of trglycerides obtained has been taken into account, as it affects critically the properties of the biodiesel (cold behaviour, resistance to oxidation, etc.). In the course of these experiments, the degradation of the polluting components of the feedstock has been followed, mainly by TOC measurements.

The overall objective of this research was to evaluate the feasibility of the production of lipids by use of oleaginous yeasts, paying special attention to the parameters that may affect the selection of the waste materials to be used as feedstock for production of biodiesel.

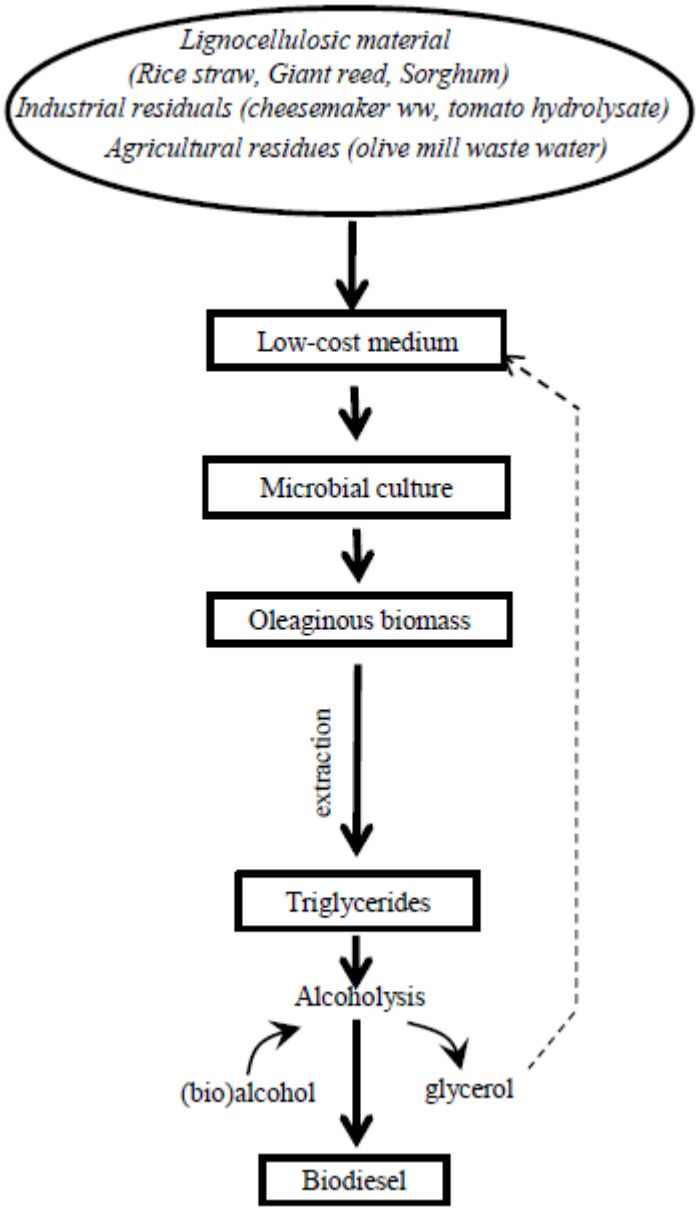

Figure 1.3 Short flow chart of experimental activity

Specific objectives of this research are as follows:

1 **-** To enhance the economic attractiveness of II generation biodiesel and the energetic use of waste materials from different sources, both agricultural and industrial;

2 **-** To optimize the pretreatment of waste materials, in order to enable their partial or total transformation by the oleaginous microorganisms. Specific attention has been devoted to the pretreatment of lignocellulosic biomasses tested (*Arundo donax, Sorghum bicolor*), requiring preliminary hydrolysis to obtain fermentable sugar. In this study, acid hydrolysis has been adopted due to its simplicity, as well as to its large diffusion in the industrial practice;

3 **-** To monitor the growth of oleaginous microorganisms in the growing media obtained from the waste materials, to enhance the accumulation rate of intracellular lipid, as well as to improve the microbial oil composition;

4 **-** To optimize the extraction of microbial lipid/oil from biomass;

5 **-** To enable the synthesis of biodiesel from microbial oil by alcoholysis.

CHAPTER-II: MATERIALS AND METHODS

2.1 Microorganisms and culture media

Lipomyces starkeyi, *Cryptococcus curvatus*, *Rhodotorula glutinis Rhodosporidium toruloides* were kept on potato dextrose agar (Sigma) at T = 5 ± 1 °C. The microorganisms were cultivated in a N-limiting medium, containing (g l^{-1}): KH_2PO_4 (Serva), 1.0; $MgSO_4 \cdot 7H_2O$ (BDH), 0.5; $(NH_4)_2SO_4$ (Carlo Erba), 2.0; yeast extract (Fluka) 0.5, glucose 70.0. The microorganisms were grown under aerobic conditions at 30°C on a rotary shaker 160 rpm (Minitron, Infors HT, Switzerland).

2.2 Olive Oil Mill Wastewater (OMW)

OMW were obtained from the Casa Olearia Italiana (Monopoli, Italy). Samples were immediately frozen at -20° C until further use. Before each experimental test, OMW were de-frozen, and the solids were removed by centrifugation (4000 rpm, 30 min, and 20°C) in a thermostatic centrifuge (Rotanta 460R, Hettich, USA).

Figure 2.1 Olive mill waste water

The pH of OMW after centrifugation was 4.68. The composition of the OMW is given in the Table 2.1. The medium of OMW was sterilized in autoclave at 121°C for 20 min. The flasks were plugged with cotton.

Table 2.1 Composition of the olive mill wastewater.

Water, %	95.3
Nonaqueous comp., %	4.7
Sugar, %	1.3
Polyphenols, %	0.9
Proteine, %	0.3
Dry weight (105 °C) %	4.64
Mineral res. (550 °C), %	1.11
Fe %	0.0013
Mg %	0.192
Ca %	0.026
K %	0.285
Na %	0.064
Cu %	0.00019
Zn %	0.000316

2.2.1 Fermentation with OMW

The fermentation tests were carried out in conical flask of 500 ml. The liquid medium was inoculated by 2 ml of microorganism suspension, obtained dissolving 10 loops of solid culture in 8 ml of physiological solution. The flasks were incubated in a rotary shaker at an agitation rate of 160 ± 5 rpm and an incubation temperature T = 30 ± 1 °C. The pH value of the medium was 4.68 before sterilization. One hundred fifty ml of the medium was transferred in a 500-ml shaking flask. After fermentation pH value of the medium was 4.56-7.5, that varies with the composition of medium.

2.2.2 Phytotoxicity tests

OMW phytotoxicity was assessed on the seeds of Lactuca sativa specie purchased by "La Semiorto Sementi" located in Sarno, South of Italy. The bioassays were carried out according to USEPA procedures (1996).

A suitable volume (5 mL) of OMW, both raw or after treatment, with *Lipomyces starkeyi*, diluted (1:10 and 1:25) with deionized water, was added to 100 mm diameter Petri dishes containing a filter paper disk (Whatman no.1, 90 mm). Ten seeds were placed on each paper disk, and for each sample tested three replicates were prepared. Controls with deionized water were also run. The plates were incubated in a growth chamber (Angelantoni HCT 120) in the dark at 23°C and after 72h the germinated seeds were counted, and the rootlet of each germinated seeds was measured with a ruler. Each experiment was repeated three times. A primary root > 2 mm was considered as the end germination point. Seed germination and root elongation at the end of the bioassays were measured, and the relative index of germination (GI) was calculated according to the following formula:

$$\% GI = (Gs/Gc) * (Ls/Lc) * 100$$

where Gs and Gc are the number of germinated seeds in the sample and in the control, respectively; Ls and Lc are the average root length of seedlings for the samples and for the control one, respectively.

2.2.3 Statistical analysis

All experiments have been carried out adopting a sample size of at least n=3. The hypothesis tests for the Germination Index data were carried out by a one-sided t-tests (Himmelblau, 1970), with significance levels of $\alpha = 0.01\%$.

2.3 Lipid extraction and measurement

Total lipids extracted according to Bligh and Dyer (1959) with little modification. In a typical test, 5 ml of methanol and 2.5 ml of chloroform were added to 200mg of dry biomass and vortexed 5 seconds. Subsequently, the cells were disrupted for 12 min in an Ultrasonic Homogenizer (Omni Ruptor 250, USA) at 50% power and 90% pulser. The cells were then filtered off with Whatman no.1 filter paper and the solvent-lipid mixture was placed in a 50 ml tube fitting with centrifuge racks. The layers were separated by centrifugation for 10 min at 2000 rpm in a thermostatic centrifuge (Rotanta 460R, Hettich, USA) at 20°C. The lower layer was then transferred to a pear-shape flask with Pasteur pipette. Again, 10 ml of 10% (v/v) methanol in chloroform were added to the residue, a new centrifugation was carried out, and the lower phase was added to that from the first extraction. The solvent in the pear-shape flask was evaporated to dryness (BÜCHI Rotavapor R-200, Switzerland) and extracted weight was finally recorded after drying at 105°C for 1 h.

2.4 Fatty acids composition

The fatty acids composition was determined by GC analysis on a Shimadzu GC 17/3 gas-chromatograph equipped with a flame ionization detector, following the method suggested by Li et al. (2007).

2.5 Biomass analysis

The biomass concentration in the synthetic medium was measured by OD determination at 600 nm. When culturing microorganisms in the OMW, OD measurement could not be carried out due to the darkness of the medium. Consequently, the total count of microorganisms was carried out by sequential dilution and insemination in plate count agar medium (Difco Laboratories, Detroit, MI, USA). The plates were put on ambient temperature (at 25-30^{O}C) and the colony forming units (CFU) counted after 24 h of culture on agar medium. The mean values obtained with three replicas per sample (Amaral et al., 2008).

Figure 2.2 Dry biomass cultured in OMW, the source of lipid

After each fermentation test, the biomass was recovered by centrifugation (3500 rpm for 10 min) and lyophilized (LYOBETA- 50, Spain), to enable the determination of the dry biomass and the lipid concentration measurement. The TOC measurements were carried out with a TOC-$V_{CSH/CSN}$ (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.

2.6 Preparation of nutrient broth with cheesmaker wastewaters

It has been tested the ability of *Lipomyces Starkeyi* to grow in watery broth made from cheese maker wastewaters. The wastewaters were supplied by the 'Ciro Amodio' factory of St. Anastasia (Napoli). The samples reached the laboratory within hours of their production and were immediately frozen and kept at -20 ° C until use. In this way it was possible to prevent the lactic acid bacteria, added during the production of dairy products, go to consume the nutrients present in them.

At the time of their use, the samples were thawed at room temperature and subsequently rundown of solid phase using a centrifuge Rotating (460R, Hettich, USA) at 4000 rpm for 10 min at 20 ° C.

2.7 Tomato waste hydrolysates (TWH)

Washed tomato was boiled 15 min (up to skin started to release). They were cut in to two pieces and cool up to ambient temperature to eliminate water. Then they were pressed and separated the juice using Tomato Squeezers (Figure 2.3). It was repeated two times. 100g Solid

tomato waste was mixed with 300ml of 2% (v/v) H_2SO_4. The mixture was then autoclaved at 121^{O} C for 2 hrs and filtered through whatman no. 1 paper (Fakas et al., 2008). Saturated KOH was added to the filtrate to adjust the pH at 6.0 and that is the TWH_{OR}.

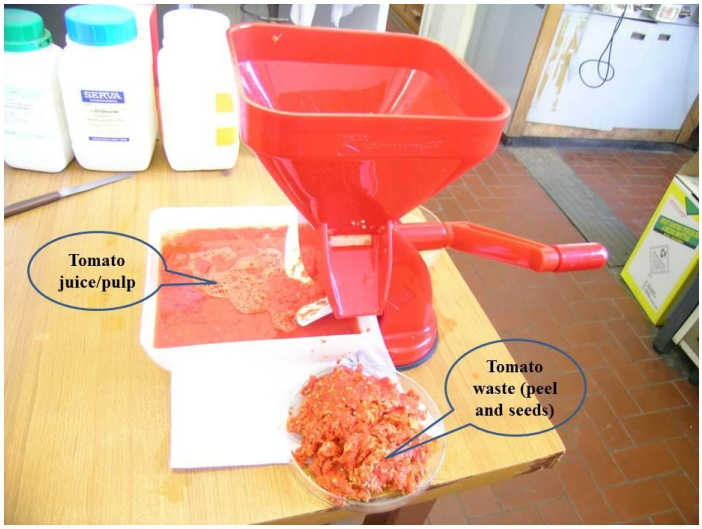

Figure 2.3 Tomato Squeezers, and tomato waste (seed and peel), after extraction of pulp

TWH_{OR} was divided in to two parts, one part was kept as it was. In to other part, 2M H_2SO_4 was added to adjust the pH at 3.0 and the mixture was centrifuged for 20 min at 4500rpm and 5^{O}C. The precipitate was removed by filtration through whatman no. 1 paper. The filtrate's pH was adjusted to 9 with saturated KOH and the mixture was centrifuged for 20 min at 4500 rpm and 5^{O}C. The precipitate was removed by filtration through whatman no. 1 paper. And finally pH was adjusted to 6 with 2M H_2SO_4 and this was the lower N containing, TWH_{LN} (Fakas et al., 2008). The higher nitrogen content sornatant, TWH_{HN} was prepared by adding $(NH_4)_2SO_4$ solution (3g in 10 ml distilled water) in TWH_{NN}.

2.7.1 Pre-adaptation of oleaginous yeasts.

The preliminary growth of the yeasts in selective media containing specific pollutants should increase their surviving and metabolizing ability in the presence of wastes containing these pollutants. In this point of view, pre-adaptation was carried out with the preparation of slant containing TWH_{NN} and agar. The ratio of TWH_{NN} and agar was 100: 1.5 (ml: g). The mixture was autoclaved and taken 10ml in each tube and stored in ambient condition for 24 hours. The pure cultured *L. starkeyi* was inoculated in the contaminated free tubes.

Figure 2.4 Slant culture of pre-adapted L.starkeyi.

2.7.2 Preparation of medium with hydrolysate

Three different culture mediums were prepared with 100%, 50% and 25% hydrolysate making dilution with physiologic solution. Then 2ml of microorganism broth was inoculated in each reactor. In addition, no commercial nutrient was added to the medium.

2.7.3 Fermentation

The fermentation tests were carried out in conical flask of 500 ml. The liquid medium was inoculated by 2 ml of microorganism suspension, obtained dissolving 10 loops of solid culture in 8 ml of physiologic solution. The flasks were incubated in a rotary shaker at an agitation rate of 160 ± 5 rpm and an incubation temperature T = 30 ± 1 °C.

2.8 Pre-treatment (acid hydrolysis) of lignocellulosic biomass

Lignocellulosic biomass such as Giant Reed (*Arundo donax*) and Sorghum (*Sorghum bicolor)* were collected from S. Angelo dei Lombardi (Campania, Italy) agro-land. Leaves were separated from stems and were cut with a hex saw at 2 cm. Then washed and dried over night at 80°C and grind with a chopper. The powdered biomasses were stored in desiccators. In a typical test, H_2SO_4 at 10%, 5%, 2.5% (w/v) was used to oven-dried biomass at a solid to liquid ratio of 1:10 with 3g of samples in 30 ml of acid solution in a 100ml glass bottle. Then they were autoclaved at 121^{0}C for 20 min (Fakas et al., 2008). After filtration (with filter paper), the filtrate was neutralized to pH 6.5 with saturated KOH solution. Hydrolysates were steriled in autoclave before inoculation.

2.9 Cellulose, hemicellulose and lignin measurement

Cellulose, hemicellulose and lignin were measured according to Ververis et al. (2004).

2.10 Measurement of reducing sugar

In each test, 0.1, 0.2, 0.3 ml of sample were up taken from each batch. All were marked up to 2 ml adding distilled water. Then added 1ml of alkaline copper-tartrate reagent and put in boiling bath for 10 min , cold and added 1 ml of arsenomolybolic acid reagent and fill up to 10 ml. Reagents were prepared according to Nelson-Somogyi method (Sadarivam and Manickam, 1996). Optical density was measured at 620nm.

Preparation of Standard solution

1. Stock solution: 100mg of sugar was dissolved in 100ml distilled water, 1 mg/ml

2. Working standard: And then 10ml of stock solution was diluted to 100ml with distilled water (0.1 mg/ml) or (100µg/ml)

3. Pipette out 0.2, 0.4, 0.6, 0.8, 1.0 ml of the working standard solution was taken into a series of test tube.

4. Made up the volume to 2ml with distilled water

5. Added 1 ml of alkaline copper tartrate reagent to each tube.

6. Placed the tubes in a boiling water bath for 10 min

7. Cold the tubes and added 1ml of arsenomolybolic acid reagent to all the tubes

8. Made up volume in each tube to 10ml with water

9. Optical density was measured at 620 nm

2.11 Analysis of Microbial Biomass

The total count of microorganisms was carried out by sequential dilution and insemination in plate count agar medium (Difco Laboratories, Detroit, MI, USA). The colonies were counted after 48 h of culture on agar medium. After each fermentation ordeal, the biomass was recovered by centrifugation (4000 rpm for 10 min) and lyophilized (LYOBETA- 50, Spain), to enable the determination of the dry biomass and the lipid content. The TOC measurements were carried out with a TOC-$V_{CSH/CSN}$ (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.

2.12 Effect of Temperature

To observe the effect of temperature on the growth of *L. starkeyi* in the hydrolysate (50%) of giant reed stem, fermenters were put at 15˚C, 20˚C, 30˚C and in fluctuated temperature -24 hrs at 15˚C and next 24 hours at 30˚C. Inoculation was performed as described in section 2.7.3.

2.13 Detoxification of hydrolysate

The detoxification treatment included over-liming, activated charcoal treatment and both together. Firstly, hydrolysate was neutralized with NaOH to pH 6.5. The activated charcoals were added to the hydrolysates at weight ratios of 0.05 (Miyafuji et al., 2003). The hydrolysates containing activated charcoal were incubated at 30^{O}C, 160 rpm for overnight then vacuum filtration to remove the adsorbent resulted in the detoxified hydrolysate. Finally, pH was adjusted to 6.5 with $Ca(OH)_2$ or 5 M H_2SO_4.

For over-liming, the pH of hydrolysate was increased to 10.0 by addition of $Ca(OH)_2$. After 1 h, the hydrolysate was filtrated under vacuum and acidified to pH 5.5 with 5M H_2SO_4 and filtrated again after 1 h for precipitate removal (Huang et al., 2009). Finaly, the over-limed hydrolysate was recovered by vacuum filtration. One fragment of over-limed hydrolysate was treated by activated charcoal as described above. Inoculation was performed as described in section 2.7.3.

So, detoxification method resulted the following mediums-

i) OL- OverLimed and neutralized with $Ca(OH)_2$

ii) AC- treated with Activated Charcoals and neutralized with NaOH

iii) OLAC- treated with OverLime and Activated Charcoals and neutralized with $Ca(OH)_2$

iv) WT- without treatment and neutralize with NaOH

2.14 Recycle of glycerin

Batch cultures were performed in 500 ml conical flasks with different initial concentrations of glycerol. In order to assess possible effects of inhibition, the liquid culture media, properly sterilized and they were characterized as follows:

-KH_2PO_4 (Serva), 1.0 g / l;

- $MgSO_4$. $7H_2O$ (BDH), 0.5 g / l;

- $(NH_4)_2SO_4$ (Carlo Erba), 2.0 g / l,

- Yeast extract (Fluka) 0.5 g / l,

- Glycerol from 35 to 140 g / lt.

The initial pH was set at 5.5 for each medium. Inoculation of *Lipomyces starkeyi*, *Yarrowia lipolytica* and *Cryptococcus curvatus* was done as described in section 2.7.3.

CHAPTER-III: RESULTS AND DISCUSSION

3.1 Fermentation in synthetic medium

In a process aimed at the production of microbial triglycerides (TG), the total amount of TG in the reactor depends from two main parameters, namely: the concentration of biomass in the reactor (X) and the fraction of TG in the biomass (Y_{LX}). The total concentration of triglycerides in the reactor (L) is obviously obtained as the product:

$$L = X \cdot Y_{LX} \qquad (3.1)$$

Consequently, the reactor optimal design should take into account non only conditions for a maximum lipid fraction to be achieved, but also the conditions to obtain a higher concentration of biomass.

It is known (Yong-Hong et al., 2006; Papanikolaou et al., 2008; Meng et al., 2009) that oleaginous yeasts accumulate lipids as storage materials only under N-limiting conditions. A problem arises as the process is aimed at obtaining higher concentrations of biomass under these conditions, in that the biomass growth is obviously stopped in the presence of very low amounts of nitrogen sources. In principle, a progressive addition of the N-source (or a fed-batch system) could allow to obtain higher concentrations of biomass and higher fractions of lipids, as well.

In order to characterize the effect of the N-source depletion, microorganisms were cultured in a synthetic media (described in the Method paragraph) with a low content of nitrogen (C/N ratio = 58). The growth profiles obtained with *Lipomyces starkeyi, Cryptococcus curvatus,*

Rhodotorula glutinis and *Rhodosporidium toruloides* are reported in the Figure 3.1. It can be seen that the growth kinetics are substantially similar.

Lipomyces starkeyi were selected for the subsequent tests, as they have been proved to store large amounts of lipids, showing only a minimal reutilization of the stored lipids (Liu et al., 2007).

In order to increase the biomass yield obtained under N-limiting conditions, we have grown *Lipomyces starkeyi* under multiple additions of the nitrogen source, restoring the initial concentration of $(NH_4)_2SO_4$ after a stationary phase was established. The Figure 3.2 describes the growth profile of *Lipomyces starkeyi* under multiple additions of the nitrogen source.

The experimental data show that, after each addition of $(NH_4)_2SO_4$, a new exponential phase starts, though the increases in biomass concentration are progressively reduced. These results demonstrate that a higher value of X can be obtained by progressive addition of the N-source, in order to limit the increase of the C/N ratio, and eventually to obtain satisfactory levels of the triglyceride concentration.

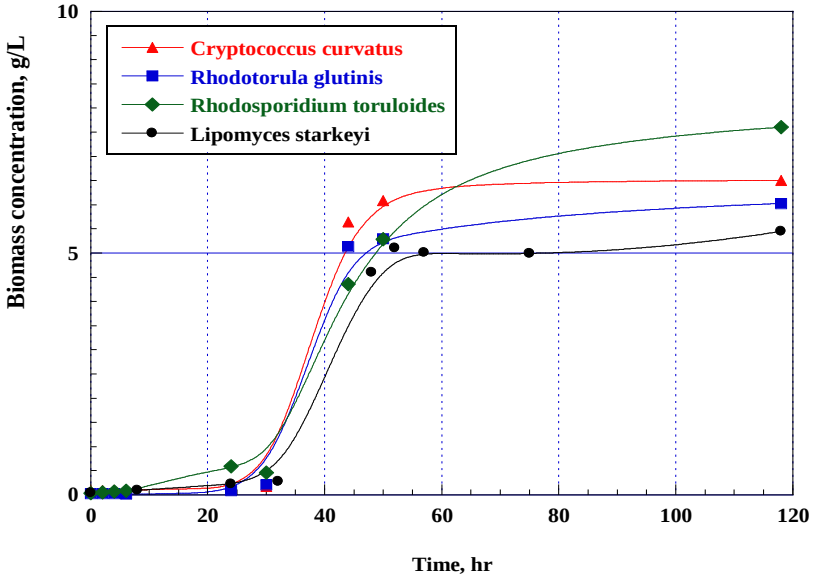

Figure 3.1 Growth kinetics of four oleaginous yeasts using an N-limiting synthetic medium in batch reactors. Operating conditions: T = 30°C, 160 rpm, medium composition as in the Method paragraph.

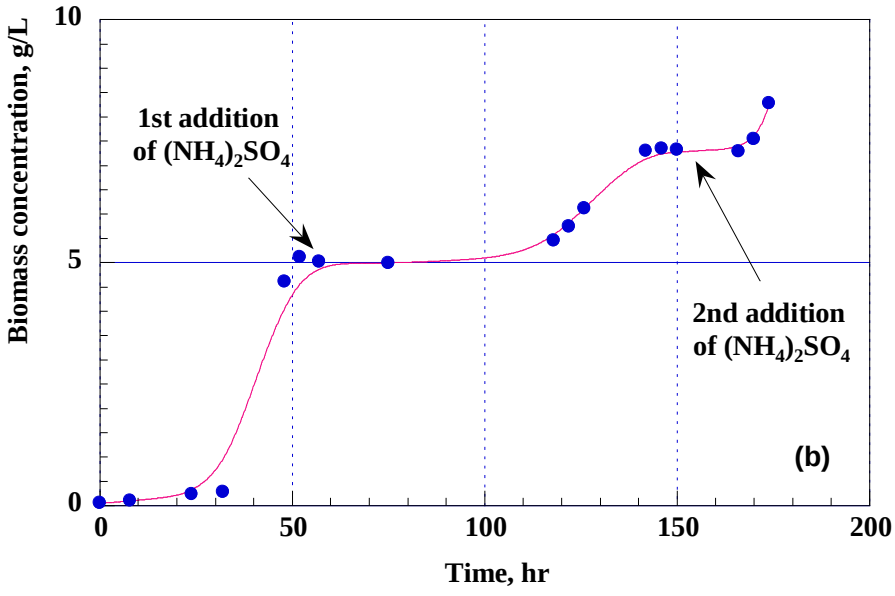

Figure 3.2 Growth of Lipomyces starkeyi using an N-limiting synthetic medium in batch reactors, under multiple additions of the nitrogen source. Operating conditions: T = 30°C, 160 rpm, medium composition as in the Method paragraph.

3.2 Olive-Mills Wastewater (OMW)

3.2.1 Economical impact of OMW

The olive oil production is a significant agricultural activity with a great economic importance particularly in Mediterranean countries. However, it generates high amounts of waste waters deriving from the olive mill process (olive oil mill wastewater or OMW). This waste causes disposal problems because of its highly polluting properties, which are documented by higher COD and BOD values (Arienzo and Capasso, 2000).

Mediterranean countries produce more than 98% of the world's olive oil, which is estimated at over 2.5 million metric tons per year. About 75% is produced in the European Union (EU). Over the last decade, olive oil production has increased about 40% worldwide and Europe has witnessed a 45% increase in production (Roig et al., 2006; FAOSTAT, 2007). Spanish olive oil production, which represents about half of European production, has shown an increase of almost 80% during that time (FAOSTAT, 2007).

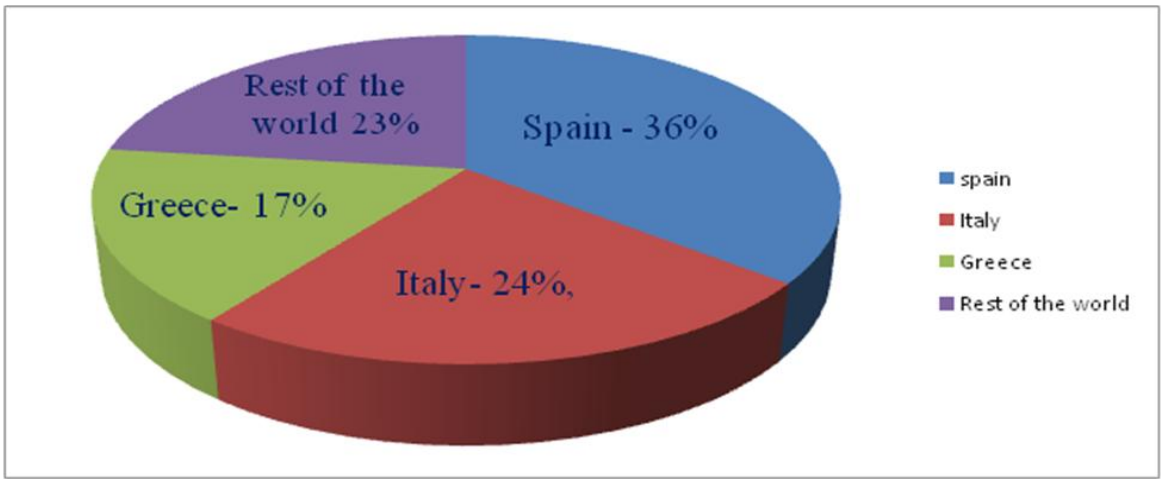

Figure 3.3 Olive oil production in EU

An estimated 7–30 million m^3 of OMW is generated every year from the production of olive oil (Niaounakis and Halvadakis, 2006) which is equivalent load of the wastewater generated from about 20 million people. Due to the presence of large amount of proteins, polysaccharides, mineral salts and other useful substances for agriculture such as humic acids, OMW has high fertilizing power. Unfortunately, besides these useful substances for agriculture, OMW also contains phytotoxic and biotoxic substances (Niaounakis and Halvadakis, 2006). For many years, olive mill wastewater (OMW) has been the most pollutant and troublesome waste produced by olive mills in all Mediterranean countries. Thus, the management of this liquid residue has been extensively investigated and some extensive and detailed reviews, which focus mainly on its management (Roig et al., 2006). It is well known that phenolic compounds are major contributors to the toxicity and the antibacterial activity of OMW. Phenols in seeds have also been proposed as germination inhibitors (Khan and Ungar, 1986). The presence of phenols causes the inhibition of germination of *Atriplex Triangularis* and *Pinus laricio* seeds (Muscolo et al., 2001). One of the phenolic compounds, gallic acid significantly reduced larval growth of *S. frugiperda* neonates (Bulla et al., 2004).

3.2.2 Fermentation in the presence of OMW

The *Lipomyces starkeyi* were cultured in the presence of the OMW, without external organic supplement. The experimental tests were carried out in the presence of raw OMW (after a preliminary centrifugation), as well as in water mixtures containing 50% OMW and 25% OMW, respectively. The growth profiles, reported in the Figure 3.4, were evaluated in terms of Colony Forming Units (CFU), as OD measurement could not be carried out due to the darkness of the medium.

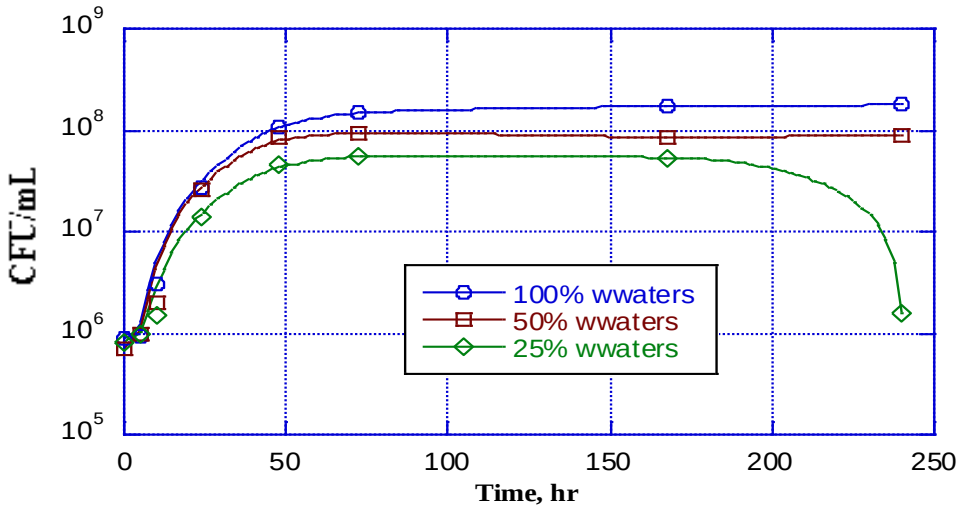

Figure 3.4 Growth of *Lipomyces starkeyi* in the presence of olive mill wastewaters (OMW) in batch reactors. Operating conditions: T = 30°C, 160 rpm. OMW composition as in the Method paragraph

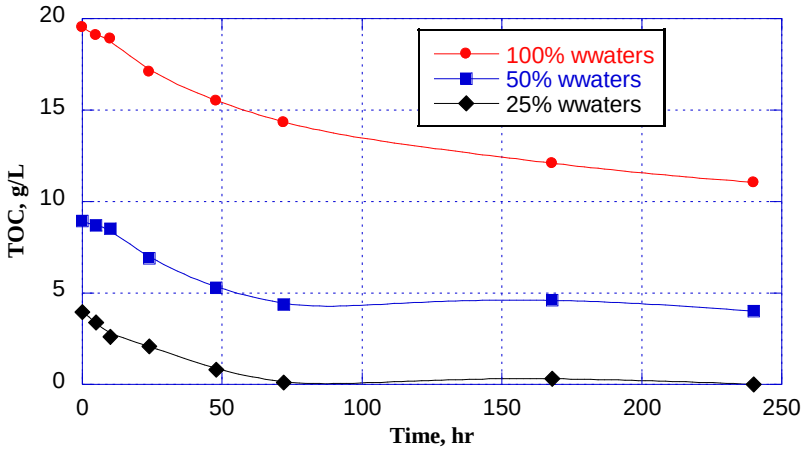

Figure 3.5 TOC measurements during the culture of *Lipomyces starkeyi* in the presence of olive mill wastewaters (OMW) in batch reactors. Operating conditions: T = 30°C, 160 rpm. OMW composition as in the Method paragraph.

The results show an initial increase of CFU limited to the first 3 days. The biomass growth was slightly slower when the yeasts were cultured in the presence of dilute OMW. Subsequently, *Lipomyces* cultured in 25% OMW were for about 170 hours in a stationary phase, followed by a progressive reduction of CFU. *Lipomyces* cultured in the presence of 100% OMW, and 50% OMW survived at constant biomass concentration for an even longer period.

The TOC levels registered in the course of the OMW treatment are reported in the Figure 3.5. A complete removal of the organic carbon was carried out only in the presence of the 25% OMW. However, in all the tests carried out, the OMW reduction was mostly achieved in the first 3 days, that is in the period of the CFU growth, showing the removal of organic carbon to be associated to the biomass increase.

The Table 3.1 describes the reduction of sugars, proteins and phenol concentrations obtained by OMW treatment using *L. starkeyi*. The experimental data demonstrate that efficiency of the yeasts in metabolising the sugars is higher as the preliminary dilution of OMW increases.

In any case, the sugars removal was uncompleted when treating 100% and 50% OMW, demonstrating that the break in the biomass growth was not due to the exhaustion of sugars.

Table 3.1 Sugars, proteins and phenols removal obtained during the *Lipomyces starkeyi* culture in the presence of olive mill wastewaters.

sample	Sugars - % change	Proteins - % change	Phenols - % change
100% OMW	-48%	-82%	-43%
50% OMW	-54%	-87%	-47%
25% OMW	-86%	-98%	-43%

This conclusion was confirmed by a further test: when adding a glucose amount corresponding to a concentration of 70 g/L in the OMW, the growth curves did not change significantly (data not shown).

The amounts of removed proteins were higher as compared to sugars. As for sugars, the highest efficiency of protein removal was obtained with the most diluted OMW sample. The consumption of phenols in the course of OMW treatment is described in the Table 3.1. In each test, more than half of the initial phenol content was removed, though the final phenol concentration was not dependent on the preliminary dilution of OMW.

3.2.3 Logistic model for the biomass growth

In order to model the biomass production and the TOC abetment, a numerical model was built based on the experimental data obtained under different experimental conditions.

The biomass production rate was obtained by a biomass balance:

$$\frac{dX}{dt} = \mu X \tag{3.2}$$

The specific growth rate was defined adopting the logistic model:

$$\mu = \mu_{max}\left(1 - \frac{X}{X_{max}}\right) \tag{3.3}$$

The TOC profiles were described adopting the hypothesis of proportionality between TOC reduction rate and biomass growth rate:

$$\frac{d[TOC]}{dt} = \mu X \frac{1}{Y_{X/TOC}} \tag{3.4}$$

Where, $Y_{X/TOC}$ is the ratio of the amount of biomass produced to the amount of TOC consumed (g biomass/g TOC). The equations (3.2), (3.3) and (3.4) were integrated using a fourth-order Runge-Kutta integration method. The least-square method was used to obtain the parameter estimates.

The model results fitted satisfactorily the experimental curves (see Figure 3.6).

A more detailed comparison between the experimental and theoretical data is given in the Table 3.2. The parameter μ_{max} slightly reduces as the OMW concentration increases. This result agrees with previously reported data, concerning the bacterial growth in the presence of OMW. The maximum values of biomass concentration (X_0) increases with the initial OMW concentration, though the X_0 value obtained with raw OMW (63.1 g/lt) is not much higher than that measured in the presence of 25% OMW (54,6 g/l). The biomass yield based on TOC consumption ($Y_{X/TOC}$) appears to be substantially constant, suggesting that changes in the OMW concentration do not cause a significant increase in the maintenance requirements.

Table 3.2 Comparison of Experimental Values of the Growth Parameters with the Theoretical Data Obtained with the Logistic Model, with Reference to the Culture of *L. starkeyi* in Batch Reactors, under Different Experimental Conditions

						g/L		l/h		g/L		g/g	
Experiment	OMW fraction (%)	Dilution factor	pH control	Addition of glucose	T (^oC)	Xo	$[TOC]_o$	μ_{max} (exp)[a]	μ_{max} (pred)	X_{max} (exp)	X_{max} (pred)	$Y_{X/TOC}$ (exp)[b]	$Y_{X/TOC}$ (pred)
E1	25	4	No control	No addition	30	0.0512	3.95	0.181	0.190	3.20	2.81	1.32	1.30
E2	50	2	No control	No addition	30	0.0488	8.93	0.171	0.175	5.90	5.81	1.29	1.25
E3	100	1	No control	No addition	30	0.0576	19.5	0.128	0.132	10.4	10.0	1.20	1.28
E4	100	1	No control		30	0.0539	20.1	0.127	0.136	11.1	10.8	1.31	1.22
E5	100	1	pH 5.5	No addition	30	0.0510	20.1	0.130	0.135	11.0	10.7	1.24	1.30
E6	100	1	pH 6.5	No addition	30	0.0491	20.0	0.127	0.134	10.6	10.5	1.30	1.27
E7	100	1	No control	No addition	35	0.0512	19.9	0.071	0.078	6.12	5.68	1.29	1.20
E8	100	1	No control	No addition	25	0.0522	20.1	0.099	0.097	11.0	10.3	1.34	1.31

[a] The experimental value of μ_{max} was calculated from the equation $\mu_{max} = \ln(X_2/X_1)/(t_2 - t_1)$, assuming a constant growth rate in the early exponential phase. [b]The experimental value of $Y_{X/TOC}$ was calculated from the slope of the curve of the biomass concentration (X) as a function of the TOC.

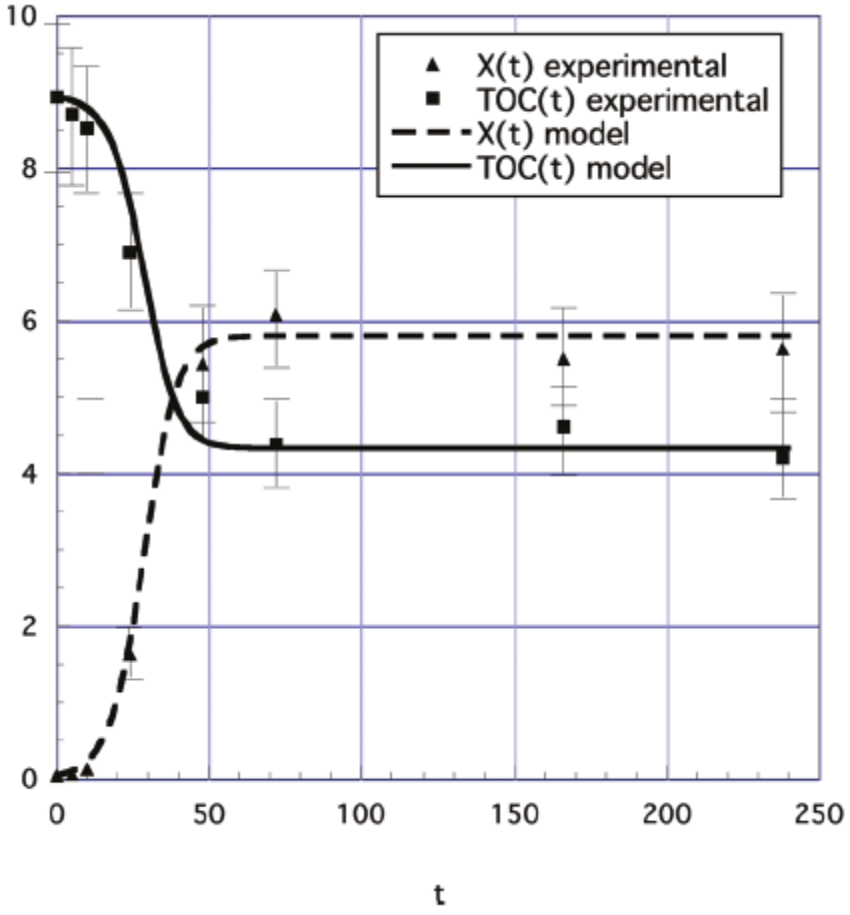

Figure 3.6 Comparison of experimental measurements of biomass concentration (X, g/L) and TOC (g/L) with the theoretical data obtained with the logistic model, with reference to the culture of *L. starkeyi* in batch cultures, in the presence of raw OMW.Op

Equations 3.2-3.4 were integrated using a fourth-order Runge-Kutta integration method. The least-squares method was used to obtain the parameter estimates. The model fitted the experimental data (see the results in the Table 3.2) with R^2 values higher than 0.95. The Figure 3.6 shows a graphic comparison between the model predictions (dashed line) and the experimental results of a typical test. Standard errors, reported in the Figure 3.6, never exceeded 14%.

3.2.4 Phytotoxicity

The results on GI of Lactuca sativa seeds of untreated and treated OMW samples are reported in the Table 3.3. When no sample dilutions were carried out before the GI test, both the untreated

OMW and the *Lipomyces*-treated OMW resulted to be completely phytotoxic. Consequently, further GI tests were carried out upon dilution of samples. When testing diluted samples (1:10 v/v), the untreated OMW remained completely phytotoxic, whereas the *Lipomyces*-treated OMW showed a significantly high germination index (125.5). In order to achieve a significant reduction of the phytotoxic activity of the untreated OMW, an higher dilution ratio (1:25) was required. The hypothesis that the GI of the OMW was actually increased in the course of the treatment was confirmed by a one-sided t-test (Himmelblau, 1970), with a significance level,

$\alpha = 0.01\%$.

As widely reported in literature (Iamarino et al., 2009; Jaouani, 2003) the phenols are considered the compounds main responsible of the OMW phytotoxicity. Consequently, the increase of germination index is likely originated by the reduction of phenols (see Table 3.3).

Table 3.3 Germination index of Latuca sativa seeds on untreated and treated OMW samples.

	GERMINATION INDEX	
Dilution ratio	**Untreated OMW**	***Lipomyces*-treated OMW**
1	0	0
10	0	125,5
25	105,0	121,0

3.2.5 Lipid yield and composition

The amounts of lipids extracted from *L. starkeyi* cultured in OMW are reported in the Table 3.4. The experimental data show that a 50% dilution of OMW results in a significant increase in the concentration of lipids (28.6% against 22.4%), though a further increase in wastewater dilution produces only a minimum improvement. The fatty acids distribution in the lipids accumulated in the *L. starkeyi* grown in OMW is described in the Table 3.5. The composition indicates a clear prevalence of oleic acid, what is expected as the lipid content of OMW is mostly made of olive oil. On the other hand, the prevalence of oleic acid has been observed also under different operating conditions (Li et al., 2007). The total content of saturated fatty acids is about 13 %, potentially leading to excellent low temperature behavior of biodiesel. On the other hands, the reduced amount of polyunsaturated residues leads to a good oxidation stability of the biodiesel.

Table 3.4 Lipid concentration in L. Starkeyi after 10 days culture in OMW

Sample	Lipid concentration (%)
Lipomyces grown in synthetic medium	12.1
Lipomyces grown in raw OMW	22.4
Lipomyces grown in 50 % OMW	28.6
Lipomyces grown in 25 % OMW	29.5

Table 3.5 Distribution of fatty acids in the lipids accumulated in the L. starkeyi grown in OMW

Fatty acid composition	Amount (%)
Myristic acid C14:0	<1 %
Palmitic acid C16:0	15.1 %
Palmitoleic acid C16:1	0.5 %
Stearic acid C18:0	5.5 %
Oleic acid C18:1	65.1 %
Linoleic acid C18:2	10.8 %
Linolenic acid C18:3	2.5 %
Arachidonic acid C20:0	0.3 %
Free fatty acids	14.5 %

3.3 Cheesmaker wastewaters

3.3.1 Economical impact of CW

Wastewater generated during cheese making comes from washing of the cheese vats, the pipelines, milk separator, milk pasteurizer, the inside of the milk trucks, and other equipment. Most dairies use a "clean in place" (CIP) system which pumps cleaning solutions through all equipment in this order: water rinse, caustic solution (sodium hydroxide) wash, water rinse, acid solution (phosphoric or nitric acid) wash, water rinse, and sodium hypo-chlorite disinfectant. These spent chemicals eventually become waste also.
The disposal of milk processing wastewater for a dairy farm is the main problem from the environmental point of view, given the significant amount of pollution produced and the high index. Suffice it to say that the treatment of 10 kg of milk produce on average 1-2 kg of cheese and 8-9 kg of waste, and that a small dairy, which produces an average of 20 m^3 of wastewater per day, causing pollution comparable to that of a population of about 10,000 inhabitants.

The problem of disposal is so deeply felt by the dairies, and in different areas of the country, the problem becomes greater and larger entity of the dairy sector. In most cases the dairies give the waste to specialized firms, which picks up them and then obtain, where possible, refined products with high added value, relocated on the Italian market and internationally. The cost of these activities is quite cheap (4-6 € per m^3) and currently falls only on the dairy industry, the risk of illegal procedures is quite high.

3.3.2 Sample specification

The wastewaters used in this thesis work were collected from three different streams:

• Serum (an intermediate waste in the production of ricotta and mozzarella)

• “Acqua di filatura” (a residual liquid of the mozzarella production)

• “Scotta” (a residual liquid of the ricotta production)

The industrial processing of milk for cheese and butter production creates large quantities of waste. In fact, by working in dairy, 10 kg of milk produce on average 1-2 kg of cheese, depending on the type, and 8 - 9 kg of liquid waste. In the specific case of the production of mozzarella, the effluent obtained after maturation of the curd is called "siero" (serum), during spinning is obtained as a residual "Aqua di filatura (water spinning)." After the first coagulation, the protein-rich serum undergoes heating (= riscaldamento) and a new coagulation, to obtain "ricotta" as precipitated phase. The "scotta" is the wastewater remaining after the separation of "ricotta".

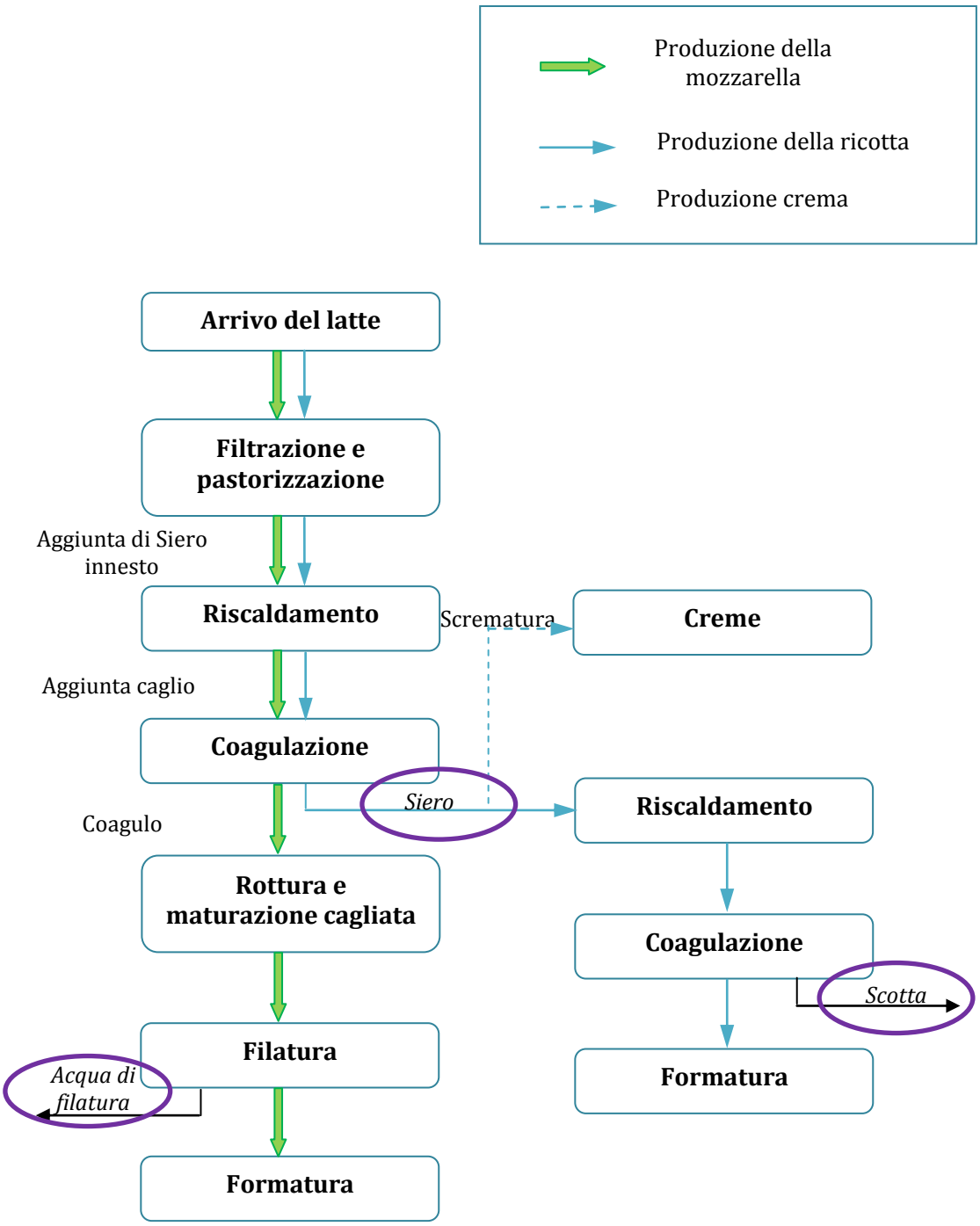

Figure 3.7 Diagram of mozzarella, cheese and dairy production with sampling points

3.3.3 Cultures of *Lipomyces* in "acque di filature"

A first series of experimental test was carried out using the "acque di filature", without any additions or treatments of cultural medium. Some physico-chemical properties of the "acque di filature" have been reported in Table 3.6.

Table 3.6 The main chemical and physical characteristics of different dairy waste stream

Parameters	Siero	Scotta	Acque di filatura
Lactose [g/l]	46	47	18
Protein [g/l]	8	3,9	2
Fats [g/l]	5	0,7	6
Ash [g/l]	5	5,3	
Lactic acid [g/l]	0,5		
Total solids [g/l]	64	56,7	
Vitamins [mg/l]	12		
BOD_5 [mgO_2/1]	20.100 - 22.000	10.000	16.800
COD [mgO_2/1]	50.800 - 6.000	20.000	36.400
pH	6.1	5.9	3.5
Volume per m^3 of treated milk [dm^3]	900		50 - 60

The experimental reported in the Figures 3.8 and 3.9 show respectively the values of TOC (Total Organic Carbon) and CFU (Colony Forming Units) as a function of the culture period.

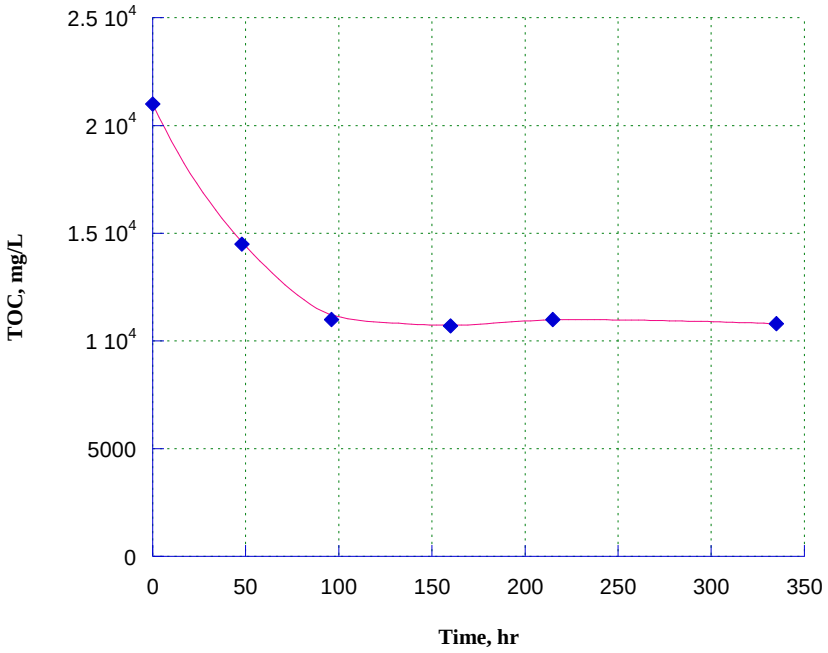

Figure 3.8 Evolution of TOC of the 'acque di filatura' during the culture of *L. starkeyi*

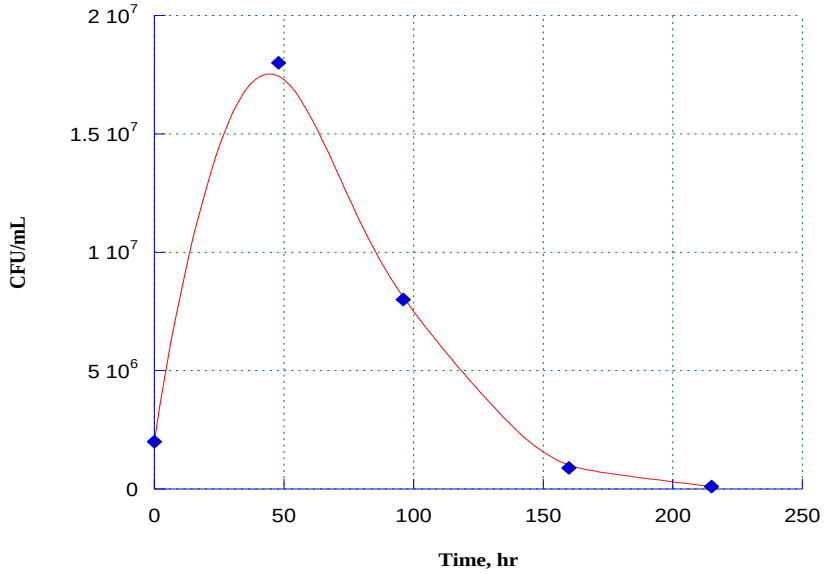

Figure 3.9 Cell growth in the medium of 'acque di filatura' during the culture of *L. starkeyi*

During the first 96 hours, the TOC progressively decreased from a value of about 20000 mg / l to a value of approximately 12000 mg / lt. In the same period, the concentration of CFU reached a maximum of 1.8x 10^7 CFU/ml, to decrease subsequently. Over 96 hours of treatment, the gradual extinction of the yeasts was observed, without further changes in the level of organic matter (TOC). Evidently, the consumption of organic substances (TOC) was strictly associated to the survival of microorganisms.

The TOC degradation has not been complete, probably due to the exhaustion of a nutrient or the accumulation of a metabolic product beyond the threshold of toxicity.

3.3.4 Culture of *Lipomyces* in diluted "acque di filature"

In order to investigate the causes of the obstruction of the degradation of organic substances, the test described in the previous paragraph was amended on track. After 96 hr of test, the medium was diluted, using 3 different media:

• A first sample (A) was diluted to 50% with saline, i.e., a 0.9% w / v NaCl in sterile deionized water. In this way, the concentration of all the chemical species present in the culture medium (hence also of potentially toxic metabolic products) was reduced by 50%;

• A second sample (B) was diluted to 25% with fresh "acque di filature", therefore adding components necessary for cell growth possibly exhausted during the first 96 hours of treatment;

• In a third sample (C), nitrogen source $(NH_4)_2SO_4$ was added to achieve the same amount of N concentration initially present in the sample.

The results reported in the Figures 3.10 and 3.11, respectively, show the values of the TOC and counting CFU as a function of the culture period.

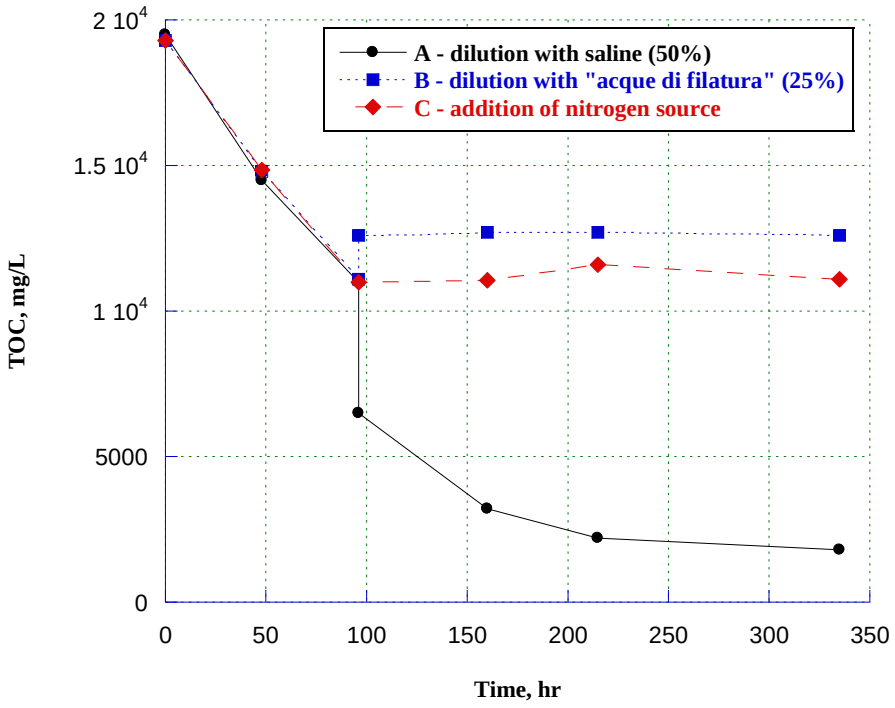

Figure 3.10 Evolution of TOC of the medium (diluted and undiluted) of 'acque di filatura' during the culture of *L. starkeyi*

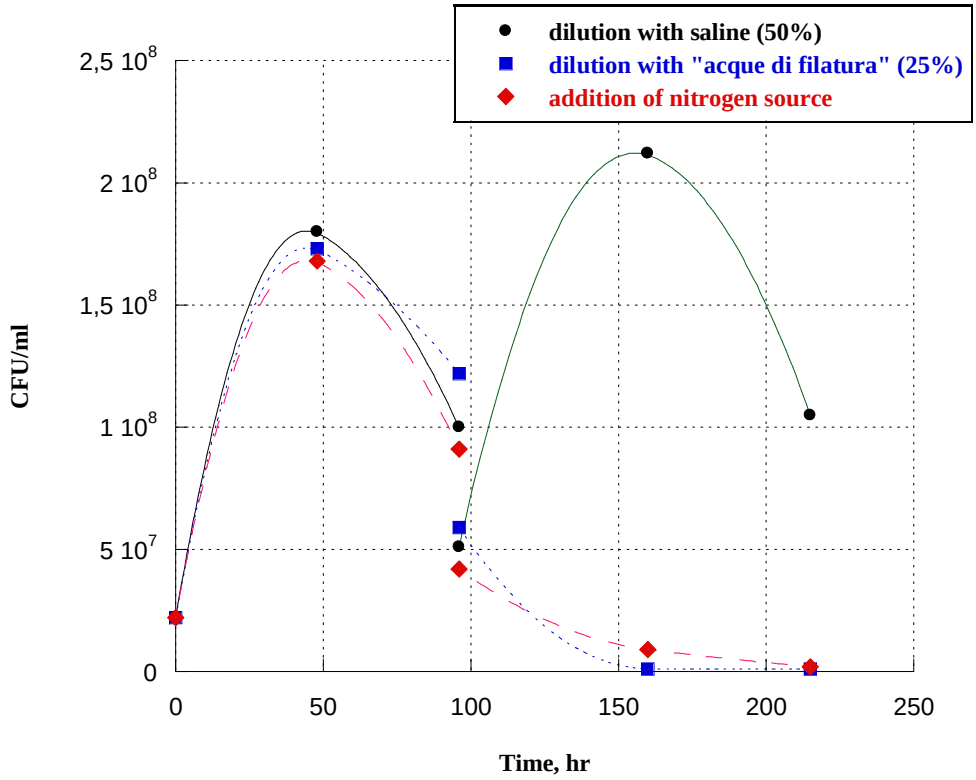

Figure 3.11 Cell growth in the diluted medium of 'acque di filatura' during the culture of *L. starkeyi*

The experimental data show that the addition of nitrogen (sample C) did not produced neither the resumption of microbial growth, nor the degradation of organic substances. Therefore, blocking the growth of yeasts was not due to the exhaustion of nitrogen source. Similar results were obtained by adding fresh "acque di filatura" (sample B), demonstrating that none of the nutrients initially present in the culture medium was lacking after 96 hrs.

Nevertheless, the medium added with saline (sample A) showed a resumption of microbial growth and the degradation of organic substances. This result suggested that blocking the growth of microorganisms was due to the accumulation of a metabolic product beyond the threshold of toxicity.

3.3.5 Cultures of *Lipomyces* in serum

Similar experimental tests were performed using serum as culture medium. In particular:

• The sample A was diluted with 50% of saline

• The sample B was diluted with 50% serum

• A reference sample (sample C) did not undergo any dilution procedure

The Figure 3.12 shows the values of TOC as a function of the culture period.

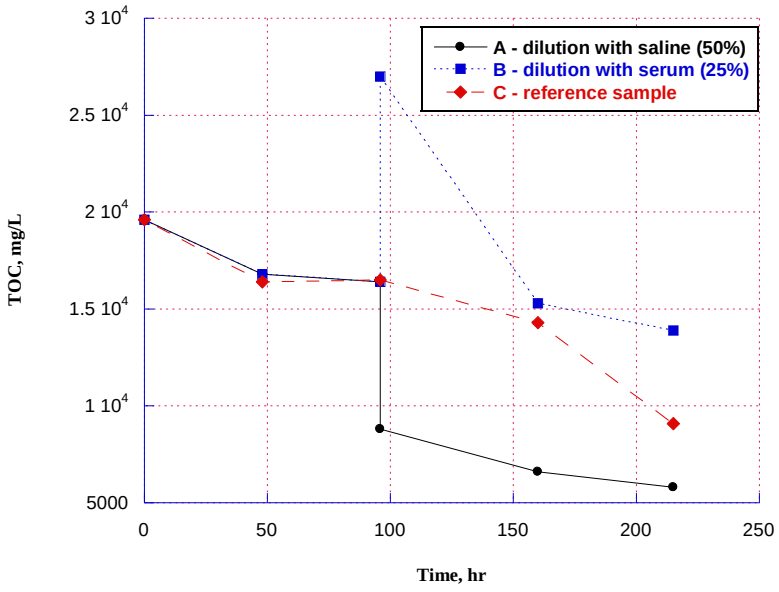

Figure 3.12 Evolution of TOC of the medium (diluted and undiluted) of 'siero' during the culture of *L. starkeyi*

In this case the TOC curve started at a higher value of TOC (about 20000 mg / l). The reference sample (curve C), not subjected to any dilution, showed a progressive reduction of the TOC. Evidently, the organic components of the siero may be degraded by microorganisms without major problems. When adding saline (sample A) or serum (sample B) the degradation of

organic matter continued even after the dilution, with no apparent improvement of the rate of consumption of the carbon source.

3.3.6 Cultures of *Lipomyces* in "scotta"

The "scotta" was tested as a possible growth medium for *Lipomyces*. The experimental data shown in Figures 3.13a, 3.13b illustrate respectively the values of TOC and CFU as a function of culture period.

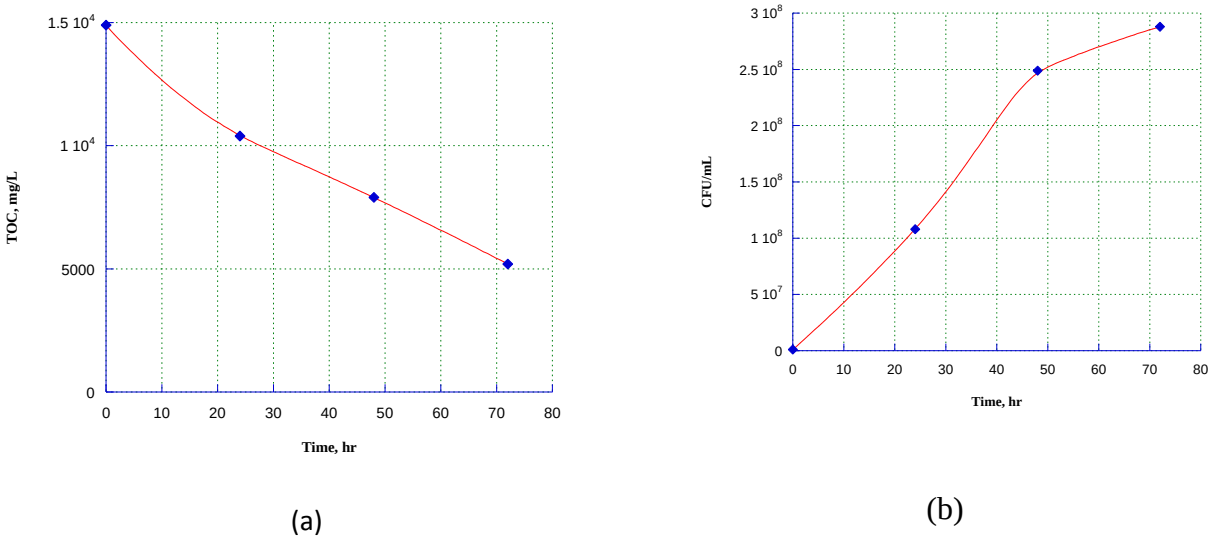

Figure 3.13 Evolution of TOC (a) and cell growth (b) of the medium of 'scotta'

In this case the TOC curve starts at a value of about 15000 mg/ L. The experimental curves showed a progressive reduction of the TOC, and a corresponding increase in the number of microorganisms. Evidently, the organic components of the "scotta" can be degraded by *Lipomyces* without major difficulties.

3.4 Lignocellulosic materials (LCM)

3.4.1 Economical impact of LCM

Lignocelluloses, which mainly produced by conventional agriculture and forestry practices is one of the cheapest and most abundant resources in the world. With the rapid increasing of the world population, the utilization of cellulose resource has induced researcher's great attention and recognition all over the world in order to solve the food and energy crisis (Zhang *et al.*, 1991). Presently, plant oils are traditionally used as triglycerides feedstock to produce biodiesel. Unfortunately, the cost of these oils is relatively high. As a result, the cost of the biodiesel, that is mainly due (70-85%) to the vegetable oils used as feedstock, still exceeds that of the mineral diesel. Therefore, cost effective microbial oil has been suggested for biodiesel production in the near future (Meng *et al.*, 2009; Zhao, 2005).

The conversion of lignocellulosic materials has been proposed by chemical and biological methods. In contrast, the biological conversion is considered environmentally friendly and less energy intensive (Thanakoses *et al.*, 2003). The recent increased interest in non-wood fiber plants is caused by the need to prevent the fast deforestation of northern (particularly North-American) countries and/or to meet the re-orientation of the West European agriculture towards non-food crops due to general food overproduction (Leminen et al., 1996; Moore, 1996).

A widely distributed perennial grass *Arundo donax* (Giant reed) is being considered among the group of more promising industrial crops (Christou et al., 2000). Giant reed has also been identified as a prime biomass source for fuel and an alternative crop for paper/pulp or wood

substitutes. The high yield potential and low input demands of Giant reed make it an attractive biomass crop (Lewandowskia et al., 2003).

It has specific feature such as annual harvesting period, high biomass productivity (up to 37 t-1 year^{-1} ha^{-1}, Vecchiet et al., 1996) ability to be intensively cultivated (Dalianis et al., 1994) and easy adaptability to different climatic and soil conditions (Perdue, 1958) which make *A. donax* one of the more promising industrial crops.

Another prospective source of lignocellulosic biomass is Sorghum and currently ranks fourth in acreage among cereal crops produced in the world. Although Sorghum (*Sorghum bicolor (L.) Moench*) has-been primarily used for grain and forage, the vegetative biomass has recently received more attention as a carbohydrate source for anaerobic fermentation to methane (McBee et al., 1987) or ethanol (McBee et al., 1988). Structural component composition of the Sorghum stem is an important consideration when vegetative biomass is the primary product. It was found to contain 43.6–58.2% soluble sucrose, glucose and fructose in the stalk (Billa et al., 1997; Dolciotti et al., 1998; Amaducci et al., 2004; Antonopoulou et al., 2008). Moreover, it can grow under more harsh climatic and soil conditions.

Table 3.7 Sorghum Production in EU

Country/ Region	Yield (Metric tons per hectare)				Production (Million metric tons)			
			2009/10 Proj.				2009/10 Proj.	
	2007/08	Prel. 2008/09	Jun	Jul	2007/08	Prel. 2008/09	Jun	Jul
EU-27	5.65	5.64	5.37	5.35	0.53	0.55	0.56	0.57
France	5.88	6.15	6	6	0.29	0.24	0.27	0.27
Italy	5.68	6.32	6	6	0.19	0.24	0.24	0.24
Others	1.01	1.12	1.05	1.05	3.24	3.71	3.46	3.46

Source: http://www.agrostats.com

The alternative of traditional raw materials, vegetable oils is the crying need to solve the huge demand of vehicle fuel. Because of the similar fatty acid composition of vegetable oil, microbial oil (also known as single cell oil) is considered as most potential alternative by Angerbauer et al., (2008) Li et al., (2007); Zhu et al., (2008) and others. In the economic point of view, the production of microbial oils from waste or renewable materials is of significant importance (Huang et al., (2009). To economic production of microbial oils, some inexpensive culture media such as cane molasses (Zhu et al., 2008), wheat straw and wheat bran (Peng and Chen, 2008), sewage sludge (Angerbauer et al., 2008), rice straw (Huang et al., 2009), sweet Sorghum (Economou et al., 2010) have been used.

In the present study the experimental activity has been aimed to the optimization of the conversion of lignocellulosic biomass into fermentable sugar, as well as the achievement of satisfactory yields in terms of triglycerides (Zhu et al., 2008). The overall objective of this research is to enhance the economic attractiveness of waste materials for production of biodiesel through the production of lipids by oleaginous yeasts.

3.4.2 Pretreatment (Hydrolysis) of LCM

In most instances, pretreatment is a prerequisite condition to use agro-industrial residues in bioconversion to fuels and chemicals. The main purpose of pretreatment is to separate the components of lignocellulosic biomass (Oh et al., 2002), as well as to reduce lignocellulosic biomass crystallinity, render cellulose accessibility, and remove lignin (Sun and Cheng, 2002). Since lignocellulosic materials are very complicated, their pretreatment is not simple either. The best method and conditions of pretreatment depend greatly on the type of lignocelluloses (Taherzadeh and Karimi, 2008). Different types of pretreatment methods were applied depending on the properties of substrate. Table 1.1 shows the most usable pretreatment methods.

Peng and Chen, (2008) studied with wheat straw applying steam explosion treatment and they found nitrogen content was markedly lower (0.56%) than that of untreated (2.62%). This pretreatment method removes most of the hemicellulose, thus improving the enzymatic digestion (Taherzadeh and Karimi, 2008). However, in order to achieve lipid accumulation in a microorganism, medium should composed with an excess of carbon and a limiting amount of nitrogen (Ratledge,2004). Ruiz *et al.* (2008) studied steam explosion for pretreatment of sunflower stalks before enzymatic hydrolysis at a temperature in the range of 180–230 °C. The highest glucose yield was obtained in steam-pretreated sunflower stalks at 220 °C, while the highest hemicellulose recovery was obtained at 210 °C. Xin and Geng (2010) pretreated horticultural waste by steam alone or by dilute NaOH solution. Steam pretreatment was conducted at 121 °C for 2 h and dilute NaOH pretreatment was carried out at varying concentrations (1-3%) at 105°C over night for enzyme production by *T. reesei*. They observed

that the highest enzymes activities were obtained when the steam-pretreated horticultural waste powder was used. Pretreatment with both steam and NaOH did not contribute much to the improvement in the enzyme (microbial) activities compared to that with steam alone. It was also observed that pretreatment with increased concentrations of NaOH, e.g. 1% to 3%, did not show significant increase in the yield of any enzymes.

Four different pre-treatment were applied on sewage sludge e.g., acid hydrolysis, alkaline hydrolysis, thermal treatment and ultrasonic treatment by Angerbauer et al. (2008). The best result was obtained with the ultrasonic treatment which have some advantages like insusceptible to impurities (little stone) and it is also convenient to the industrial application. Few data are available in the experience in lab-use, pilot-plants and industrial pilot-plants (Muller et al., 2001).

A wide range of organic or aqueous-organic solvents as well as catalysts such as oxalic, salicylic, and acetylsalicylic acid can be used in organosolv pretreatment of lignocellulosic materials at temperatures of 150-200 °C. In addition, the solvent may accompany acetic acid released from acetyl groups developed by hydrolysis of hemicelluloses. A variety of organic solvents such as alcohols, esters, ketones, glycols, organic acids, phenols, and ethers have been used. However, the price of solvent and simplicity in recovery of solvent should also be considered (Taherzadeh and Karimi, 2008). The operational cost could be reduced by recover (evaporation and condensation) and reuse of the solvents. Removal of solvents from the pretreated cellulose is usually necessary because the solvents might be inhibitors to the enzymatic hydrolysis and fermentation or digestion of hydrolysate (Sun and Chen, 2008). For economic reasons, the use of low-molecular-weight alcohols such as ethanol and methanol has been favored over alcohols with higher boiling points, e.g. ethylene glycol, tetrahydrofurfuryl

alcohol (Chum, 1985; Sun and Chen, 2008; Arato et al., 2005). Ethanol is a common solvent, although it inhibits hydrolytic enzymes (Wyman, 1996). It should therefore be removed from the solid fraction before enzymatic hydrolysis. The main advantage of the use of solvents over other chemical pretreatments is that relatively pure, low-molecular-weight lignin is recovered as a by-product (Sun and Chen, 2008).

Table 3.8 Variation of hydrolysis parameters implemented on agro-industrial residues

Residual Materials	**Hydrolysis with**		**Solid:liquid**	**% of solution**	**Temperature (°C)**	**Treatment time (minute or hour)**	**References**
	Acid	**Alkali**					
Rice straw	H_2SO_4		1:10	1.5	121	90 min	Huang et al., 2009
Sewage sludge	H_3PO_4		1:4	4.5	125	40 min	Angerbauer et al., 2008
Sewage sludge		KOH	1:4	0.625	125	40 min	Angerbauer et al., 2008
Tomato waste	H_2SO_4		1:3	2	121	2	Fakas et al., 2008
Oil palm waste	H_2SO_4		1:15	75	50	1h	Cheng et al. ,2007
Corn stalk, *populus euramevicana* leaves and rice straw	H_2SO_4		1:10			8h	Dai et al., 2007
Cane molasses	H_2SO_4		N/A	49	60	2h	Liu et al., 2008
Horticulture waste		NaOH	N/A	1-3	105	overnight	Xin and Geng, 2010
Cassava bagasse, sugarcane bagasse,wheat bran, rice straw		NaOH	N/A	0.4	N/A	12h	Singhania et al.,2006

Acid hydrolysis is the mostly usable pretreatment method for the lignocellulosic materials and Sulfuric acid is the most applied acid, while other acids such as HCl, phosphoric and nitric acid were also reported (Taherzadeh and Karimi, 2007). Table 3.8 shows that the acid pretreatment can operate either under a high temperature and low acid concentration (dilute-acid pretreatment) or under a low temperature and high acid concentration (concentrated-acid pretreatment). Lenihan et al. (2010) performed hydrolysis of hemicellulosic biomass in the form of potato peel using dilute phosphoric acid and obtained optimum yield at 135 °C and 10% (w/w) acid concentration. 55.2 g sugar/100 g dry potato peel was produced after a time of 8 min.

In the present study, we carried out hydrolysis with dilute H_2SO_4, since it is at present more convenient, applicable, and suggested for our selective biomass. In a preliminary experiment, we arbitrarily selected one lignocellulosic biomass to observe the effect of the H_2SO_4 concentration on the hydrolysis process. It was observed that, for our autoclave setting, i.e., 121˚C for 20 min, a lower concentration of acid was required (Figure 3.14) to maximize the amount of fermentable sugars obtained from the biomass degradation. The formation of reducing sugar from cellulosic biomass reached a maximum at 5% of H_2SO_4 in the hydrolysis process. This behavior can be explained by considering that higher concentrations of acid may lead to further sugar degradation that's why most of the hydrolysis was conducted at lower concentration of acid (Saha et al., 2005; Saha and Bothast, 1999; Chung et al., 2005).

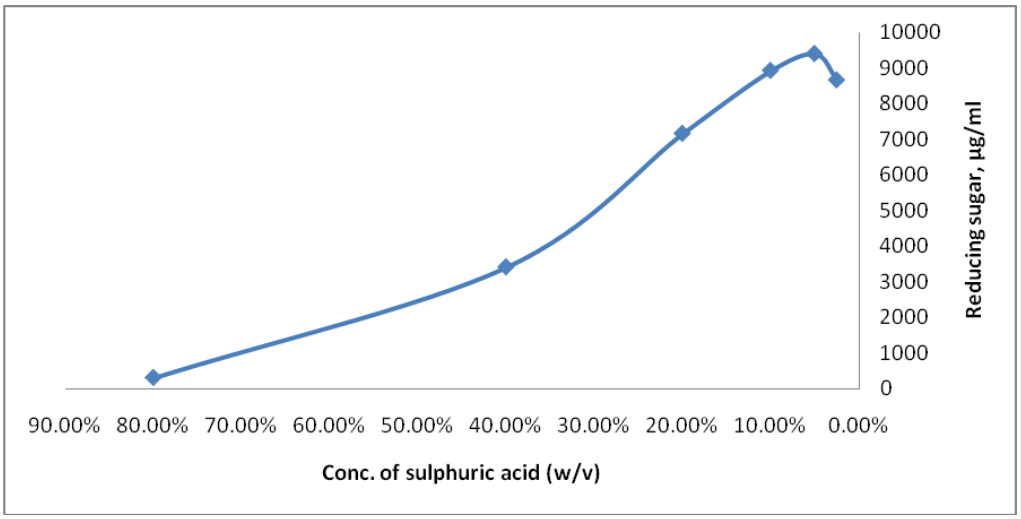

Figure 3.14 Effect of concentration of H2SO4 on hydrolysis process at condition-121C, 20 min

Subsequently, selected biomass (Giant reed stem, Sorghum, Tomato waste) were hydrolyzed with H_2SO_4 of 10%, 5% and 2% respectively . This experiment also showed almost similar result (Figure 3.15) as before. The optimum acid concentration was lower (2%) in the tests carried out with tomato wastes, due to the lower content of lignin in the tomato peel. Consequently, we selected 5% H_2SO_4 for giant reed stem and Sorghum and 2% for tomato waste in subsequent experiments.

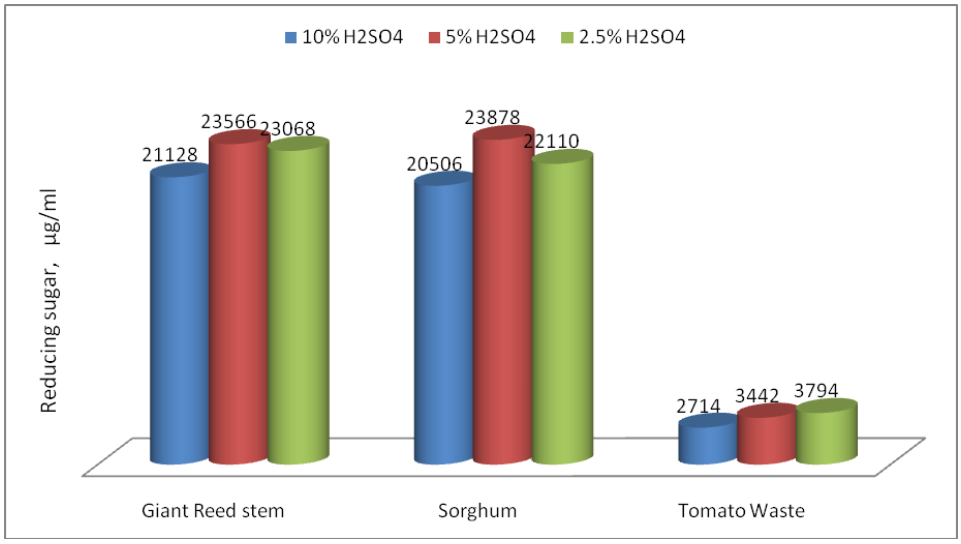

Figure 3.15 Optimization of acid hydrolysis.

3.4.3 Detoxification

Another major challenge in biological conversion of lignocellulosic biomass is the removal of antimicrobial agent from hydrolysate. Undesirably, during the hydrolysis process some non-carbohydrate compounds such as acetic acid, furfural, 5-hydroxymethylfurfural (HMF), and water soluble lignin generated with fermentable monosaccharides. Mainly, furfural and HMF originated from the decomposition of pentoses and hexoses, acetic acid from the acetyl group in hemicellulose and phenolic compounds including syringaldehyde, p-hydroxybenzaldehyde, vanillin, etc derived from lignin (Almeida et al., 2007; Hu et al., 2009).

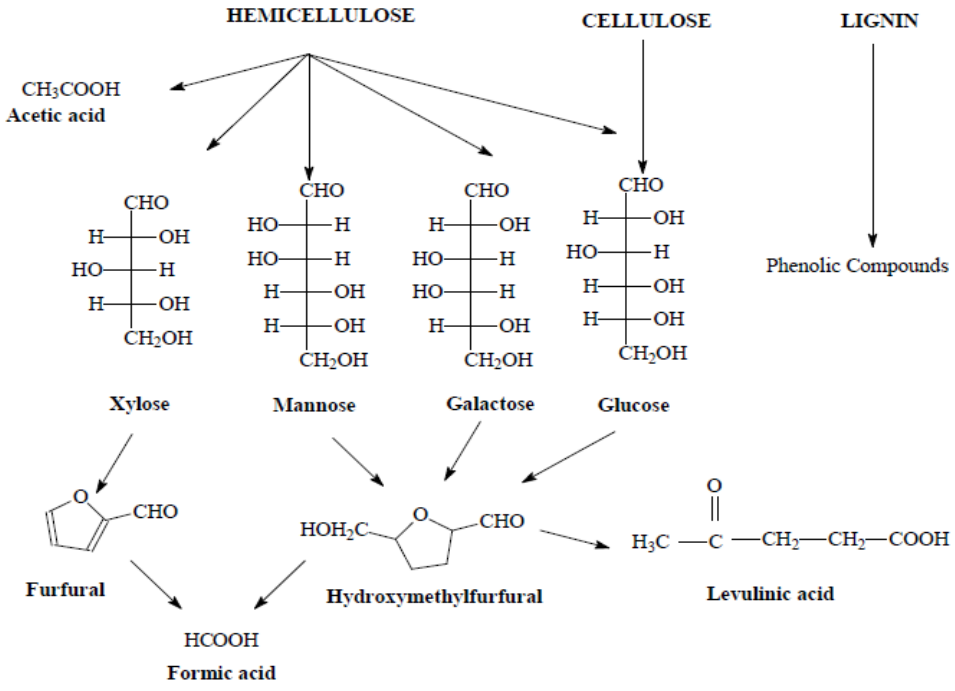

Figure 3.16 Formation of inhibitors during hydrolysis of lignocellulosic materials (adapted from Palmqvist and Hahn-Hagerdal, 2000).

These byproducts had various effects on microbial cell growth, metabolism, sugar utilization and lipid accumulation (Almeida et al., 2007, Huang et al., 2009). To remediate these problems, different detoxification methods have studies such as overliming, vacuum evaporation,

adsorption with active charcoal, ion-exchange resins or inhibitor degrading microorganisms (Huang et al., 2009, Nichols et al., 2005). Of course, all types of inhibitors are not responsible for every hydrolysate; their distribution depends on raw materials as well as operational condition employed for hydrolysis (Schirmer-Michelet al., 2008).

It was reported (Huang et al., 2009) that the classical overliming and vacuum evaporation could remove furfural completely while adsorption was effective in HMF removal. Moreover, combination of the three methods not only lowered the concentration of the inhibitors but concentrated the fermentable sugars in sulphuric acid treated rice straw hydrolysate. Similar result was explored by Parajo et al. (1998) and they stated that the concentration of inhibitors in rice straw hydrolysate was much lower than in other lignocellulosic hydrolysates and rice straw was so considered to be a good raw material for microbial oil production.

3.4.4 Liquid fermentation -Tomato waste hydrolysate (TWH)

3.4.4.1 Composition of Tomato waste

A number of authors have studied the composition of tomato waste and the most recent data we found from Valle et al. (2006). The Figure 3.17 has been composed using data from Abaza et al. (1987), Alvarado et al. (1999), Bhargava and Talapatra5 (1968), Cabrera et al. (1984), Valle et al. (2004), Edwards et al. (1952), Gupta et al. (1985), Latlief and Knorr (1983) and Valle et al. (2006). All of them agreed that fiber is the major component of tomato by-product up to 59% dwb (dry weight basis). TWHs also contain significant amount of protein, as well as remarkable amount of minerals (Figure 3.17). The TWH is sensibly composed with nutrient of microbial culture, especially for *L. starkeyi*. Yong-Hong et al. (2006) studied the optimal culture conditions for the lipid production by *Rhodosporidium toruloides* and investigated that fermentable C-compound should be present higher than that of N-compound in the culture media. They clearly stated that adding $ZnSO_4$, $CaCl_2$, $MnCl_2$, and $CuSO_4$ into the medium, to some extent, could increase lipid production, but the high metal ion concentration inhibited lipid accumulation too. Due to alike composition of TWH, *L. starkeyi* was able to grow in that medium without addition of any commercial nutrient.

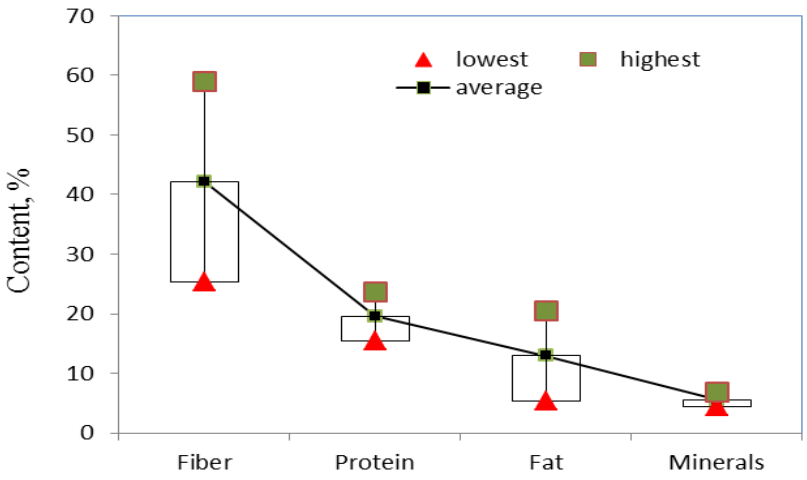

Figure 3.17 Composition of tomato waste (% dry weight basis)

3.4.4.2 Effect of Nitrogen content

Tomato waste hydrolysate (TWH) was categorized into three types (Fakas et al., 2008) depending on the nitrogen content- TWH_{HN}, TWH_{NN} and TWH_{LN}. Firstly, the influence of nitrogen was determined counting colony in three different nitrogen containing broth (Figure 3.11). It was observed that microbial growth was faster and maximum in TWH_{LN}. When growth became in equilibrium stage, additional nitrogen was introduced (not shown in the Figure 3.18) in all reactors after 139 hrs but no significant change was viewed, which indicated that lower nitrogen content was suitable condition for the growth of *L. starkeyi*.

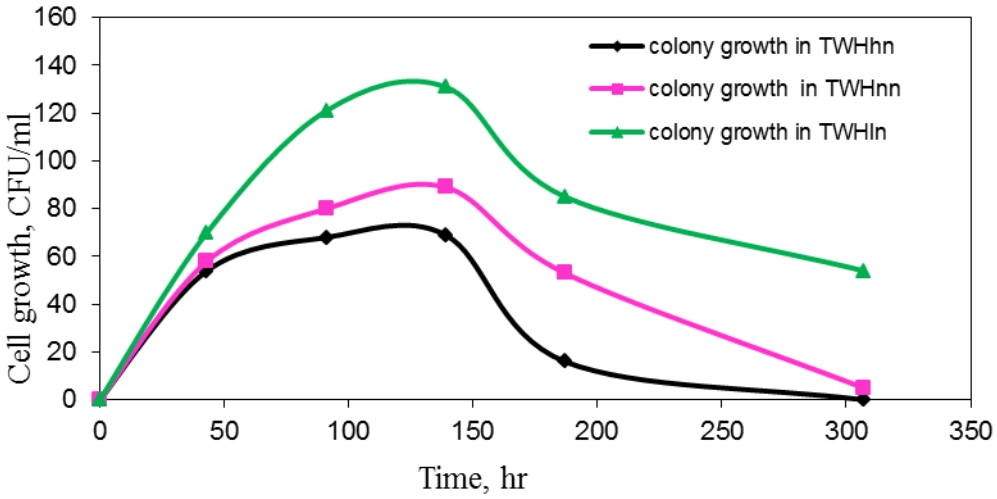

Figure 3.18 Effect of N content on the culture of *Lipomysis sterkeyi* in the broth of TWH

3.4.4.3 Effect of the dilution

The effect of the dilution was studied to enhance the growth rate of *L. starkeyi* is shown in the Figure 3.19. Dilution would help to reduce the concentration of antimicrobial agent if present in the broth. Growth rate was observed with 100% TWH (TWH-100), 50% TWH (TWH-50) and 25% TWH (TWH-25). In the case of TWH-100 and TWH-50, growth rate was almost similar but biomass production was maximum in TWH-100.

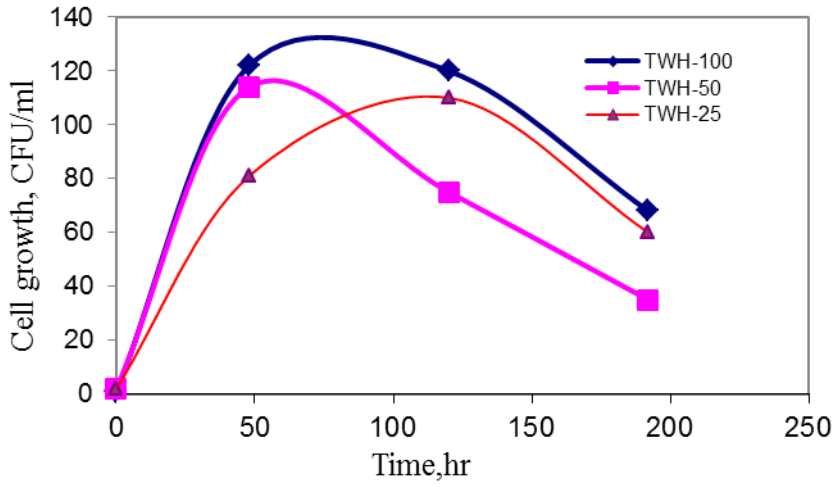

Figure 3.19 Effect of dilution on the culture of *Lipomysis sterkeyi* in the broth of TWH

On the other hand, since microorganism grew faster and biomass production was higher in 100% broth of TWH, we tried to accelerate the cell growth with making dilution after stationary phase. It seems to us, this dilution would reduce the concentration of inhibitors- initially present and produced by the metabolic function. We considered the stationary phase from 72 to 168 hrs and the Figure 3.20 showed that dilution after stationary phase didn't had any effect on the cell growth.

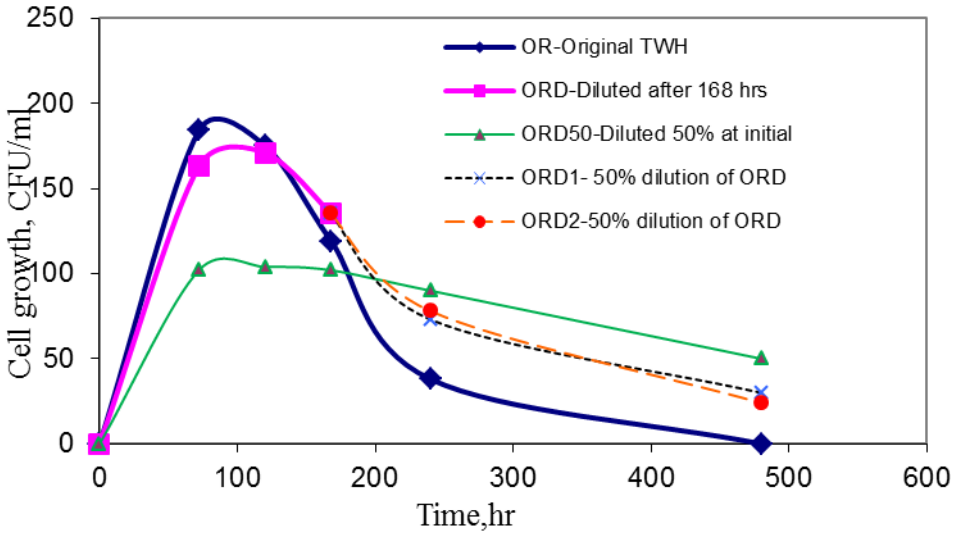

Figure3.20 Effect of dilution on the culture of *Lipomysis sterkeyi in* the broth of TWH_{OR}

3.4.4.4 Biomass and lipid yield

From Table 3.9, it was clear that with more dilution of medium biomass yield and lipid content gradually decreased. This may cause of lowering of nutrient in per unit volume of fermentation broth. There was another possibility, the effect of inhibitors was minor or absent. On the other hand, lowering of nitrogen had no significant effect on the biomass yield as well as lipid content. Though, biomass production was not satisfactory level compared to Fakas et al. (2008). But they described similar result, TWH_{NN} medium was suitable in terms of biomass and lipid production. However, it was promising that *L. starkeyi* was able to grow in the medium of TWH without addition of any commercial nutrition. Even, biomass yield was lower when external N-source was added to medium (TWH_{HN}).

Table 3.9 Biomass yield in the medium of TWH

Medium	Biomass yield, g/l	Lipid content, %
TWH-100	2.32	10.08
TWH-50	1.69	8.48
TWH-25	0.50	7.78
TWH_{NN}	2.68	9.23
TWH_{LN}	2.50	9.87
TWH_{HN}	2.18	8.43

3.4.5 Sorghum (*Sorghum bicolor)*

3.4.5.1 Cultivation and Composition of Sorghum plant

One of the prime sources investigated as energy crops is sweet Sorghum (*Sorghum biocolor*). Although a native to the tropics, sweet Sorghum is well adapted to temperate climates (Gnansounou et al., 2005; Kangama and Rumei, 2005). It is also highly resistant to drought and salinity, and has a remarkable yield potential even in marginal environments (Cosentino, 1996; Foti et al., 1996; Steduto et al., 1997; Amaducci et al., 2004). Sorghum is the fifth leading cereal in terms of world production and one of the coarse grain cereals grown as a rain fed crop in the semi arid areas (Push Pamma, 1993; Dendy, 1995). It is grown in approximately 50 million hectares with a production of 70 million tons (Food and Agriculture Organization, 1994; National Research Council, 1996). More than 300 million people in more than 30 countries depend on Sorghum as the main source of energy and protein (NRC, 1996). Almost 30% of the harvested Sorghum area is in sub Saharan Africa (Food and Agriculture Organization, 1993). In eastern Africa, more than 70% of Sorghum is cultivated in the dry and hot low lands (Mukuru, 1993). The sweet Sorghum cultivars exhibited the production of Stems dry weight (SDW) increased from 5.5– 23.9 t ha^{-1} (Zhao et al., 2009)

Sweet Sorghum has been recognized as one of the most promising ethanol crops in China (FAO, 2002; Gnansounou et al., 2005; Kangama and Rumei, 2005). Although dry matter and sugar accumulation of sweet Sorghum have been documented, little is known about differences in total soluble sugar, cellulose, hemicellulose, and grain yield as a whole for energy purposes. Moreover, it will be crucial to plant cultivars with different crop cycles in order to increase the harvest period for industrial scale production of ethanol from sweet Sorghum. It was found to

contain 43.6–58.2% soluble sucrose, glucose and fructose in the stalk (Billa et al., 1997; Dolciotti et al., 1998; Amaducci et al., 2004; Antonopoulou et al., 2008) and 22.6–47.8% insoluble cellulose and hemicellulose (Dolciotti et al., 1998; Rattunde et al., 2001; Antonopoulou et al., 2008).

In the present study, the composition of Sorghum was found as 44.4% cellulose, 41.9% hemicellulose and 13.7% lignin regarded in the first approximation as a mixture of cellulose, hemicellulose, and lignin. In the acid hydrolysis process cent percent hemicellulose was decomposed to hydrolysate (Figure 3.21) and little fraction of cellulose (7.5%) disintegrated to liquid medium. But total fraction of lignin remained in the solid residue and also most of the part of cellulose.

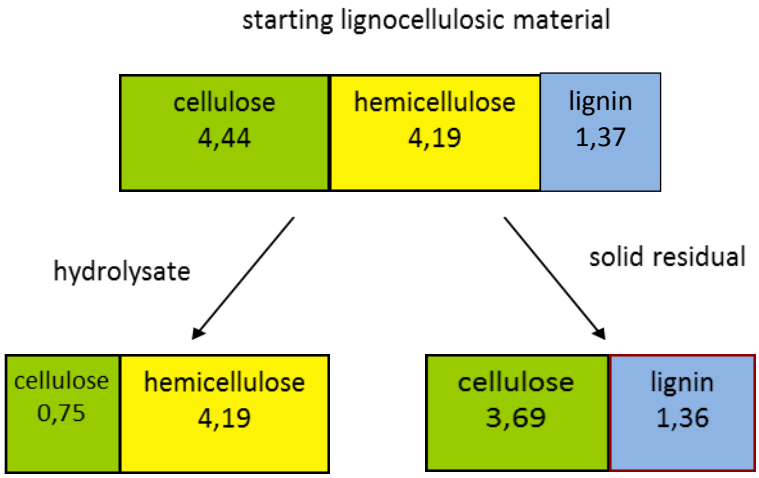

Figure 3.21 Composition of stem of Sorghum and their distribution to hydrolysate and residual fraction (values on basis 10)

3.4.5.2 Optimization of cultural medium

Microorganism was cultured in the broth containing 100%, 50% and 25% of hydrolysate of Sorghum stem. There was no microorganism grown in 100% hydrolysate medium. It might occur due to higher concentration of inhibitors. Growth rate was higher in 50% broth than 25% and reached at maximum within 7 days (150-200 hrs) (Figure 3.22). Higher concentrated broth consumed lower amount of fresh water. This concept leads to produce microbial oil through solid-state fermentation (Economou et al., 2010; Fakas et al., 2009).

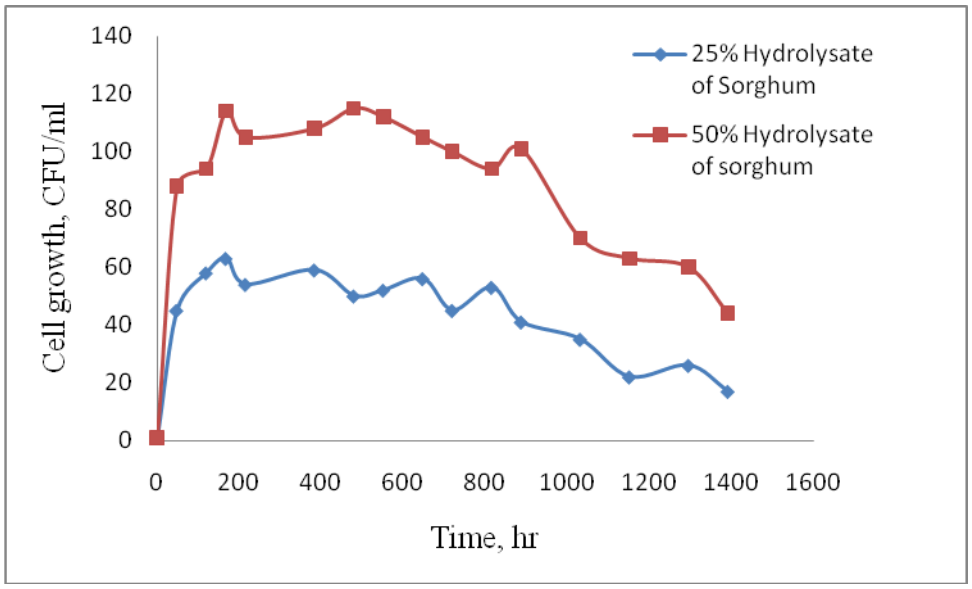

Figure 3.22 Optimization of cultural medium (Sorghum) in terms of dilution

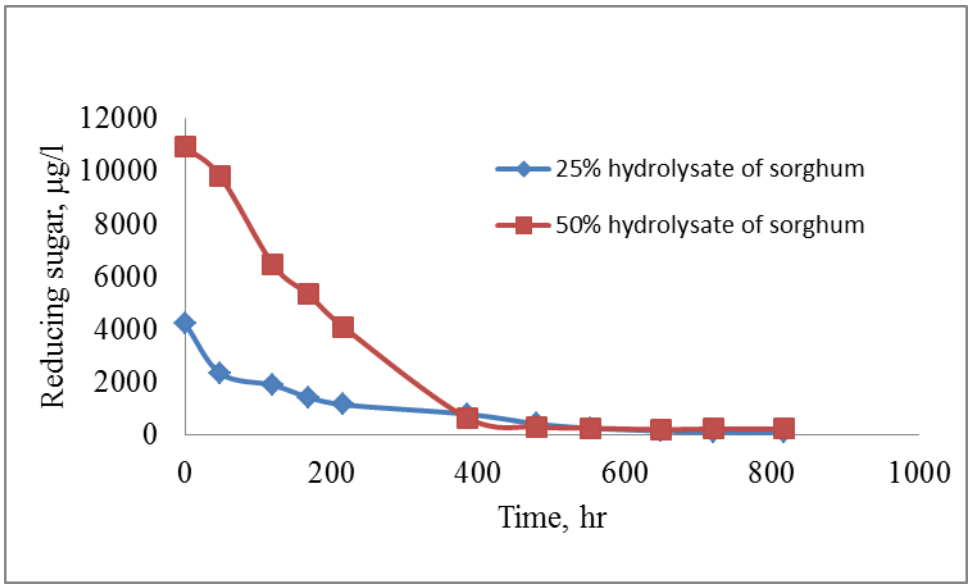

Figure 3.23 Change of reducing suagr during the growth of *L. starkeyi* in the medium of Sorghum hydrolysate

3.4.5.3 Growth kinetics

Kinetics growth profiles of GRS and SGM were almost similar in sequence. However, colony formation unit (CFU) was a little bit higher for GRS and survival period was also longer. On the other hand, dry biomass yield was lower than that of SGM, even lipid content. The possible reason of the different kinetics behavior is the effect inhibitors (Hu et al., 2009).

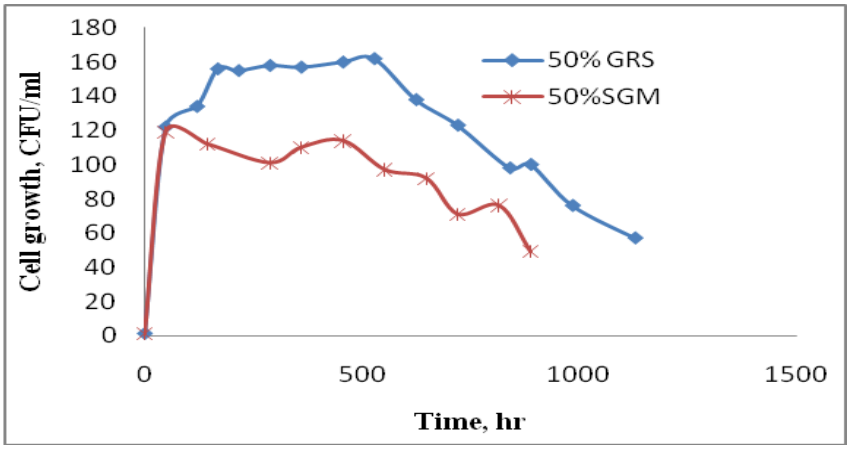

Figure 3.24 Growth kinetics of microorganism in cellulosic hydrolysate

3.4.5.4 Nutrient consumption by *L. starkeyi*

Consumption of fermentable sugar by *L. starkeyi* in hydrolysate medium of SGM and GRS is illustrated in the Figure 3.25 and 3.29 respectively. In both cases, cell growth was correlated with the sugar utilization from the beginning of the fermentation. Sugar concentration was gradually declining with respect to the microbial cell growth up to 200-300hrs. When cell growth was stopped, no significant change of reducing sugar was observed. Similar growth profile was

described for *Trichosporon fermentans* cultured in cane molasses (Zhu et al., 2008) and *Mortierella isabellina* cultured in sweet Sorghum (Economou et al., 2010). So, sugar was the main C nutrient and growth factor of *L. starkeyi*.

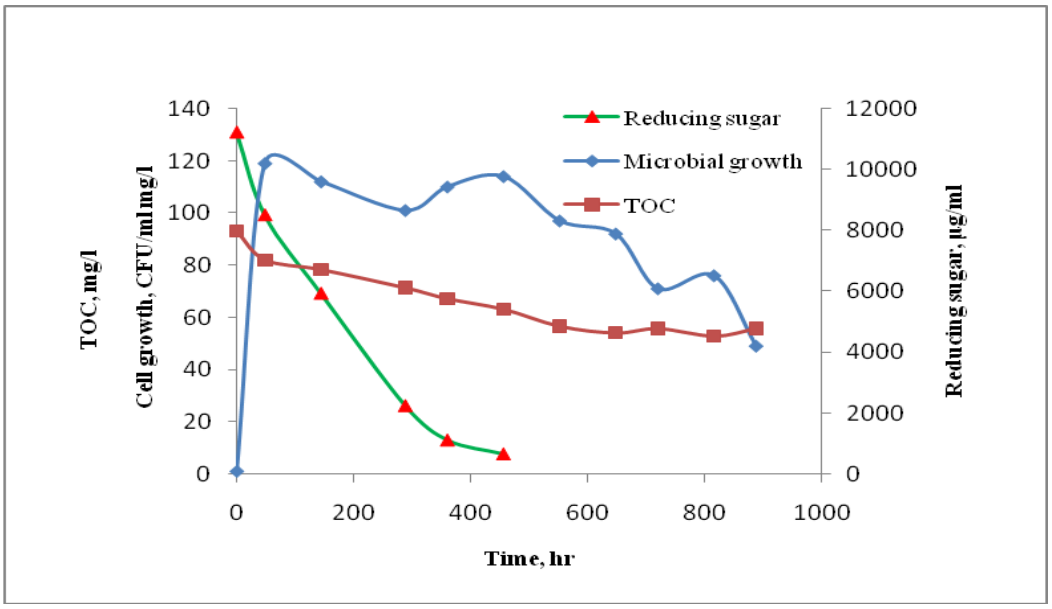

Figure 3.25 Change of C sources with microbial growth of *L. starkeyi* in SGM

In the same time, TOC values were also falling with growth of microbes but it never be finished. It seemed to almost constant when growth of cell was stopped. This phenomena of TOC indicated that hydrolysate medium contain other C sources which were not metabolized by *L. starkeyi*.

3.4.5.5 Biomass and Lipid yields

Figure 3.26 Microbial biomass, cultured on hydrolysate of lignocellulosic materials

Optimistic result of lipid accumulation by *L. starkeyi* was observed using GRS and SGM hydrolysate, considering them as alternative C and N sources. Superior lipid accumulation was occurred in the bioconversion of SGM hydrolysate due to the comparatively higher C/N ratio (53.28) than that of GRS hydrolysate (Table 3.10).

Table 3.10 Comparison between Giant reed stem and Sorghum in terms of biomass and lipid production

Carbon sources	Initial C/N	Dry Biomass yield (g/l)	Lipid content (%)	Lipid yield (g/l)
Giant reed stem	30.95	2.76	7.08	0.196
Sorghum	53.28	2.89	15.53	0.449

Whereas Angerbauer et al., mentioned that lipid productivity of *L. starkeyi* at C/N ratio 150 and 60 were similar (6.4g/l and 5.9 g/l). Though cell growth was faster in GRS (Figure 3.17), lipid yield (0.449 g/l) was significantly higher in SGM than that of GRS hydrolysate medium. It may cause of favorable composition (e.g. C/N ratio, lower inhibitors) of hydrolysate. Our next paper will exemplify the reason very precisely.

3.4.6 Giant Reed Stem (*Arundo donax)*

3.4.6.1 Optimization of cultural medium

As same as Sorghum, Microorganism was cultured in the broth containing 100%, 50% and 25% of hydrolysate. There was no microorganism grown in 100% hydrolysate medium. It might occur due to higher concentration of inhibitors. Growth rate was higher in 50% broth than 25% and reached at maximum within 7 days (150-170 hrs) (Figure 3.27). Higher concentrated broth consumed lower amount of fresh water. So, it was tried to keep the medium at higher concentration to avoid loss of fresh water an extra cost.

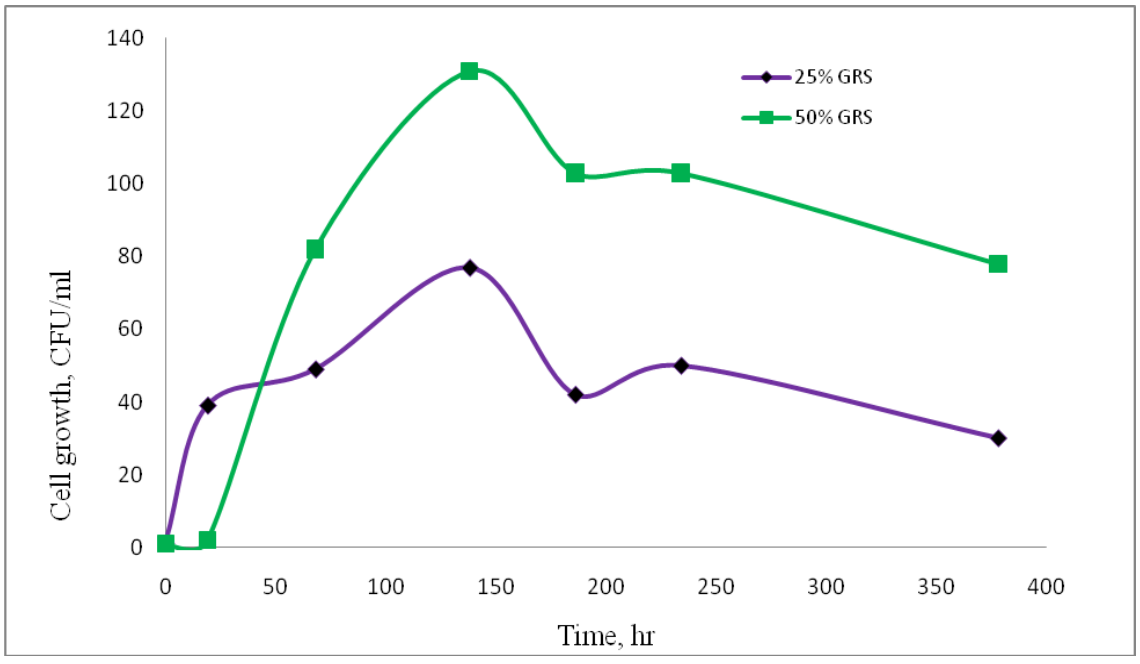

Figure 3.27 Optimization of cultural medium of hydrolysate of Giant Reed Stem in terms of dilution

3.4.6.2 Effect of cultivation conditions

Arundo donax is a hydrophyte, growing along lakes, streams, drains and other wet sites. It uses prodigious amounts of water, as much as 2,000 L/meter of standing *A. donax*, to supply its incredible rate of growth (Purdue 1958; Iverson 1994). Under optimal conditions it can grow more than 5 cm per day (Purdue 1958). *Arundo donax* stands are among the most biologically productive of all communities. Under ideal growth conditions they can produce more than 20 tons per hectare above-ground dry mass (Perdue 1958). Samples of Giant reed stem (GRS) were collected from different area cultivating with various conditions. In the Table 3.11, water '0' means, no water was sprayed from artificial sources (tap or shallow) and nitrogen fertilizer '100' means, synthetic nitrogen fertilizer was used in cultivation.

Table 3.11 Sample description

Sample	**Area**	**Cultivation conditions**		**Total nitrogen in hydrolysate medium, mg/lt**	**Hemicellulose Content (%)**
		Water	N fertilizer		
A	S.A. Lombardi	0	100	223	28
B	Torre Lama	100	100	125	30
C	Torre Lama	0	100	312	33

Growth rate was observed in 50% diluted hydrolysate of all three samples in triplicate. Sample C showed better performance (Figure 3.28) in terms of CFU/ml.

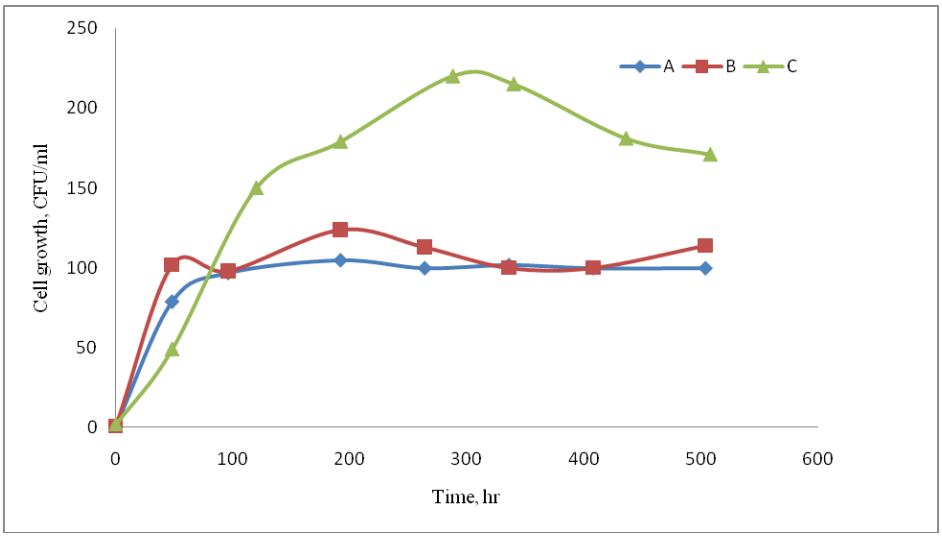

Figure 3.28 Effect of cultivation condition on cell growth of *L. starkeyi*

Table 3.12 Biomass and lipid yield

Sample	Biomass (g/l)	Lipid content (%)	Lipid yield (g/l)
A	1.00	9.725	0.195
B	1.12	12.525	0.281
C	1.82	11.425	0.415

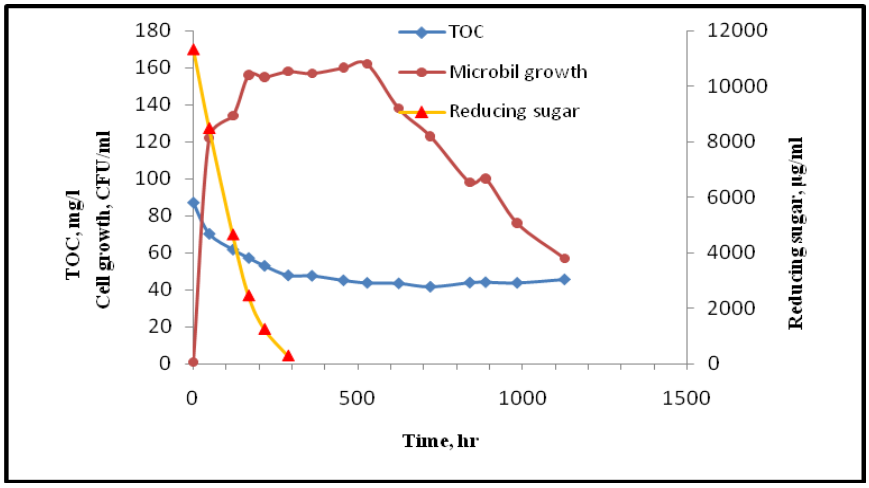

Figure 3.29 Change of C sources with microbial growth of *L. starkeyi* in GRS

3.4.6.3 Effect of Temperature

Microbial growth was studied at different temperature like 15˚C, 20˚C, 30˚C and fluctuated (T-F). According to Figure 3.30, growth rate of *L. starkeyi* was slower at lower temperature (T-15, T-20) than that of higher and fluctuated temperature (T-30, T-F). It is interesting that growth profile of *L.starkeyi* was almost similar at T-30 and T-F, which indicated that it would be cultured in open environment or in uncontrolled temperature.

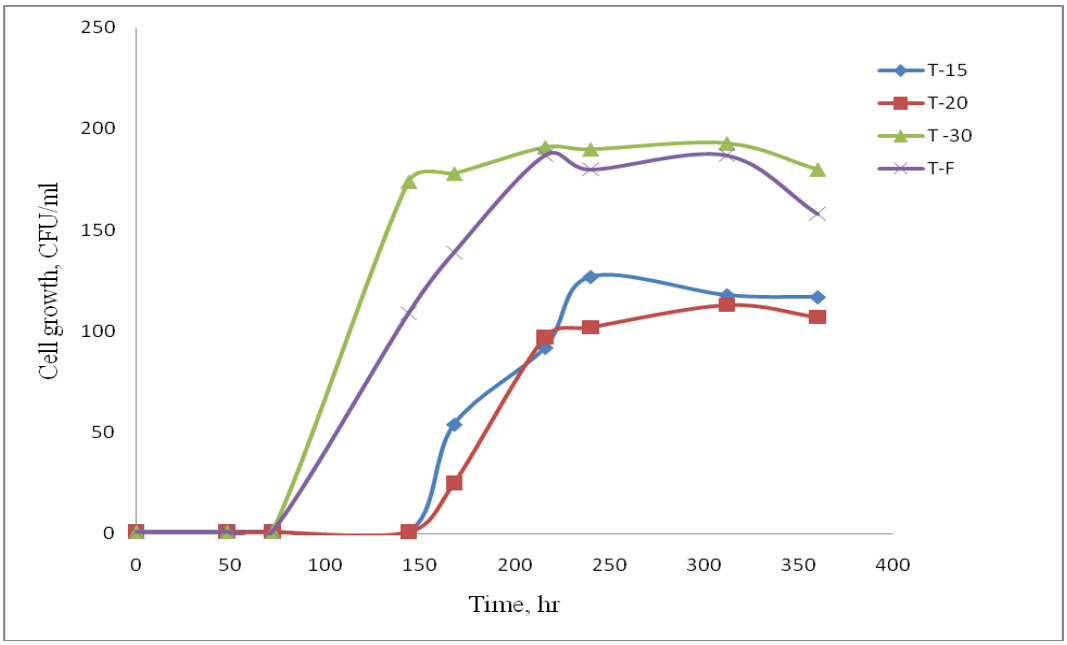

Figure 3.30 Growth profile of *L. starkeyi* at temp. 15˚C, 20˚C, 30˚C and fluctuated (15-30˚C)

The influence of temperature on biomass and lipid yield was not significantly high (Table 3.13). Only it varies in fluctuated temperature. May be this condition interrupted the metabolic function of *L. starkeyi.*

Table 3.13 Effect of temperature on Biomass and Lipid yield

Temperature	Biomass yield, g/lt	Lipid content, %	Lipid yield, g/lt
T-30	4.3	8	0.344
T-F	3.8	7.2	0.275
T-20	4.5	7.6	0.345
T-15	4.9	7.7	0.385

3.4.6.4 Efficiency of detoxification methods

To improve the fermentability of the lignocellulosic hydrolysates, various detoxification methods have been studied such as extraction with organic solvents (Wilson et al., 1989), overliming (Palmqvist et al., 1996; Larsson et al., 1999; Martinez et al., 2001), evaporation (Larsson et al., 1999), steam stripping (Maddox and Murray, 1983; Yu et al., 1987), sulfite treatment (Larsson et al., 1999; Parajó et al., 1997), ion-exchange (Nilvebrant et al., 2001), enzyme treatment (Jönsson et al., 1998; Palmqvist et al.,1997), zeolite treatment (Eken-Saraçoˇglu and Arslan, 2000) and activated carbon treatment (Maddox and Murray, 1983,Gong et al., 1993).

In this study, we implemented overlime and activated charcoal to remove inhibitors like furfural and phenolic compounds. In the previous experiments, microorganisms were not able to grow in 100% hydrolysate of *Arundo donax.* As a result, hydrolysates were diluted to 50% and it

was fermentable but this process consumed huge amount of fresh water. Therefore, for achieving high fermentability, detoxification of hydrolysates is necessary before the fermentations to remove the inhibitory compounds.

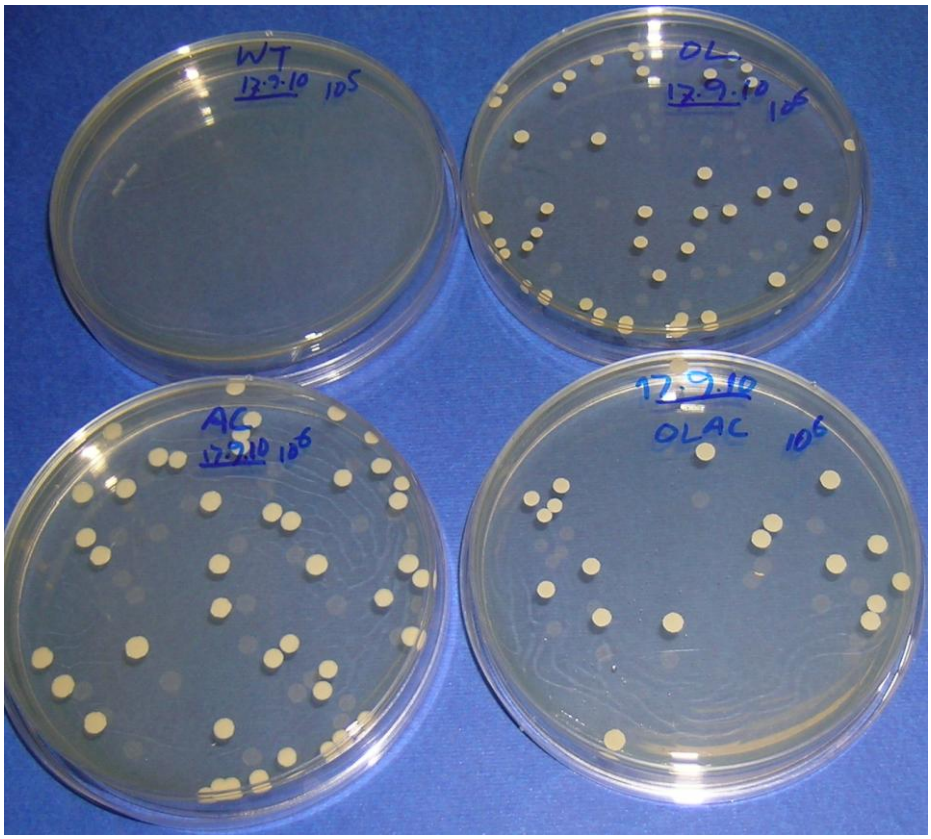

Figure 3.31 Growth of microorganisms on different Detoxified medium (WT-, OL, AC, OLAC)

The hydrolysates was detoxified in three ways i) overlimed ii) treated with activated charcoal and iii) both together. Figure 3.32 illustrated the influence of these three methods on the growth of *L. starkeyi*. Untreated hydrolysate was totally unfermentable (see Figure 3.31) and *L. starkeyi* was unable to survive due to high inhibitory activity. Activated charcoal showed better influential role to remove toxic compounds than others.

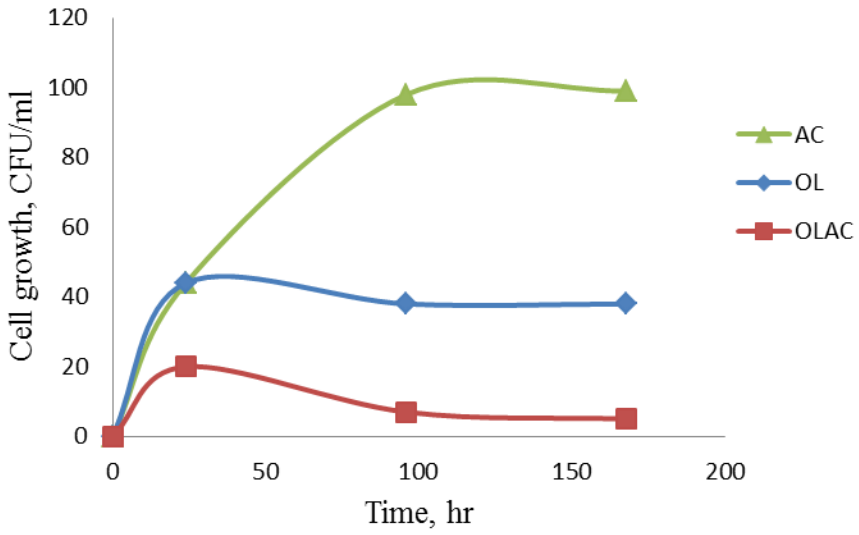

Figure 3.32 Growth of *L. starkeyi* in the detoxified mediums

In a previous report on the detoxification of the hydrolysates by using activated carbon with high absorptivity, not only removed inhibitors that can affect fermentations but also some amounts of the fermentable sugars. On the other hand, Miyafuji et al. (2003) found almost the same concentrations of various sugars in the hydrolysates treated with the wood charcoals and in the untreated hydrolysate. These results indicated that the wood charcoals were capable in selectively removing the inhibitors such as furan and phenolic compounds without removing the fermentable sugars. Whereas in the present study, activated charcoals and overliming removed inhibitors as well as significant amounts of reducing sugars (Table 3.14). But growth kinetics of *L. starkeyi* (Figure 3.32) and biomass yield proved that activated charcoals efficiently removed inhibitors than others.

Table 3.14 The influence of different detoxification methods on RS, TOC and biomass yield

Sample	Reducing Sugar, µg/ml	TOC, mg/lt	Biomass yield, g/l
WT	32415	39600	00
AC	25041	29840	4.073
OL	25433	35164	1.808
OLAC	18204	20580	1.958

Furthermore, Martinez et al. (2001) demonstrated that overliming was effective for removing 51±9% of the furan and 41±6% of the phenolic compounds. However, sugars were also removed from the hydrolysates after overliming treatment at about 8.7 ± 4.5%. Similar effects of the overliming on the hydrolysates were confirmed in other research on the overliming combined with zeolite treatments (Eken-Saraçoˇglu and Arslan , 2000). It was also reported that the detoxification with lime was a costly method and may contribute to total ethanol production costs up to 22% (Von et al., 1994). When hydrolysate was treated with both OL and AC, reducing sugar was removed by 44%. As a result microbial growth was unfavorable.

3.5 Recycle of glycerol as carbon source

3.5.1 Recover of the co-produced glycerol

Glycerol is a co-product of the triglyceride alcoholysis, carried out to obtain biodiesel. The reduction of the price of the glycerol, caused by the increase of the biodiesel production, is a main concern for the commercial feasibility of the process. This also an alternative way of glycerol valorization to its biotransformation to single cell oil (SCO) by oleaginous yeast and moulds (Papanikolaou and Aggelis , 2002). A number of investigations dealing with the utilization of glycerol as carbon source by various yeast and moulds is restricted and majority of these studies are referred to production of biomass and various extracellular and intracellular proteins (D'Anjou and Daugulis ,2000; Minning et al.,2001). We have recycled glycerol as a carbon source for different oleaginous yeasts, such as *Lipomyces starkeyi, Yarrowia lipolitica and Criptococcus curvatus,* in order to increase the biodiesel yield of the process.

The recycle of the co-produced glycerol increases the flexibility of the process, offering a method to modulate the C/N ratio of different waste materials. This is an important aspect because, as already pointed out previously, the lipid yield of the oleaginous yeasts is critically affected by the C/N ratio. In principle, a feedstock with high nitrogen content can be making suitable for oleaginous yeasts culture by suitable addition of glycerol.

3.5.2 Culture of Lipomyces starkey in the presence of glycerol

The *Lipomyces starkeyi* have been cultured in liquid media, using glycerol as the sole source of carbon. The Figure 3.33 reports the growth curves.

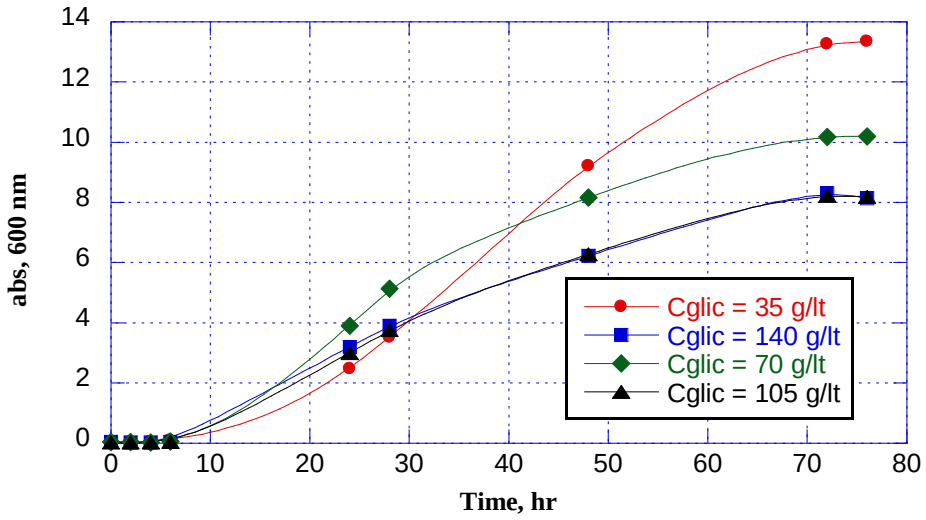

Figure 3.33 Growth profile of *Lipomyces starkeyi* using glycerol as the sole carbon source

The experimental data show that, in the presence of glycerol concentrations higher than 35 g/lt, inhibition phenomena occur, as demonstrated by the lower concentration of biomass obtained.

3.5.3 Culture of *Yarrowia lipolytica* in the presence of glycerol

The tests carried out growing *Yarrowia lipolytica* using glycerol as the sole carbon source are described in the Figure 3.34.

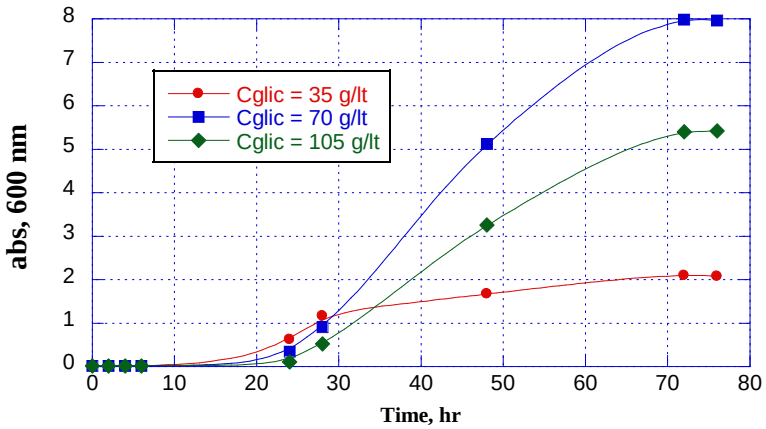

Figure 3.34 Growth profile of *Yarrowia lipolytica* using glycerol as the sole carbon source

The experimental data show that *Yarrowia* growth is affected by inhibition phenomena in the presence of glycerol concentrations higher than 70 g/lt. Consequently, *Yarrowia lipolytica* appears to be more suitable for the use of glycerol as carbon source. Similar growth was observed when industrial glycerol was used as feedstock for the growth of *Y. lipolytica* (Papanikolaou and Aggelis , 2002). They observed the growth rate of 6.0-7.5 g/l in increased industrial glycerol concentration media (45,80 and 120 g/l) and during flask cultures low amount of storage lipid accumulated inside the yeast cell (0.05-0.10 g/g).

3.5.4 Culture of *Cryptococcus curvatus* in the presence of glycerol

The Figure 3.35 describes the growth of *Cryptococcus curvatus* using glycerol as the sole carbon source.

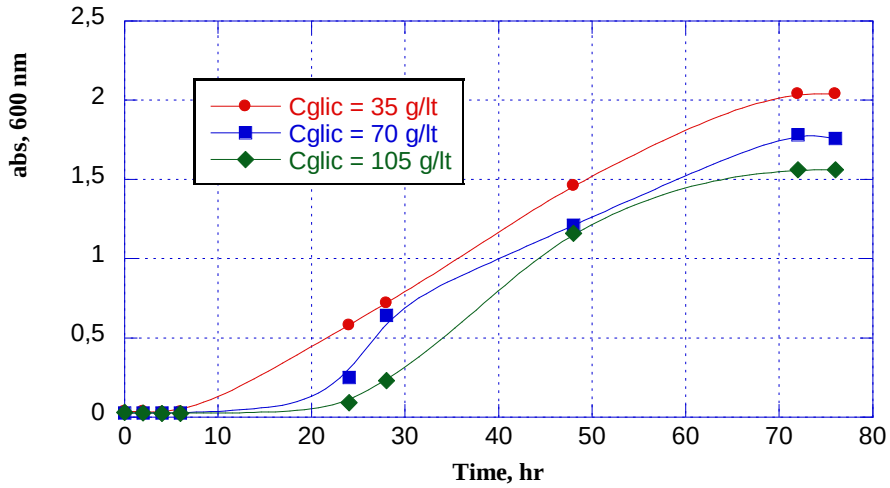

Figure 3.35 Growth profiles of *Cryptococcus curvatus* using glycerol as the sole carbon source

In this case, inhibition phenomena are observed at glycerol concentrations higher than 35 g/lt, though the reduction of the biomass concentration obtained is not significant. Whereas Meeesters et al. (1996) described that *Cryptococcus curvatus* strain showed restricted cell growth, when glycerol concentration was higher than 64 g/l. There is possibility having the long array of glycerol tolerance of C. *curvatus*. So, it needs further study to optimize the initial glycerol concentration varying other parameters like pH, C/N, agitation, temperature etc to make able *C curvatus* for consumption of higher amount of glycerol.

3.5.5 Lipid fraction obtained by the culture in the presence of glycerol

The lipid fractions of the oleaginous yeasts grown using glycerol as the sole carbon source are reported in the Table 3.15.

Table 3.15 Lipid fractions using glycerol as the sole carbon source

Microbes	Lipid fraction, %
Lipomyces starkey	5.0
Yarrowia lipolytica	9.2
Cryprococcus curvatus	11.5

The lipid fractions are relatively low probably better results can be obtained mixing glycerol with different waste materials.

CONCLUSIONS

The experimental results obtained demonstrate the following conclusions:

– Different waste materials, such as olive-mill wastewaters, as well as different lignocellulosic materials, can be used as starting materials for the production of microbial oils, i.e. for the growth of oleaginous microorganisms.

– Oleaginous yeasts have been grown also using glycerol as the sole carbon source, offering a useful application of the glycerol, co-produced when biodiesel is obtained by triglyceride alcoholysis. In this case, however, the lipid fractions obtained were quite low.

– In all instances, the experimental data demonstrate that microorganisms generate triglycerides for energy storage only when the ratio C/N is sufficiently high. Consequently, the amount of nitrogen in the culture medium should be optimized, as excess nitrogen concentrations lead to reduced triglyceride yields, whereas in absence of nitrogen the biomass growth is stopped. As regards the concentration of sugars, these should never be too low, as the oleaginous microorganisms, in the absence of sugars, tend to metabolize the lipids as nutrient, therefore reducing the triglyceride yield.

– The culture of oleaginous microorganisms in the presence of olive-mill wastewater (OMW) was of special significance as regards the environmental benefits. As a matter of facts, the microbial activity has lead to a significant reduction of phenols in the OMW, and eventually to a decrease of their phytotoxicity.

– The fermentation of hydrolysates of lignocellulosic materials is of great strategically importance, due to the abundance of the agricultural and forestal residues, offering a renewable feedstock for the production of biofuels.

– The microbial oils can be used as feedstock for the synthesis of biodiesel by methanolysis. The quality of the biodiesel produced is satisfactory as regards both the cold behavior and the oxidation stability.

The future development of the research work will be aimed to achieve the following objectives:

i) Development of methods for the hydrolysis of lignocellulosic biomass, allowing higher sugar yields. In this view, the enzymatic hydrolysis appears very promising, though improvements are required as regards different aspects of the process, in particular the enzyme separation and reutilization after the biomass treatment.

ii) A more complete characterization of the waste materials potentially useful for the production of microbial oils. Currently, II nd generation biofuels are sought, to produce with no utilization of fertile soils. Consequently, lignocellulosic materials should be obtained from plants able to grow in the presence of semi-fertile soils, usually not exploited for agriculture.

iii) Optimization of the fermentation of oleaginous microorganisms, to obtain higher levels of lipid concentrations.

iv) Optimization of the methanolysis of the microbial oils, to increase the biodiesel yields. As the microbial oils contain high levels of free fatty acids (FFA), suitable catalysis are required, in that the traditional alkaline catalyst (NaOH) may interact with FFA, leading to soap formation and reducing the biodiesel yield, as well as the quality of the co-produced glycerol. The enzymatic synthesis of the biodiesel, based on the use of microbial lipases, offer significant improvements in the process efficiency.

Appendix A

Remediation of Waters Contaminated with MCPA by the *Lipomyces starkeyi* Entrapped in a Sol–Gel Zirconia Matrix

A.1 Introduction

Most experiments reported in this thesis have been carried out using the yeast *Lipomyces starkey*. The natural habitat of *Lipomyces starkey* is soil and ensilage (Lodder, 1970), where the microorganisms degrade carbohydrates using extracellular carbohydrolases, and contribute to the biodegradation of herbicides (Nishimura et al., 2002). The ability of *Lipomyces* to metabolize herbicides has been studied as this property of the yeasts can be useful in some instances, e.g. to use wastewaters polluted with herbicides as feedstock for the production of biodiesel.

A.2 Herbicide

4-chloro-2-methylphenoxyacetic acid (MCPA) is a phenoxy acid herbicide widely used in agriculture for post-emergence control of weeds in cereals, grasslands, trees, and turf. It is relatively soluble (273.9 mg L^{-1} in water at neutral pH), highly mobile, and can leach from soil. Consequently, this compound has been found in well water in some countries and has been classified by the U.S. Environmental Protection Agency (EPA) as a potential groundwater contaminant (Walker et al., 1992). For this reason, the risk of environmental pollution of soil and surface waters must be considered, and the development of new remediation technologies appears to be of primary importance.

A.3 Immobilization of yeasts in sol-gel materials

Cells immobilization offers a valid strategy for cleaning up the environment from herbicides (Wang et al., 2007, Chen et al. 2002) As a matter of facts, it is generally thought that microbial metabolism provides a safer, more efficient, and less expensive alternative to physico-chemical methods for pollution abatement (Kandimalla, 2006). The immobilization of cells within a stable matrix system may lead to the efficient use of their physiological capabilities (Bottcher, 2004), e.g. for producing secondary metabolites, or in biotransformation/biocatalysis reactions, so it can be very advantageous for several biotechnological applications. Further benefits stemming from the use of immobilized cells are the easier preparation of some metabolic products, as well as their protection from environmental stresses. In addition, the use of immobilized cells helps in recovery and reuse of the embedded cells, allowing a repeated use of the biocatalyst in batch bioreactors. Finally, immobilized cells can be easily segregated in a tank, allowing increased throughput in continuous reactors with reduced risk of washout, therefore lea ding to obvious economical benefits.

Numerous immobilization techniques such as physical adsorption, covalent attachment, and entrapment in polymer or inorganic matrices have been explored over the years to achieve high-yield, reproducible, and robust immobilization techniques preserving the activity of microbial cells. So far, no single method or material has emerged as the better for every application and ongoing efforts strive to optimize these methods to render them adequate for specific applications.

Sol–gel nanomaterials are gaining a growing importance as solid supports for the immobilization of biomolecules to be used for biocatalysis, biosensors, and biomedical

applications, offering an useful alternative to the traditional polymer technology (Avnir, 2006). Due to their porous structure in a nanometric scale, sol–gel materials offer unique intrinsic properties, such as high surface to volume ratio, large surface area and porosity. In addition, the sol–gel process offers higher flexibility as regards the surface functional groups, and the matrix composition. Nanoporous materials, in particular the inorganic oxide materials obtained using metallorganic precursors, are usually non-toxic, chemically and thermally stable, so they have wide applications where biocompatibility and thermal stability requirements are essential. Moreover, the chemical nature of the surface of nanopores can be tailored in order to increase the stability and catalytic activity of the biomolecules. .

A.4 Experimental

The *Lipomyces starkeyi* cultivated in a synthetic medium, containing (g L^{-1}): KH_2PO_4 (Serva), 1.0; $MgSO_4$ $7H_2O$ (BDH), 0.5; $(NH_4)_2SO_4$ (Carlo Erba), 2.0; yeast extract (Fluka) 0.5; glucose 70.0 (Fluka) under aerobic conditions at 30 °C on a rotary shaker at 160 rpm. The yeasts were harvested during their exponential growth phase to ensure the highest activity and number.

In a typical immobilization test, 200 mg of lyophilized *Lipomyces* were dissolved in 3.0 mL of physiologic solution and the mixture was stirred in ultrasonic bath up to obtain a suspension with a good dispersion degree. A 5.5 mL volume of 1-propanol (>99.80%) was then added to such suspension that was subsequently mixed with a solution containing 10 mL of Zirconium (IV) propoxide (70 wt.% in 1-propanol), 1.5 mL of acetylaceton (>99%) and 3.0 mL of 1-propanol. Under these conditions, gelation occurred after about 20 min at room temperature, keeping a vigorous stirring. A homogeneous slightly yellow coloured gel was produced. The gel was left at room temperature for 3 h and than lyophilized, in vacuum at -50 °C for 20 h, to give

the final material. Zirconia gel was prepared and dried under the same conditions. Moreover, gels were also obtained starting from an equivalent amount of not lyophilized yeasts according to the above procedure in order to check the possible influence of this parameter on the MCPA removal.

The characterization of the Zirconia gel was characterized by Fourier-transform infrared (FTIR) spectroscopy, as describred in Sannino et al., 2010.

Specific tests were carried out to evaluate the ability of *Lipomyces* to remove the 4-chloro-2-methylphenoxyacetic acid (MCPA). In a typical MCPA removal experiment, a stock solution of herbicide was prepared dissolving 100 mg of MCPA in 500 mL of Milli-Q ultrapure water (final concentration 200 mg L^{-1}), and subsequently kept refrigerated. MCPA-removal experiments were performed in batch conditions incubating 10 mg of *Lipomyces* entrapped in zirconia (ZrO_2-*Lipomyces*) with 1 mL of MCPA 200 mg L^{-1}; it is noteworthy that this value is very close to the MCPA solubility. Tests were also carried out using immobilized biomass preliminary incubated at 100 °C for 24 h. In addition, control experiments were made using 10 mg of not immobilized *Lipomyces* or 10 mg of pure zirconia.

Blanks of MCPA in aqueous solution were analyzed in order to check pesticide stability and possible sorption to vials. After incubation in a thermostatic rotary shaker at 30 °C, the samples were centrifuged at 7000 *rpm* for 20 min.

The MCPA was analyzed with an Agilent 1200 Series HPLC apparatus. The removal (%) of MCPA (X) was calculated by the following balance equation:

where c_0 is the initial concentration of MCPA (mg L^{-1}) and c_1 the MCPA concentration at the end of the incubation (mg L^{-1}).

Kinetic tests were conducted adopting incubation times of 1, 2, 3, 4, 8 and 24 h at 30 °C. After centrifugation, the supernatants were analyzed as described above.

Sorption isotherm was obtained adding different volumes of a stock solution of herbicide (200 mg L^{-1}) to gel-derived ZrO_2 to give an initial concentration ranging from 0.05 to 200 mg L^{-1} of MCPA. After incubation and centrifugation, the supernatants were analyzed as described above.

Degradation/sorption tests were performed adopting both different incubation times (from 1 to 24 h) and temperatures (from 30 to 50 °C). Because the almost complete removal of the herbicide occurred within 1 h and no significant variations were observed with the temperature, in all experiments an incubation period of 1 h and a temperature of 30 °C have been utilized.

In order to verify the practical applicability of the immobilized biocatalyst, repeated batch tests of sorption/degradation were carried out with entrapped *Lipomyces*. In these tests, both lyophilized and not lyophilized were used, to evaluate the influence of the hydration level of catalyst on the yeasts performance. After each batch test, the liquid phase was removed and replaced by an equal volume of MCPA solution at the initial concentration of 200 mg L^{-1}.

Results and Discussion

MCPA removal. The results of the MCPA removal tests performed adopting an incubation time of 1h are displayed in the Figure A1.

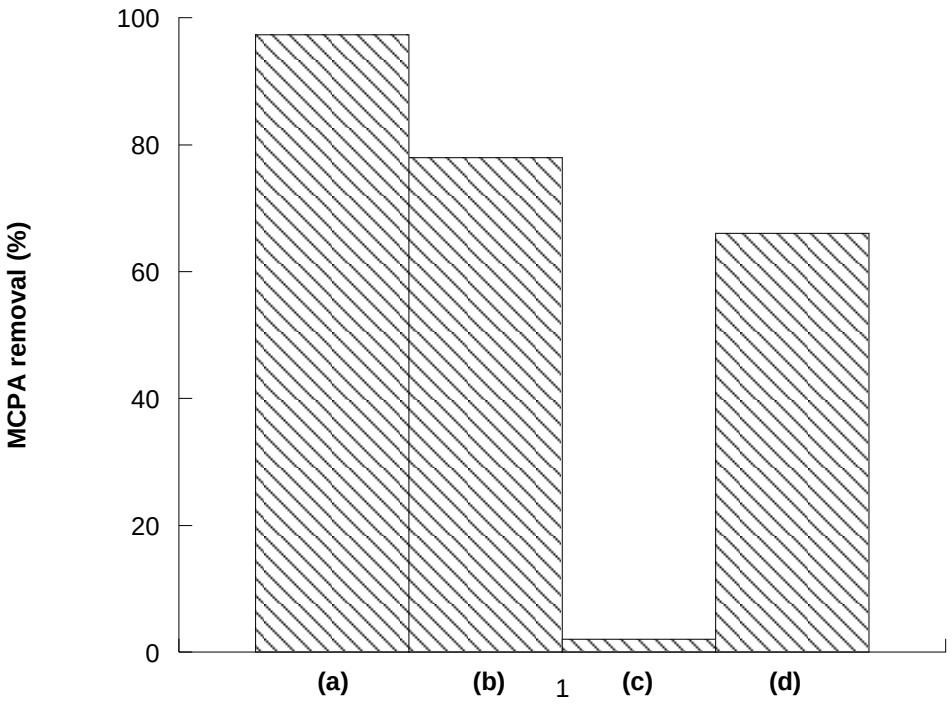

Figure A1. MCPA percentage removal after 1 h of incubation time and at 30 °C. (a) ZrO_2-*Lipomyces*, (b) pure ZrO_2 (c) not immobilized *Lipomyces*, (d) ZrO_2-*Lipomyces* preliminary incubated at 100 °C for 24 h.

Although the initial MCPA concentration was very high, ZrO_2-*Lipomyces* (sample **a**) were able to remove the herbicide almost completely (97.3%). A remarkable removal (78%) was also observed when using ZrO_2 (sample **b**), whereas a very little degradation (2%) was seen for not immobilized *Lipomyces* (sample **c**). ZrO_2-*Lipomyces* preliminary incubated at 100 °C for 24 h (sample **d**) exhibited a reduced remediation activity (66%). These results indicate that:

f) the ZrO_2 matrix shows a significant removal capacity due to sorption;

g) *Lipomyces* entrapped into the ZrO_2 matrix show a degradative activity towards MCPA.

The degradative activity of the entrapped yeasts was further confirmed by comparing the MCPA removal efficiencies of the samples **a** and **d** (FigureA1). In fact, the herbicide removal was almost complete for ZrO_2-*Lipomyces*, whereas the ZrO_2 matrix preliminary subjected to

thermal treatment exhibited a removal efficiency lower than 70%, that is likely due to the adsorption of MCPA on the zirconia surfaces.

The reduced degradation of the herbicide, observed when the microorganisms not immobilized were suspended in a liquid solution of MCPA (200 mg L^{-1}), suggests that the metabolic activity of the microorganism was enhanced under the environmental conditions produced by the entrapment inside the zirconia matrix, i.e. local pH, local concentration of MCPA (affected by the mass transfer resistances), local concentration of water and other components of the reaction system. To validate this hypothesis, the activity of not immobilized microorganisms was tested in the presence of a lower concentration (20 mg L^{-1}) of MCPA (data not shown), finding a MCPA removal efficiency significantly higher (37%). This result confirmed that the local MCPA concentration and the eventual local acidity is a key factor in the degradation mechanism, so indicating that the entrapped state is essential to preserve the degradative ability of yeasts.

On the basis of the above discussion it is possible to suppose that, when a concentration of 200 mg L^{-1} of MCPA is utilized, the degradative activity of the *Lipomyces* is explicated only on the herbicide molecules that are sorbed on the surfaces of the ZrO_2 matrix. In other words, a sequential mechanism can be envisaged, described by the following scheme:

$$(MCPA)_{solution} \underset{}{\overset{sorption}{\rightleftharpoons}} (MCPA)_{sorbed} \xrightarrow{microbial\ degradation} (degradation\text{-}products)$$

To validate the aforesaid mechanism, as well as to highlight the contribution of the entrapped microorganisms, sorption experiments were conducted using the zirconia matrix without biomass and varying the concentration of herbicide added. The sorption isotherm of MCPA on the gel-derived zirconia is displayed in Figure A2 and it is well-fitted by the equation

$$C_e = 2517 + 1057\ C_s \quad (2)$$

where C_e is the equilibrium concentration of MCPA (mg L^{-1}), and C_s the loading of sorbed MCPA (mg kg^{-1}).

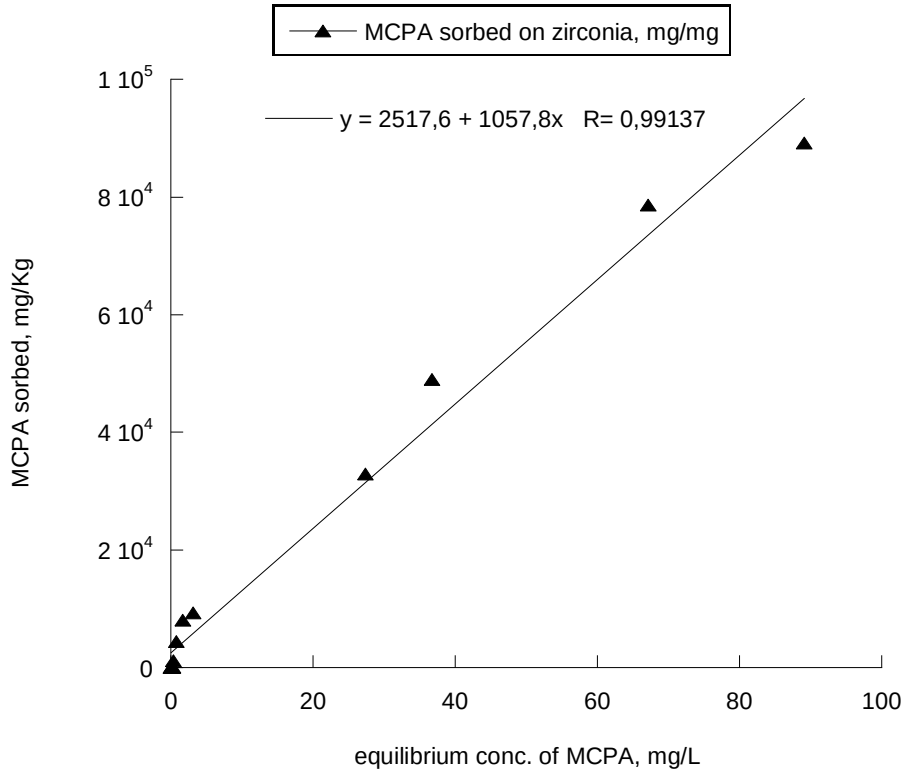

Figure A2. Sorption isotherm of MCPA on the pure ZrO_2 after 1 h of incubation time and at 30 °C.

The isotherm of MCPA on ZrO_2 was a C-type one, characterized by a straight line trend, indicative of a partitioning mechanism whereby the molecules were distributed between the interfacial phase and the bulk solution phase without any specific bonding between the sorbent

and sorbate. Such a process occurs only if penetration into the solid takes place (*24*). Consequently, as the herbicide is sorbed there is a proportional increase on the surface of sorbent.

MCPA removal tests by repeated batches of lyophilized and not lyophilized *Lipomyces* are displayed in Figure A3, where the removal (%) of MCPA after each batch treatment is reported as a function of the batch number.

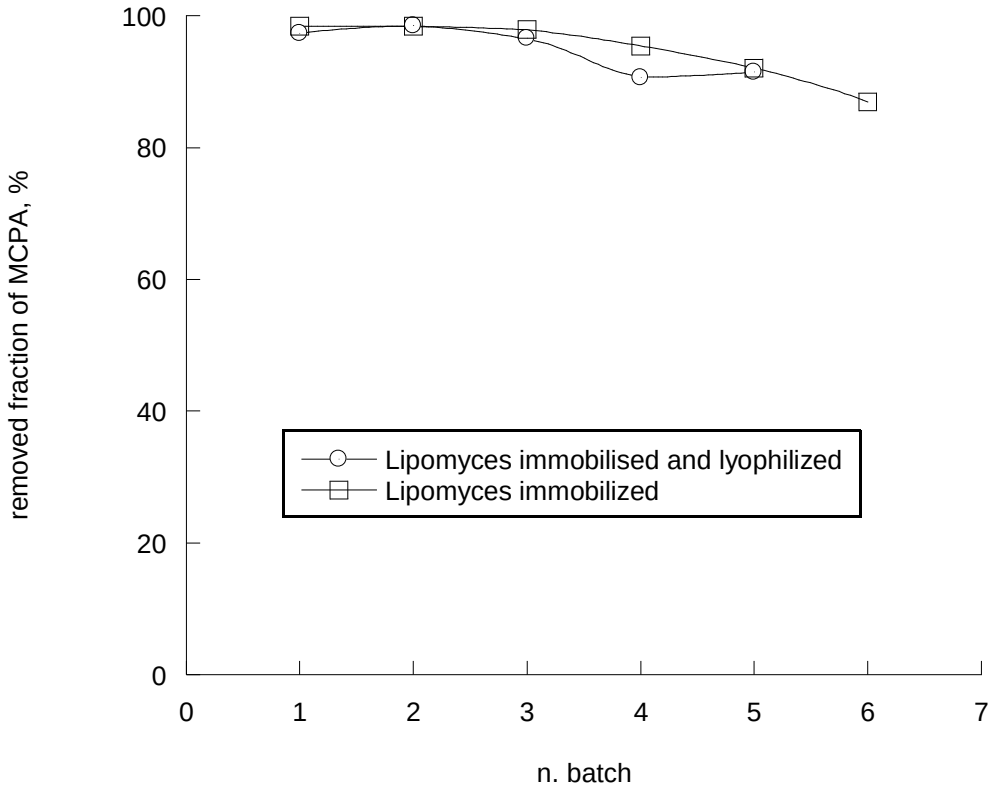

Figure A3. MCPA removal tests by repeated batches of lyophilized (■) and not lyophilized *Lipomyces* (●).

A removal efficiency of about 98% was observed for both the biocatalysts up to the third batch test, with only a subsequent slight decrease, probably due to the progressive saturation of the zirconia, leading to a lower sorption capacity, though a value still higher than 85% was kept after the sixth batch test (see Figure A3). At each stage both the biocatalysts exhibited almost the same removal efficiency, suggesting that the initial hydration level of the yeasts does not affect the biocatalyst activity.

The products of the MCPA degradation were characterized by GC-MS, as described in Sannino et al. (2010), demonstrating that the herbicide was actually metabolized by the yeasts.

In conclusion, we have demonstrated that immobilized *Lipomyces* were able to degrade the herbicide MCPA. The sol-gel zirconia matrix hold promise as a biocompatible scaffold for encapsulation of cells for bioremediation applications. The reusability of encapsulated cells makes it possible to conduct continuous operations in small-scale, as well as industrial-scale treatment systems.

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Microbial Conversion of Olive Oil Mill Wastewaters into Lipids Suitable for Biodiesel Production

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Lipomyces starkey were able to survive and proliferate in the presence of olive oil mill wastewaters (OMW), a medium difficult to process by biological treatments, due to the antimicrobial activities of their phenolic components. The microorganisms were grown in the presence of undiluted OMW, without external organic supplements, producing a significant reduction of both the total organic carbon (TOC) and the total phenols content. The OMW treated by *L. starkey* showed a significant increase of the germination index. The preliminary dilution of OMW enhanced the reduction of polluting components of OMW, leading to a complete TOC removal, as well as to lower levels of residual phenols. The activities of extracellular lipases and esterases significantly increased in the course of the OMW fermentation. A significant increase in lipid yield was observed in *L. starkey* in the course of the OMW treatment, particularly enhanced when the feedstock was preliminarily diluted. The fatty acid distribution showed a prevalence of oleic acid, demonstrating the potential of *L. starkeyi* as a source of lipids to be used as a feedstock for the synthesis of II generation biodiesel.

KEYWORDS: Yeasts; lipids; biodiesel; olive oil mill wastewaters

INTRODUCTION

Biodiesel is attracting increasing interest as a substitute for petroleum-based diesel, due to the negative environmental effects of fossil fuels combustion and the concerns about petroleum supplies. As a matter of fact, biodiesel is a biodegradable, nontoxic, and clean biofuel that can be obtained from renewable sources.

Unfortunately, the starting materials traditionally used for biodiesel synthesis, namely, vegetable oils, animal fats, and (more recently) waste cooking oils, cannot realistically satisfy the demand for biodiesel at the current rate of consumption (*1*, *2*). In addition, the cost of the biodiesel, which is mainly due to (70−85%) the vegetable oils used as feedstocks, still exceeds that of the mineral diesel. Alternative sources of triacylglycerols (TAGs) are also necessary to reduce the social cost of biodiesel production, as the increase of the latter is leading to significant increases in the price of vegetable oils, a basic food in many underdeveloped countries, as well as to the deforestation of large areas.

Thus, new cheaper sources of lipids are needed for biodiesel to be a competitive and sustainable fuel, and the development of nontraditional processes for the production of TAGs, to be used as feedstock for biodiesel production, is presently targeted by a growing number of research work (*1*−*3*).

Oleaginous microorganisms, which have the ability to produce more than 20% of their weight in the form of lipids, are attracting increasing interest as a potential source of TAGs (*4*−*8*). The basic physiology of lipid accumulation in such microorganisms has been well-studied (*9*−*12*). It is known that lipid production requires nitrogen-limiting conditions.

An important advantage offered by the application of the oleaginous microorganisms stems from their ability to produce aerobically lipids from residual organic matters. Consequently, to optimize the cost of the process, as well as to increase its environmental benefit, residual materials have been tested as possible nutrients for the oleaginous microorganisms, such as sewage sludge (*8*), lignocellulosic materials (*7*), and hydrolyzed tomato waste (*13*).

In this study, we demonstrate that the oleaginous yeast *Lipomyces starkeyi* can be grown in the presence of olive oil mill wastewaters (OMW). The objective of this work was to investigate the conversion of OMW into microbial lipids as an alternative feedstock for the synthesis of biodiesel.

Olive oil production is a significant agricultural activity with great economic importance, particularly in Mediterranean countries. However, it generates high amounts of waste waters derived from the olive mill process (OMW). This waste causes disposal problems because of its highly polluting properties, which are documented by high chemical oxygen demand (COD) and biological oxygen demand (BOD) values (*14*).

Different studies have been so far devoted to the application of biological treatments for the reduction of the high organic carbon contents of the OMW (*15*). Most of these studies describe treatments based on the use of yeasts (*16*, *17*) or white rot fungi (*18*−*20*). It has been shown (*21*) that the phenolic components of OMW may inhibit the growth of microorganisms, limiting the efficiency of the digestion processes. As far as we know, no previous scientific papers have focused on the biological synthesis of lipids starting from OMW-based media.

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Table 1. Chemical Composition of OMW

	concentration (g/L)
water	965.40
nonaqueous components	4.70
sugars	12.79
phenols	9.14
proteins	3.41
lipids	0.72
dry weight (105 °C)	47.20
mineral residue (550 °C)	11.00
Fe	0.01
Mg	0.19
Ca	0.26
K	2.85
Na	0.65
Cu	0.002
Zn	0.003

The natural habitat of *L. starkeyi* is soil and ensilage (*22*), where the microorganisms degrade carbohydrates using extracellular carbohydrolases and contribute to the biodegradation of herbicides (*23*). *L. starkeyi* have been proved to store large amounts of lipids (*9*), showing only a minimal reutilization of the stored lipids (*10*). Lipid accumulation is affected by the concentration of some ions like Zn^{2+} and Mn^{2+} lipids (*11*, *12*). So far, sewage sludge has been the only residual material tested as a culture medium for *L. starkeyi* growth (*8*).

MATERIALS AND METHODS

Microorganisms and Culture Medium. All of the oleaginous yeasts used within the present work (*L. starkeyi*, *Cryptococcus curvatus*, *Rhodotorula glutinis*, and *Rhodosporidium toruloides*) were obtained by the collection of the Dipartimento di Biologia Vegetale of the Perugia University (Italy). The microorganisms were kept on potato dextrose agar (Sigma) at $T = 5 \pm 1$ °C and cultivated in a synthetic N-limiting medium, containing (g/L): KH_2PO_4 (Serva), 1.0; $MgSO_4 \cdot 7H_2O$ (BDH), 0.5; $(NH_4)_2SO_4$ (Carlo Erba), 2.0; yeast extract (Fluka), 0.5; and glucose, 70.0. The growth was carried out under aerobic conditions at 30 °C on a rotary shaker at 160 rpm (Minitron, Infors HT, Switzerland).

OMW. OMW was obtained from the Casa Olearia Italiana (Monopoli, Italy). Samples were immediately frozen at −20 °C until further use. Before each experimental test, OMW samples were defrozen, and the solids were removed by centrifugation (4000 rpm, 30 min, 20 °C) in a thermostatic centrifuge (Rotanta 460R, Hettich, United States). The pH of OMW after centrifugation was 4.68. The composition of the OMW is given in **Table 1**.

Fermentation in OMW. The fermentation tests were carried out using a fixed volume (150 mL) of OMW (both raw or diluted), without external organic supplement, in a 500 mL conical flask. A preliminary centrifugation of OMW (2000 rpm, 10 min) was carried out before each test. The liquid medium was inoculated with 2 mL of microorganism suspension, obtained by dissolving 5 loops of solid culture in 8 mL of physiological solution. The flasks were incubated in a rotary shaker at an agitation rate of 160 ± 5 rpm and an incubation temperature of $T = 30 \pm 1$ °C.

Lipid Extraction and Measurement. Methanol (5.0 mL) and chloroform (2.5 mL) were added to 200 mg of dry biomass and vortexed for 5 s. Subsequently, the cells were disrupted for 12 min in an Ultrasonic Homogenizer (Omni Ruptor 250, United States) at 50% power and 90% pulser. The cells were then filtered off with Whatman no. 1 filter paper, and the solvent−lipid mixture was placed in a 50 mL tube fitting with centrifuge racks. The layers were separated by centrifugation for 10 min at 2000 rpm in a thermostatic centrifuge (Rotanta 460R, Hettich) at 20 °C. The lower layer was then transferred to a pear-shaped flask with a Pasteur pipet. Again, 10 mL of 10% (v/v) methanol in chloroform was added to the residue, a new centrifugation was carried out, and the lower phase was added to that from the first extraction. The solvent in the pear-shaped flask was evaporated to dryness (BÜCHI Rotavapor R-200, Switzerland), and the extracted weight was finally recorded after drying at 105 °C for 1 h.

Biomass Analysis. The biomass concentration in the synthetic medium was measured by OD determination at 600 nm. When microorganisms were cultured in the OMW, the OD measurement could not be carried out due to the darkness of the medium. Consequently, the total count of microorganisms was carried out by sequential dilution and insemination in plate count agar medium (Difco Laboratories, Detroit, MI). The colonies were counted after 24 h of culture on agar medium. After each fermentation test, the biomass was recovered by centrifugation (3500 rpm for 10 min) and lyophilized (LYOBETA- 50, Spain), to enable the determination of the dry biomass and the lipid concentration measurement.

Fatty Acids Composition. The fatty acids composition was determined by GC analysis on a Shimadzu GC 17/3 gas chromatograph equipped with a flame ionization detector, following the method suggested by Li and co-workers (*6*). Briefly, wet cell pellets from 1 mL of culture were treated with 0.5 mL of a 5% KOH−methanol solution at 65 °C for 50 min. After the addition of 0.2 mL of BF_3 diethyl etherate and 0.5 mL of methanol, the mixture was refluxed for 10 min, cooled, diluted with distilled water, and extracted with petroleum ether. The organic layer was collected and washed with distilled water. The GC analysis of the fatty acid methyl esters was carried out using N_2 as the carrier gas (40 mL/min), an injection temperature of 230 °C, an oven temperature of 190 °C, and a detector temperature of 230 °C.

Chemical Analyses. The chemical analyses of OMW samples before and after treatment with microorganisms were performed according to the Rodier methods (*24*), and each test was performed in triplicate. In particular, the cations (Fe, Mg, Ca, K, Na, Cu, and Zn) were determined by atomic absorption spectrometry (Perkin-Elmer Analyst 700). The total sugar and protein contents were measured according to anthrone (*25*) and Bradford (*26*) methods, respectively, by using in the first analysis an equimolar standard solution of galactose and mannose (50% w/v) and, for the second, a bovine sieroalbumine solution as the standard. The total phenolic content was estimated according to the Folin method (*27*), using gallic acid as the standard.

The total organic carbon (TOC) measurements were carried out with a TOC-$V_{CSH/CSN}$ (Shimadzu, Japan), upon suitable dilution of a culture medium sample. The TOC values were obtained by subtracting the IC (inorganic carbon) value from the TC (total carbon) value.

Extracellular Lipase and Esterase Activity. The activity of lipases and esterases in the culture medium was measured after biomass removal by centrifugation, using *p*-nitrophenyl-butyrate (*p*NPB) in 10 mM sodium phosphate buffer, pH 7.0, at 37 °C. One unit of activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute.

Phytotoxicity Test. The OMW phytotoxicity was assessed on the seeds of *Lactuca sativa* species purchased by "La Semiorto Sementi" located in Sarno, South of Italy. The bioassays were carried out according to U.S. EPA procedures (*28*).

A suitable volume (5 mL) of OMW, both before or after treatment, with *L. starkeyi*, diluted (1:10 and 1:25) with deionized water, was added to 100 mm diameter Petri dishes containing a filter paper disk (Whatman no. 1, Ø 90 mm). Ten seeds were placed on each paper disk, and for each sample tested, three replicates were prepared. Controls with deionized water were also run. The plates were incubated in a growth chamber (Angelantoni HCT 120) in the dark at 23 °C, and after 72 h, the germinated seeds were counted, and the rootlet of each germinated seeds was measured with a ruler. Each experiment was repeated three times. A primary root >2 mm was considered as the end germination point. Seed germination and root elongation at the end of the bioassays were measured, and the relative index of germination (GI) was calculated according to the following formula:

$$\%GI = (Gs/Gc) \times (Ls/Lc) \times 100$$

where Gs and Gc are the number of germinated seeds in the sample and in the control, respectively, and Ls and Lc are the average root length of seedlings for the samples and for the control, respectively.

Statistical Analysis. All experiments have been carried out adopting a sample size of at least $n = 3$. The hypothesis tests for the GI data were carried out by a one-sided *t* tests (*29*), with significance levels of $\alpha = 0.01\%$.

RESULTS AND DISCUSSION

Fermentation in Synthetic Medium. Oleaginous yeasts accumulate lipids as storage materials only under N-limiting conditions.

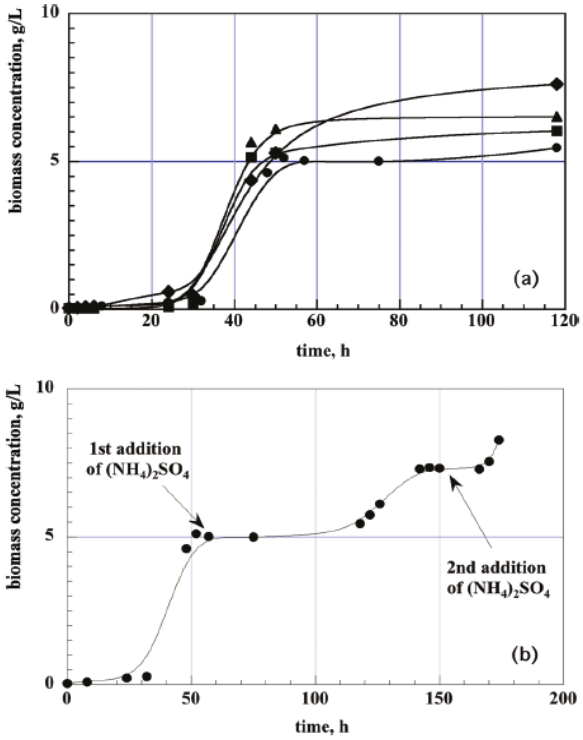

Figure 1. (**a**) Growth kinetics of four oleaginous yeasts using a N-limiting synthetic medium in batch cultures: *C. curvatus* (▲), *R. glutinis* (■), *R. toruloides* (♦), and *L. starkeyi* (●). Operating conditions: $T = 30$ °C and 160 rpm. The medium composition is as in the Materials and Methods. (**b**) Growth of *L. starkeyi* using a N-limiting synthetic medium in batch cultures, under multiple additions of the nitrogen source. Operating conditions: $T = 30$ °C and 160 rpm. The medium composition is as in the Materials and Methods.

Consequently, different oleaginous microorganisms (*L. starkeyi*, *C. curvatus*, *R. glutinis*, and *R. toruloides*) were preliminary cultured in a synthetic medium (described in the Materials and Methods) with a C/N ratio = 58. The growth profiles reported in the **Figure 1a** demonstrate that the growth kinetics are substantially similar. The lipid yield obtained with *L. starkeyi* after 120 h of growth (12.4%) was higher as compared to these pertaining to the other microorganisms. In addition, *L. starkeyi* has been proved to store large amounts of lipids, showing only a minimal reutilization of the stored lipids (*10*). For these reasons, *L. starkeyi* was selected for the subsequent tests.

Figure 1b describes the growth profile of *L. starkeyi* under multiple additions of fixed amounts (3 g each) of the nitrogen source $(NH_4)_2SO_4$ after a stationary phase was established. The experimental data show that, after each addition of $(NH_4)_2SO_4$, a new exponential phase starts, leading to an increase in the biomass concentration obtained under N-limiting conditions, although the increases in the biomass concentration are progressively reduced. The lipid yields obtained after the first and the second additions of $(NH_4)_2SO_4$ (see **Figure 1b**) were 14.1 and 15.5, respectively, showing that operation under N-limiting conditions also allows an increase in the lipid fraction of the biomass.

Fermentation in the Presence of OMW. *L. starkeyi* was cultured in the presence of the OMW, without external organic supplement. Experimental tests were carried out in the presence of raw OMW (after a preliminary centrifugation), as well as in water mixtures containing 50 and 25% OMW, respectively. The biomass concentration was evaluated in terms of colony-forming units (CFU), as OD measurements could not be carried out due to the darkness of the medium. Typical growth profiles are reported in **Figure 2a**.

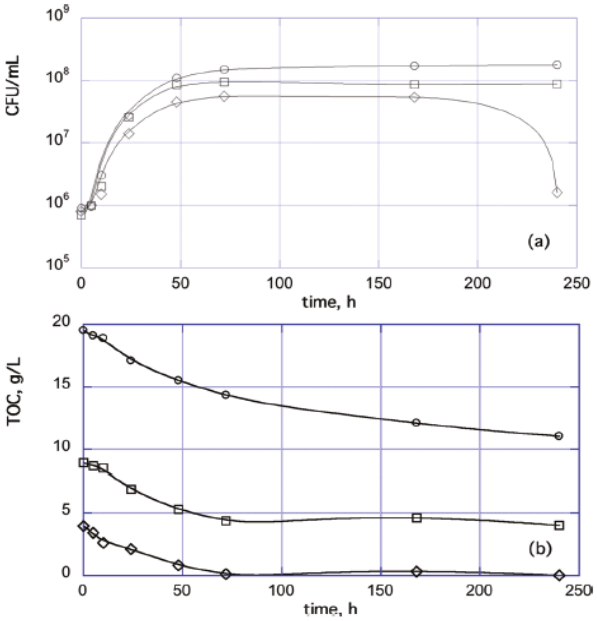

Figure 2. (**a**) Growth of *L. starkeyi* in batch cultures, in the presence of raw OMW (○), or in water mixtures containing 50% OMW (□) and 25% OMW (◇). Operating conditions: $T = 30$ °C and 160 rpm. The OMW composition is as in the Materials and Methods. (**b**) TOC measurements during the culture of *L. starkeyi* in batch cultures, in the presence of raw OMW (○), or in water mixtures containing 50% OMW (□) and 25% OMW (◇). Operating conditions: $T = 30$ °C and 160 rpm. The OMW composition is as in the Materials and Methods.

The results show an initial increase of CFU limited to the first 3 days. The biomass growth was slightly slower when the yeasts were cultured in the presence of diluted OMW. Subsequently, *Lipomyces* cultured in 25% OMW were for about 170 h in a stationary phase, followed by a progressive reduction of CFU. *Lipomyces* cultured in the presence of 100 and 50% OMW survived at a constant biomass concentration for an even longer period.

The OMW are a very complex medium (see **Table 1**). Consequently, the biomass growth in OMW could be affected by different C sources (sugars, lipids, and phenols). Although it is likely that the microorganisms degrade preferentially the C sources that are more easy to metabolize (sugars), we first measured the degradative activity of the yeasts in terms of TOC levels, to evaluate subsequently the variation of the different classes of components. The TOC levels registered in the course of the same tests are reported in **Figure 2b**. A complete removal of the organic carbon was carried out only in the presence of 25% OMW. However, in all of the tests carried out, the OMW reduction was mostly achieved in the first 3 days, that is, in the period of the CFU growth, showing the use of the organic carbon as a primary carbon source (*30*).

Logistic Model for the Biomass Growth. To model the biomass production and the TOC abatement, a numerical model was built based on the experimental data obtained under different experimental conditions. The biomass production rate was obtained by a biomass balance:

$$\frac{dX}{dt} = \mu X \qquad (1)$$

Table 2. Comparison of Experimental Values of the Growth Parameters with the Theoretical Data Obtained with the Logistic Model, with Reference to the Culture of *L. starkeyi* in Batch Reactors, under Different Experimental Conditions

						g/L		1/h		g/L		g/g	
experiment	OMW fraction (%)	dilution factor	pH control	addition of glucose	T (°C)	X_0	$[TOC]_0$	μ_{max} (exp)[a]	μ_{max} (pred)	X_{max} (exp)	X_{max} (pred)	$Y_{X/TOC}$ (exp)[b]	$Y_{X/TOC}$ (pred)
E1	25	4	no control	no addition	30	0.0512	3.95	0.181	0.190	3.20	2.81	1.32	1.30
E2	50	2	no control	no addition	30	0.0488	8.93	0.171	0.175	5.90	5.81	1.29	1.25
E3	100	1	no control	no addition	30	0.0576	19.5	0.128	0.132	10.4	10.0	1.20	1.28
E4	100	1	no control	C_{gluc} = 70 g/L	30	0.0539	20.1	0.127	0.136	11.1	10.8	1.31	1.22
E5	100	1	pH 5.5	no addition	30	0.0510	20.1	0.130	0.135	11.0	10.7	1.24	1.30
E6	100	1	pH 6.5	no addition	30	0.0491	20.0	0.127	0.134	10.6	10.5	1.30	1.27
E7	100	1	no control	no addition	35	0.0512	19.9	0.071	0.078	6.12	5.68	1.29	1.20
E8	100	1	no control	no addition	25	0.0522	20.1	0.099	0.097	11.0	10.3	1.34	1.31

[a] The experimental value of μ_{max} was calculated from the equation $\mu_{max} = \ln(X_2/X_1)/(t_2 - t_1)$, assuming a constant growth rate in the early exponential phase. [b] The experimental value of $Y_{X/TOC}$ was calculated from the slope of the curve of the biomass concentration (X) as a function of the TOC.

The specific growth rate was defined adopting the logistic model:

$$\mu = \mu_{max}\left(1 - \frac{X}{X_{max}}\right) \quad (2)$$

The TOC profiles were described adopting the hypothesis of proportionality between TOC reduction rate and biomass growth rate:

$$\frac{d[TOC]}{dt} = \mu X \frac{1}{Y_{X/TOC}} \quad (3)$$

where $Y_{X/TOC}$ is the ratio of the amount of biomass produced to the amount of TOC consumed (g biomass/g TOC).

Equations 1−3 were integrated using a fourth-order Runge−Kutta integration method. The least-squares method was used to obtain the parameter estimates. The model fitted the experimental data (see the results in the **Table 2**) with R^2 values higher than 0.95. **Figure 3** shows a graphic comparison between the model predictions (dashed line) and the experimental results of a typical test. Standard errors, reported in the **Figure 3**, never exceeded 14%.

Growth Parameters of *L. starkeyi* in the Presence of OMW. A detailed comparison between the experimental and the theoretical data is given in **Table 2**. As higher OMW concentration values are adopted (experiments E1, E2, and E3 in **Table 2**), the specific growth rate (μ_{max}) slightly reduces. This result agrees with previously reported data, concerning the bacterial growth in the presence of OMW (*30*), and is due to the higher concentration of the phenolic components of OMW. In principle, another component of OMW that may inhibit microbial growth is the olive oil. However, it was ascertained (**Table 3**) that the lipase activity of *L. starkeyi* in the presence of OMW increases significantly. Consequently, the yeasts should be able to metabolize olive oil residuals.

The maximum values of biomass concentration (X_{max}), reported in the **Table 2**, increase with the initial OMW concentration, with the maximum value of X_{max} obtained when using raw OMW (10.4 g/L). The biomass yield based on TOC consumption ($Y_{X/TOC}$) appears to be substantially constant, suggesting that changes in the OMW concentration do not cause a significant increase in the maintenance requirements.

Table 4 describes the reduction of sugars, proteins, and phenols concentration obtained during the *L. starkey* cultures at different concentrations of OMW (experiments E1, E2, and E3 in **Table 2**). The experimental data demonstrate that efficiency of the yeasts in metabolizing the phenols is higher as the preliminary dilution of OMW increases, yielding phenol removal efficiencies of 43, 47, and 53% in the presence of OMW volumetric fractions of 100, 50, and 25%, respectively. These results indicate the critical concentration of phenols in OMW, preventing the growth of *Lipomyces*, to be higher than 9 g/L.

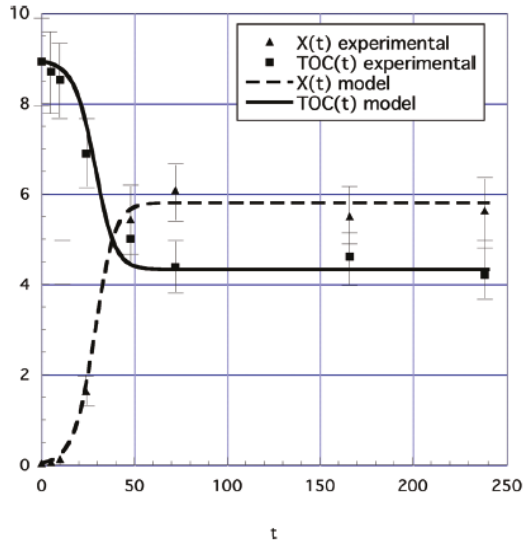

Figure 3. Comparison of experimental measurements of biomass concentration (X, g/L) and TOC (g/L) with the theoretical data obtained with the logistic model, with reference to the culture of *L. starkeyi* in batch cultures, in the presence of raw OMW. Operating conditions: T = 30 °C and 160 rpm. The OMW composition is as in the Materials and Methods. Standard error bars are reported.

Table 3. Extracellular Lipase Activity before and after *L. starkeyi* Culture in the Presence of OMW

		U/mL	
dilution factor	OMW fraction (%)	lipase activity before test	lipase activity after test
1	100	145	1040
2	50	134	827
4	25	122	710

The higher concentrations of OMW also reduce the removal of sugars and proteins (**Table 4**). Again, this detrimental effect is attributed to the higher initial concentration of phenols. The fraction of protein removed was in any case higher than 80%, reaching 98% in the presence of OMW volumetric fraction of 25%.

The highest efficiency of sugars removal was obtained with the most diluted OMW sample. In any case, the sugars removal was never complete, demonstrating that the break in the biomass

Table 4. Sugars, Proteins, and Phenols Content Removal (%) Obtained during the *L. starkeyi* Culture in the Presence of Undiluted and Diluted OMW as Compared with Untreated OMW

		%		
dilution factor	OMW fraction	removal of sugars content	removal of proteins content	removal of phenols content
1	100	48	82	43
2	50	54	87	47
4	25	86	98	53

Table 5. GI of *L. sativa* Seeds on Untreated and Treated Samples of Raw OMW

	GI	
dilution ratio	untreated OMW	*Lipomyces*-treated OMW
1	0	0
10	0	125.5
25	105.0	121.0

growth was not due to the exhaustion of sugars. This conclusion was confirmed by a further test: A glucose amount corresponding to a concentration of 70 g/L was added to raw OMW before the test beginning. In this case, the growth curves did not change significantly (experiment E4 in **Table 2**).

To check whether the growth of *L. starkeyi* in the presence of OMW was carried out under N-limiting conditions, supplementary additions (3 g) of the nitrogen source $(NH_4)_2SO_4$ were tried once a stationary phase was established during the culture in the presence of OMW (data not shown). In the latter tests, no further increases were observed in the biomass concentration, neither reductions in the TOC levels.

The initial pH of OMW (both raw or diluted) used in the experimental tests was between 4.7 and 5.0. A slow pH increase was observed in the course of the OMW fermentations, although the pH change in a single test was never higher than 1.2 pH units. To ascertain the actual effect of the pH, specific tests (experiments E5 and E6 in **Table 2**) were carried out, making every day pH adjustments to constant pH values (pH 5.5 and pH 6.5). The results reported in the **Table 2** show that the pH control does not affect appreciably the growth kinetics (i.e., μ_{max}) and stoichiometry ($Y_{X/TOC}$ and X_{max}).

Experimental tests (experiments E7 and E8 in **Table 2**) were carried out to evaluate the effect of the temperature. When the OMW fermentation was carried out at 35 °C, both μ_{max} and X_{max} were lower as compared to the corresponding values observed at 30 °C. The data obtained at a temperature of 25 °C showed a significantly lower value of μ_{max}, although the X_{max} value was close to that obtained at 30 °C.

In the course of OMW fermentation, the extracellular activity of lipases and esterases increases significantly (**Table 3**), in agreement with the literature data (*31*, *32*). Consequently, a further potential benefit of the OMW treatment with *L. starkey* is related to the production of these enzymes. The lipase and esterase activities are higher as the OMW concentration increases, probably due to the induction produced by the TAGs (olive oil) contained in the OMW.

Phytotoxicity. The results on GI of *L. sativa* seeds of untreated and treated samples of raw OMW are reported in the **Table 5**. When no sample dilutions were carried out before the GI test, both the untreated OMW and the *Lipomyces*-treated OMW were completely phytotoxic. Consequently, further GI tests were carried out upon dilution of samples. When testing diluted samples (1:10 v/v), the untreated OMW remained completely phytotoxic, whereas the *Lipomyces*-treated OMW showed a significantly high GI (125.5).

Table 6. Lipid Concentration in *L. starkeyi* after 10 Days of Culture in OMW

sample	lipid yield (%)
L. starkeyi grown in synthetic medium	12.1
L. starkeyi grown in undiluted OMW	22.4
L. starkeyi grown in 50% OMW	28.6
L. starkeyi grown in 25% OMW	29.5

Table 7. Distribution of Fatty Acids in the Lipids Accumulated in the *L. starkeyi* Grown on OMW

fatty acids composition	%
myristic acid C14:0	<1
palmitic acid C16:0	19.1
palmitoleic acid C16:1	0.5
stearic acid C18:0	8.5
oleic acid C18:1	49.1
linoleic acid C18:2	18.8
linolenic acid C18:3	3.5
arachidonic acid C20:0	0.3
free fatty acids	14.5

To achieve a significant reduction of the phytotoxic activity of the untreated OMW, a higher dilution ratio (1:25) was required. The hypothesis that the GI of the OMW was actually increased in the course of the treatment was confirmed by a one-sided *t* test (*29*), with a significance level of $\alpha = 0.01\%$. As widely reported in the literature (*33*, *34*), the phenols are considered the compounds mainly responsible for the OMW phytotoxicity. Consequently, the increase of GI likely originated from the reduction of phenols (see **Table 5**).

Lipid Yield and Composition. The amounts of lipids extracted from *L. starkeyi* cultured in OMW are reported in **Table 6** in terms of lipid yield. The experimental data show that a 50% dilution of OMW results in a significant increase in the concentration of lipids (28.6 against 22.4%), although a further increase in wastewater dilution produces only a minimum improvement.

The fatty acids distribution in the lipids accumulated in the *L. starkeyi* grown in OMW is described in the **Table 7**. The composition indicates a clear prevalence of oleic acid, in agreement with results obtained using different oleaginous microorganisms (*6*, *35*, *36*). The total content of saturated fatty acids is low enough to allow an excellent cold behavior of biodiesel, reducing its tendency of crystallization or gelling at low temperatures.

In conclusion, the accumulation of lipids by *L. starkeyi* may occur in the presence of OMW with no preliminary treatments and without external organic supplement. The growth of the yeasts is associated with a significant reduction of both the TOC and the total phenols content. The increase of the GI of the OMW after the biological treatment demonstrates the environmental benefits that can be achieved by this process.

The use of diluted wastewaters increases the fraction of the organic compounds (TOC, total phenols, and proteins) removed, although the kinetics of the biomass growth is slower. The lipid concentration (between 20 and 30%) in the microorganisms increases in the course of the OMW treatment, particularly in the presence of the preliminary diluted feedstock. The fatty acids distribution demonstrates the usability of the lipids accumulated in the *L. starkeyi* as feedstock for biodiesel synthesis.

ABBREVIATIONS USED

t, time (h); TOC, total organic carbon (g/L); *X*, cell concentration (dry weight) (g/L); X_{max}, maximum value of cell concentration (dry weight) (g/L); $Y_{X/TOC}$, biomass yield on TOC consumed

(g of biomass formed per g of TOC consumed); μ, specific growth rate (h^{-1}); μ_{max}, maximum value of the specific growth rate obtained by the logistic model (h^{-1}).

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Remediation of Waters Contaminated with MCPA by the Yeasts *Lipomyces starkeyi* Entrapped in a Sol–Gel Zirconia Matrix

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A single-stage sol–gel route was set to entrap yeast cells of *Lipomyces starkeyi* in a zirconia (ZrO_2) matrix, and the remediation ability of the resulting catalyst toward a phenoxy acid herbicide, 4-chloro-2-methylphenoxyacetic acid (MCPA), was studied. It was found that the experimental procedure allowed a high dispersion of the microorganisms into the zirconia gel matrix; the ZrO_2 matrix exhibited a significant sorption capacity of the herbicide, and the entrapped cells showed a degradative activity toward MCPA. The combination of these effects leads to a nearly total removal efficiency (>97%) of the herbicide at 30 °C within 1 h incubation time from a solution containing a very high concentration of MCPA (200 mg L^{-1}). On the basis of the experimental evidence, a removal mechanism was proposed involving in the first step the sorption of the herbicide molecules on the ZrO_2 matrix, followed by the microbial degradation operated by the entrapped yeasts, the metabolic activity of which appear enhanced under the microenvironmental conditions established within the zirconia matrix. Repeated batch tests of sorption/degradation of entrapped *Lipomyces* showed that the removal efficiency retained almost the same value of 97.3% after 3 batch tests, with only a subsequent slight decrease, probably due to the progressive saturation of the zirconia matrix.

Introduction

The widespread use of pesticides in agricultural activities increases the residue levels of these chemicals in soils and waters, thus becoming an environmental problem. Pesticides are generally applied in higher amounts than those needed for pest control and they are swept away by various transport processes such as leaching and runoff (*1*, *2*).

4-Chloro-2-methylphenoxyacetic acid (MCPA) is a phenoxy acid herbicide widely used in agriculture for postemergence control of weeds in cereals, grasslands, trees, and turf. It is relatively soluble (273.9 mg L^{-1} in water at neutral pH), highly mobile, and can leach from soil. Consequently, this compound has been found in well water in some countries and has been classified by the U.S. Environmental Protection Agency (EPA) as a potential groundwater contaminant (*3*). For this reason, the risk of environmental pollution of soil and surface waters must be considered and the development of new remediation technologies appears to be of primary importance.

Cell immobilization offers a valid strategy for cleaning up the environment from herbicides and several studies have been performed on this subject with immobilized yeast cells (*4*, *5*). As a matter of fact, it is generally thought that microbial metabolism provides a safer, more efficient, and less expensive alternative to physicochemical methods for pollution abatement (*6*). The immobilization of cells within a stable matrix system may lead to the efficient use of their physiological capabilities (*7*), e.g., for producing secondary metabolites, or in biotransformation/biocatalysis reactions, so it can be very advantageous for several biotechnological applications. Further benefits stemming from the use of immobilized cells are the easier preparation of some metabolic products, as well as their protection from environmental stresses. In addition, the use of immobilized cells helps in recovery and reuse of the embedded cells, allowing a repeated use of the biocatalyst in batch bioreactors. Finally, immobilized cells can be easily segregated in a tank, allowing increased throughput in continuous reactors with reduced risk of washout, therefore leading to obvious economic benefits.

In some applications, enzymatic systems are used instead of living cells to reduce the problems raised by the diffusional resistances through cellular membranes, as well as the unwanted transformations caused by the activity of thousands of enzymes (*8*). Nevertheless, living cells are to be preferred in some instances as they reduce the costs of the enzyme concentration and purification, in particular when multienzymatic systems are required to carry out the transformations of interest (e.g., in the case of multistep degradation of pollutants) (*8*).

Numerous immobilization techniques such as physical adsorption, covalent attachment, and entrapment in polymer or inorganic matrices have been explored over the years to achieve high-yield, reproducible, and robust immobilization techniques preserving the activity of microbial cells (*9*, *10*). So far, no single method or material has emerged as the best for every application and ongoing efforts strive to optimize these methods to render them adequate for specific applications.

Sol–gel nanomaterials are gaining a growing importance as solid supports for the immobilization of biomolecules to be used for biocatalysis, biosensors, and biomedical applications, offering a useful alternative to the traditional polymer technology (*11*, *12*). Due to their porous structure on a nanometric scale, sol–gel materials offer unique intrinsic properties, such as high surface-to-volume ratio, large surface area, and porosity. In addition, the sol–gel process offers higher flexibility with regard to the surface functional groups and the matrix composition. Nanoporous materials, in

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particular the inorganic oxide materials obtained using metallorganic precursors, are usually nontoxic, chemically and thermally stable, so they have wide applications where biocompatibility and thermal stability requirements are essential. Moreover, the chemical nature of the surface of nanopores can be tailored to increase the stability and catalytic activity of the biomolecules.

So far, few examples of whole cell immobilization in sol–gel matrices can be found in the literature, whereas a wide range of enzymes have already been immobilized within sol–gel matrices (*13*–*15*).

In a pioneering work (*16*), spores of the yeasts *Saccharomyces cerevisiae* were immobilized in silica gels, and their viability was demonstrated. Living cells of *Saccharomyces cerevisiae* were then immobilized in silica gels (*17*), exhibiting a significant activity in the conversion of carbohydrates into ethyl alcohol and CO_2, and showing increased viability and resistance against ethanol damage. *Saccharomyces* were also immobilized in alginate–silicate sol–gels (*18*), to convert fumaric acid into L-malic acid. Another yeast, *Kluyveromyces marxianus*, was immobilized in silica aerogel (*19*), and used for sucrose hydrolysis. Again, the immobilized yeast showed increased stability and prolonged viability.

Recently, we have shown (*20*) that the entrapment of lipases in a zirconia sol–gel matrix can be easily carried out with reduced denaturation of the enzyme. In fact, moderate values of temperature and pH can be adopted for the sol–gel synthesis of the zirconia matrix, thanks to the high rates of hydrolysis and polycondensation of the zirconium(IV) propoxide precursor.

In the present study, the yeasts *Lipomyces starkeyi* have been encapsulated in zirconia-based sol–gel materials to remove MCPA from polluted waters.

The natural habitat of *Lipomyces starkeyi* is soil and ensilage (*21*), where the microorganisms degrade carbohydrates using extracellular carbohydrolases, and contribute to the biodegradation of herbicides (*22*). The interest toward *Lipomyces starkeyi* has increased recently, since they have been discovered to be oleaginous microorganisms, having the ability to produce more than 20% of their weight in the form of lipids (*23*, *24*). In particular, *Lipomyces starkeyi* are able to metabolize residual organic matters to produce triglycerides that can be used as feedstock for alcoholysis, to obtain II-generation biodiesel.

Different approaches can be taken for the immobilization of biomolecules within a sol–gel material: (i) by a postsynthesis treatment or (ii) in situ during sol–gel processing. In this study, an experimental procedure has been set up to carry out a single-step entrapment of the yeasts, starting from a suspension containing both the matrix precursors and the cells, so that when the gelation occurs the microorganisms remain entrapped in the gel.

Experimental Section

Materials. 4-Chloro-2-methylphenoxyacetic acid (MCPA) was purchased from Sigma-Aldrich Chemical Co. (Poole, Dorset, UK; 99.0% purity). All solvents were of HPLC grade (Carlo Erba, Milan, Italy) and were used without further purification. Zirconium(IV) propoxide (70 wt.% in 1-propanol), acetylaceton (>99%), and 1-propanol (>99.80%) were provided by Sigma-Aldrich.

Microorganisms and Growth Conditions. The oleaginous yeasts, *Lipomyces starkeyi*, used in this study were obtained by the collection of the Department of Vegetable Biology of the Perugia University (Italy). The microorganisms were kept on potato dextrose agar (Sigma) at $T = 5 \pm 1$ °C and cultivated in a synthetic medium, containing (g L^{-1}): KH_2PO_4 (Serva), 1.0; $MgSO_4 \cdot 7H_2O$ (BDH), 0.5; $(NH_4)_2SO_4$ (Carlo Erba), 2.0; yeast extract (Fluka), 0.5; glucose (Fluka), 70.0. The growth was carried out under aerobic conditions at 30 °C on a rotary shaker at 160 rpm (Minitron, Infors HT, Switzerland). The yeasts were harvested during their exponential growth phase to ensure the highest activity and number.

Lipomyces Entrapment in Sol–Gel Material. In a typical synthesis, 200 mg of lyophilized *Lipomyces* was dissolved in 3.0 mL of physiologic solution and the mixture was stirred in an ultrasonic bath to obtain a suspension with a good dispersion degree. 1-Propanol (5.5 mL, >99.80%) was then added to such suspension that was subsequently mixed with a solution containing 10 mL of zirconium(IV) propoxide (70 wt.% in 1-propanol), 1.5 mL of acetylaceton (>99%), and 3.0 mL of 1-propanol. Under these conditions, gelation occurred after about 20 min at room temperature, while keeping a vigorous stirring. A homogeneous slightly yellow colored gel was produced. The gel was left at room temperature for 3 h and than lyophilized, in vacuum at −50 °C for 20 h, to give the final material.

Pure zirconia gel, without *Lipomyces*, was also prepared and dried under the same conditions. Moreover, gels were obtained starting from an equivalent amount of non-lyophilized yeasts according to the above procedure to check the possible influence of this parameter on the MCPA removal.

The structure of the prepared materials was studied by Fourier-transform infrared (FTIR) spectroscopy. FTIR absorption spectra were recorded, in the 4000–400 cm^{-1} range, using a Nicolet spectrometer, Nexus model, equipped with a DTGS KBr (deuterated triglycine sulfate with potassium bromide windows) detector. A spectral resolution of 2 cm^{-1} was chosen. A 2.0-mg aliquot of each sample was mixed with 200 mg of KBr in an agate mortar and then pressed into pellets of 13-mm diameter. The spectrum for each sample represents an average of 64 scans, which were corrected for the spectrum of the blank KBr pellet.

Analytical Determination of MCPA. MCPA was analyzed with an Agilent 1200 Series HPLC apparatus (Wilmington, DE), equipped with a DAD and a ChemStation Agilent Software. The detailed analysis is described in the Supporting Information.

MCPA Removal Experiments and Sorption Isotherms. A stock solution of herbicide was prepared by dissolving 100 mg of MCPA in 500 mL of Milli-Q ultrapure water (final concentration 200 mg L^{-1}), and subsequently kept refrigerated. MCPA-removal experiments were performed in batch conditions by incubating 10 mg of *Lipomyces* entrapped in zirconia (ZrO_2–*Lipomyces*) with 1 mL of MCPA 200 mg L^{-1}; it is noteworthy that this value is very close to the MCPA solubility. Tests were also carried out using immobilized biomass preliminarily incubated at 100 °C for 24 h. In addition, control experiments were made using 10 mg of non-immobilized *Lipomyces* or 10 mg of pure zirconia.

Blanks of MCPA in aqueous solution were analyzed to check pesticide stability and possible sorption to vials. After incubation in a thermostatic rotary shaker at 30 °C, the samples were centrifuged at 7000 rpm for 20 min. The removal (%) of MCPA (X) was calculated by the following balance equation:

$$X = \frac{c_0 - c_1}{c_0} \qquad (1)$$

where c_0 is the initial concentration of MCPA (mg L^{-1}) and c_1 is the MCPA concentration at the end of the incubation (mg L^{-1}).

Kinetic tests were conducted adopting incubation times of 1, 2, 3, 4, 8, and 24 h at 30 °C. After centrifugation, the supernatants were analyzed as described above.

Sorption isotherm was obtained by adding different volumes of a stock solution of herbicide (200 mg L^{-1}) to gel-derived ZrO_2 to give an initial concentration ranging from

0.05 to 200 mg L^{-1} of MCPA. After incubation and centrifugation, the supernatants were analyzed as described above.

Degradation/sorption tests were performed by adopting both different incubation times (from 1 to 24 h) and temperatures (from 30 to 50 °C). Because the almost complete removal of the herbicide occurred within 1 h and no significant variations were observed with the temperature, in all experiments an incubation period of 1 h and a temperature of 30 °C have been utilized.

To verify the practical applicability of the immobilized biocatalyst, repeated batch tests of sorption/degradation were carried out with entrapped *Lipomyces*. In these tests, both lyophilized and non-lyophilized were used, to evaluate the influence of the hydration level of catalyst on the yeasts' performance. After each batch test, the liquid phase was removed and replaced by an equal volume of MCPA solution at the initial concentration of 200 mg L^{-1}.

Analysis of Degradation Products of MCPA by GC-MS. Identification of degradation products of MCPA was performed by GC-MS analyses. MCPA standard and the samples obtained after incubation with 10 mg of *Lipomyces* immobilized in ZrO_2 were derivatized with Na_2HPO_4 (8 mL; 0.05 M; pH 8.0), pentafluorobenzyl bromide in dichloromethane (2 mL; 0.1%) and tetrabutylammonium sulfate (150 μL; 0.15 M), shaken for 30 min, and centrifuged at 3000 rpm for 5 min. One mL of the dichloromethane phase was transferred into a 2-mL glass vial, evaporated under nitrogen gas, and dissolved in cyclohexane phase (0.5 mL) before GC-MS analysis. The samples were analyzed using a Perkin-Elmer AutoSystem XL gas chromatograph, equipped with a programmed-temperature split/splitless injector with programmable pneumatic control kept at a constant temperature of 250 °C, a Restek Rtx-5MS capillary column, and a Perkin-Elmer TurboMass Gold mass spectrometer. The oven temperature was programmed to run at 80 °C for 2 min and then to increase by 12 °C min^{-1} to a final temperature of 280 °C. Under these conditions the retention time for MCPA was 14.52 min.

A NIST mass spectral library version 1.7 was used for peak identification. Calibration curves for MCPA were obtained by plotting peak areas against concentrations for diluted standards (0.01−20.0 mg L^{-1}). A linear relationship between chromatographic peak area and amount of chemical was found over this concentration range with $R^2 > 0.994$.

Analysis of the Data. All the experiments were performed in triplicate and the relative standard deviation was lower than 4%. The hypothesis test concerning the degradative activity of the immobilized *Lipomyces* was carried out by a two-tailed *t* test (*25*) with a significance level of $\alpha = 0.01\%$.

Results and Discussion

Sol−Gel Synthesis. Gelation process giving wet gel is the result of both hydrolysis and condensation reactions. The process conditions must be selected from time to time, depending on the desired characteristics of the required material. Particularly, zirconium alkoxides show a very high reactivity toward the hydrolysis−condensation reactions due to the high polarity of Zr−O bond which in turn generates a positive partial charge on the zirconium atom, making it very susceptible to nucleophilic attack. Therefore, β-diketones, among them acetylacetone, are generally used to control the hydrolysis reaction rate of zirconium alkoxide (*26*, *27*). The synthesis parameters selected in this work were optimized on the basis of our recent work concerning the entrapment of lipase in ZrO_2 (*20*), obtaining even in this case an effective yeasts entrapment. The dried gel-derived material appears as glass-like pieces, the size of which is about 1 mm as displayed in Figure S1(a) of Supporting Information.

The FTIR spectra displayed in Figure S2 and reported in Supporting Information, showed no remarkable differences between the spectrum of pure zirconia and of ZrO_2−*Lipomyces*, demonstrating the entrapment of the yeasts does not affect the microstructural characteristics of the zirconia matrix. A similar result was obtained when lipases were entrapped in the same material (*20*). Besides, the bands lying in the 450−700 cm^{-1} range are related to the vibrational modes of the Zr−O bonds. Both the spectra indicate the presence of the residual 1-propanol and acetylacetone physically adsorbed or coordinated to the zirconia network (bands in the 700−1700 cm^{-1} range) (*28*, *29*), as well as of adsorbed water ($\nu_{OH} \approx 3400$ cm^{-1}).

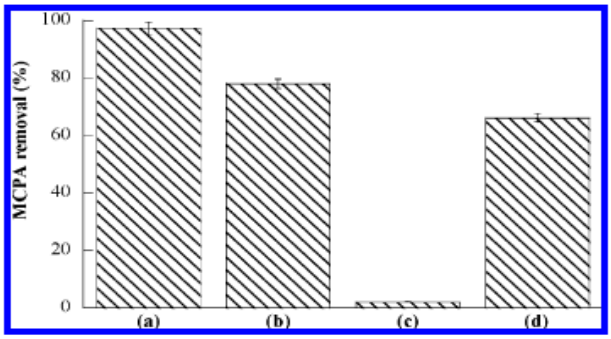

FIGURE 1. MCPA percentage removal after 1 h of incubation time at 30 °C: (a) ZrO_2−*Lipomyces*, (b) pure ZrO_2, (c) non-immobilized *Lipomyces*, and (d) ZrO_2−*Lipomyces* preliminarily incubated at 100 °C for 24 h.

No absorption bands can be clearly related to the presence of *Lipomyces*, because the majority of them, such as proteins and water, lies in the same range as the other components and, more probably, because their intensity is too low due to the small amount.

MCPA Removal. The results of the MCPA removal tests performed adopting an incubation time of 1 h are displayed in Figure 1. Although the initial MCPA concentration was very high, ZrO_2−*Lipomyces* (sample a) was able to remove the herbicide almost completely (97.3%). A remarkable removal (78%) was also observed when using ZrO_2 (sample b), whereas very little degradation (2%) was seen for non-immobilized *Lipomyces* (sample c). ZrO_2−*Lipomyces* preliminary incubated at 100 °C for 24 h (sample d) exhibited a reduced remediation activity (66%). These results indicate that the ZrO_2 matrix shows a significant removal capacity due to sorption and *Lipomyces* entrapped in the ZrO_2 matrix show a degradative activity toward MCPA, as shown by the significant difference between the removal levels observed in the samples a and b. This difference was confirmed by a two-tailed *t* test (*25*), with a significance level $\alpha = 0.01\%$.

The degradative activity of the entrapped yeasts was further confirmed by comparing the MCPA removal efficiencies of the samples a and d (Figure 1). In fact, the herbicide removal was almost complete for ZrO_2−*Lipomyces*, whereas ZrO_2−*Lipomyces* preliminarily incubated at 100 °C for 24 h exhibited a removal efficiency lower than 70%, which is likely due to the sorption of MCPA on the zirconia surfaces.

The reduced degradation of the herbicide, observed when the microorganisms not immobilized were suspended in a liquid solution of MCPA (200 mg L^{-1}), suggests that the metabolic activity of the microorganism was enhanced under the environmental conditions produced by the entrapment inside the zirconia matrix, i.e., local pH, local concentration of MCPA (affected by the mass transfer resistances), local concentration of water, and other components of the reaction system. To validate this hypothesis, the activity of non-immobilized microorganisms was tested in the presence of a lower concentration (20 mg L^{-1}) of MCPA (data not shown), finding a MCPA removal efficiency significantly higher (37%). This result confirmed that the local MCPA concentration

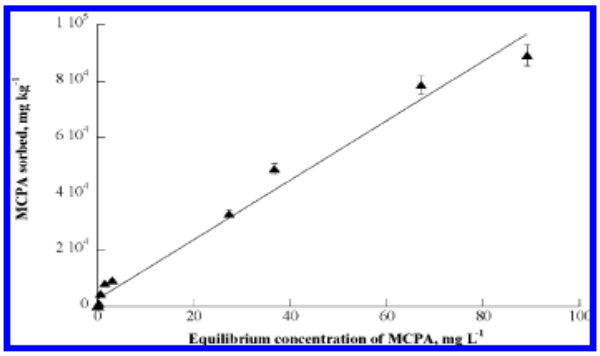

FIGURE 2. Sorption isotherm of MCPA on the pure ZrO_2 after 1 h of incubation time at 30 °C.

and the eventual local acidity is a key factor in the degradation mechanism, indicating that the entrapped state is essential to preserving the degradative ability of yeasts.

On the basis of the above discussion it is possible to suppose that, when a concentration of 200 mg L^{-1} of MCPA is utilized, the degradative activity of the *Lipomyces* is explicated only on the herbicide molecules that are sorbed on the surfaces of the ZrO_2 matrix. In other words, a sequential mechanism can be envisaged, described by the following scheme:

$$(\text{MCPA})_{\text{solution}} \overset{\text{sorption}}{\rightleftharpoons} (\text{MCPA})_{\text{sorbed}} \xrightarrow{\text{microbial degradation}} \text{(degradation products)}$$

To validate the aforesaid mechanism, as well as to highlight the contribution of the entrapped microorganisms, sorption experiments were conducted using the zirconia matrix without biomass and varying the concentration of herbicide added. The sorption isotherm of MCPA on the gel-derived zirconia is displayed in Figure 2 and it is well-fitted by the equation

$$C_e = 2517 + 1057C_s \qquad (2)$$

where C_e is the equilibrium concentration of MCPA (mg L^{-1}), and C_s is the loading of sorbed MCPA (mg kg^{-1}). The isotherm of MCPA on ZrO_2 was a C-type one, characterized by a straight line trend, indicative of a partitioning mechanism whereby the molecules were distributed between the interfacial phase and the bulk solution phase without any specific bonding

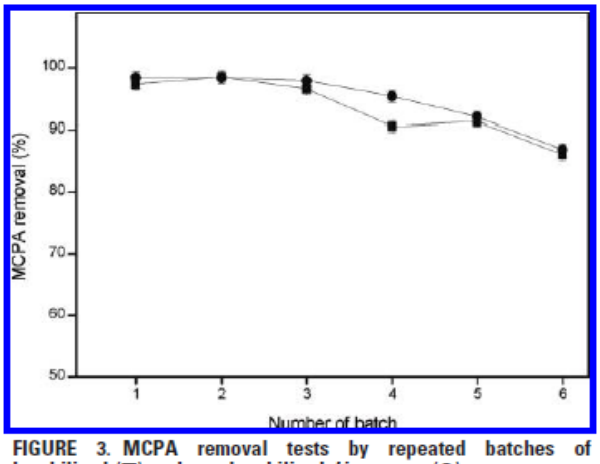

FIGURE 3. MCPA removal tests by repeated batches of lyophilized (■) and non-lyophilized *Lipomyces* (●).

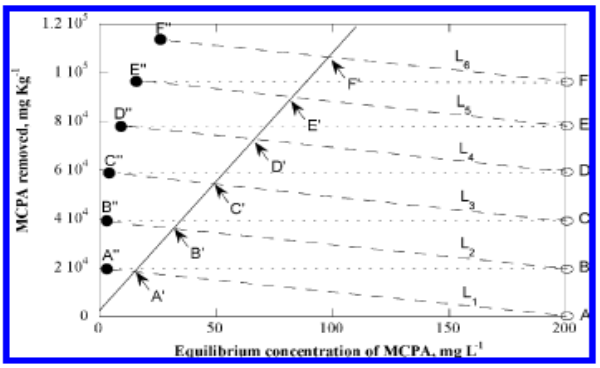

FIGURE 4. Modeling of the MCPA removal tests by repeated batches of ZrO_2–*Lipomyces* (●) and pure ZrO_2 (from sorption isotherm reported in Figure 2) (—).

between the sorbent and sorbate. Such a process occurs only if penetration into the porous solid takes place (*30*). Consequently, as the herbicide is sorbed there is a proportional increase on the surface of sorbent.

MCPA removal tests by repeated batches of lyophilized and non-lyophilized *Lipomyces* are displayed in Figure 3. A removal efficiency of about 98% was observed for both the biocatalysts up to the third batch test, with only a subsequent slight decrease, probably due to the progressive saturation of the zirconia, leading to a lower sorption capacity, though a value still higher than 85% was kept after the sixth batch test (see Figure 3). At each stage both the biocatalysts exhibited almost the same removal efficiency, suggesting that the initial hydration level of the yeasts does not affect the biocatalyst activity.

The results discussed can be interpreted on the basis of the following model. Assuming that the MCPA removal is only due to a sorption mechanism, the sequence of the physical states of the system during each batch test could be described by the following balance:

$$V \cdot (c_0 - c) = M \cdot (x - x_0) \qquad (3)$$

where V is the volume (L) of MCPA solution, M is the weight (kg) of zirconia matrix containing *Lipomyces*, c_0 is the initial MCPA concentration (mg L^{-1}), x_0 is the initial loading of sorbed MCPA (mg kg-1), and c (mg L^{-1}) and x (mg kg^{-1}) are the MCPA concentration and the loading of sorbed MCPA at a given time in the course of the test, respectively.

The point A in the Figure 4 represents the initial condition of the batch experiment no. 1 ($V = 10^{-3}$ L, $M = 10^{-5}$ kg, $c_0 = 200$ mg L^{-1}, $x_0 = 0$). Consequently, if the MCPA removal were produced by only the sorption mechanism, the sequence of the physical states of the system at each stage of the batch experiment no. 1 would be described by eq 3, which corresponds to the dashed line L_1 in Figure 4. In this hypothesis, the final equilibrium point A' [intersection between the line L_1 and the sorption isotherm described by eq 1] would be reached, corresponding to a residual MCPA concentration of 14 mg L^{-1}. On the contrary, the final value of MCPA concentration measured in the batch experiment no. 1 was 3 mg L^{-1} (point A″ in Figure 4). With the final concentration (point A'') lower than the equilibrium value (point A'), it can be inferred that *Lipomyces* performed their MCPA-degradative activity, enhancing the effect of the pure sorption mechanism.

After the batch experiment no. 1, the liquid phase was removed and replaced with an equal volume of MCPA solution at the initial concentration of 200 mg L^{-1}. Consequently, point B (see Figure 4) represents the starting

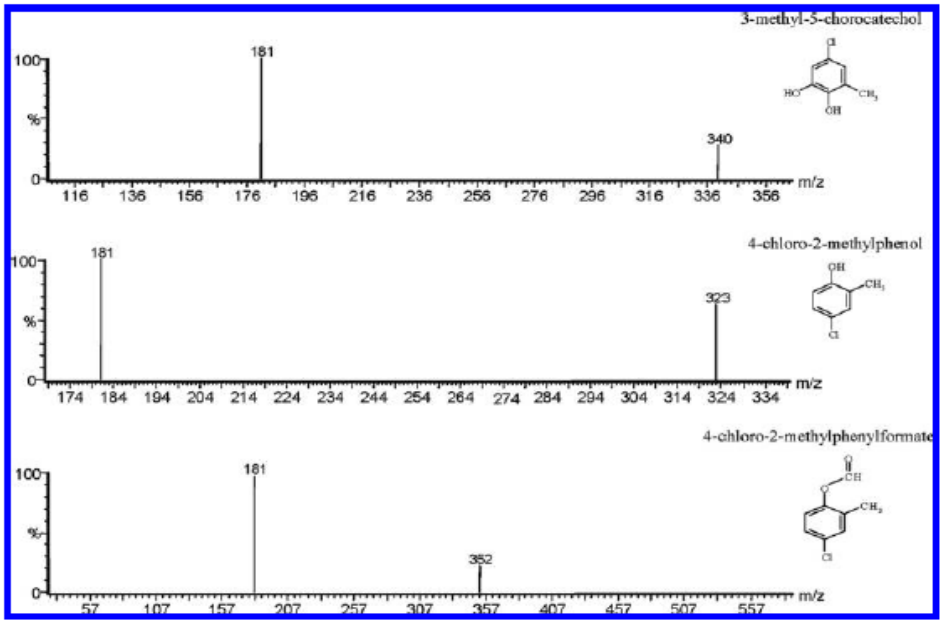

FIGURE 5. Mass spectra of the pentafluorobenzyl bromide derivatives of ZrO_2–*Lipomyces* degradation products.

conditions of the batch experiment no. 2. The dashed line L_2 describes the sequence of the physical states of the sorption system in the course of batch experiment n. 2. Once again, B' is the equilibrium point to be reached if sorption were the only removal mechanism, corresponding to a residual MCPA concentration of 31 mg L^{-1}. As the final value of MCPA concentration measured in the batch experiment no. 2 is 5 mg L^{-1} (point B'' in Figure 4), this result shows that *Lipomyces* were still performing their degradative activity toward MCPA.

The same conclusions can be drawn for all the subsequent batch experiments, which are described by the lines L_1–L_6 in Figure 5.

Identification of the Degradation Products. In Figure 5 the mass spectra of the pentafluorobenzyl bromide derivatives of degradation products of MCPA are reported. GC peaks of the derivatives were found by reconstructed mass chromatograms with selected ion *m/z* 181 (pentafluorobenzyl bromide) which was the base peak. In particular, the main degradation products found were 3-methyl-5-chloro-catechol, 4-chloro-2-methyl-phenol, and 4-chloro-2-methylphenylformate. The total concentration of these molecules was around 3.2%, which together with other secondary degradation products present at concentrations below the detection limit of the method did not exceed 5%. This result confirms that a degradative mechanism contributes to the MCPA removal observed in the presence of the *Lipomyces* immobilized in the ZrO_2 matrix.

The presence of Cl^- ions (data not shown) in the samples of *Lipomyces* immobilized in the ZrO_2 matrix detected by ion chromatography (the analytical determination is described in the Supporting Information) demonstrates that a partial mineralization of the MCPA degradation products occurred (*31*).

It should be noted that a portion of the MCPA molecules initially dissolved in the solution were subjected to a partial degradation, generating the derivatives reported in Figure 5, and some of these degradation products were fully mineralized. Smith et al. (*31*) demonstrated the degradation of MCPA and other phenoxyalkanoic acid herbicides by using an isolated soil bacterium. In particular, MCPA metabolism was accompanied by the complete release of chloride, confirming the total herbicide breakdown.

Immobilized microorganisms were employed for the bioremediation and bioaccumulation of environmentally hazardous compounds. Fielder et al. (*32*) used *Rhodococcus rhodochrous* cells and *Aspergillus versicolor* spores to degrade phenol and glycerol. The cells embedded in sol–gel matrices showed high biocatalytic activity, high compactness and low shrinkage during drying. In the literature, several studies revealed the entrapped cells to be viable within sol–gel. Due to the good diffusional properties of these materials, microorganisms were enabled to respond to the nutrients and inducers along with improved stability in terms of a long-term activity (*33*).

Briefly, in this paper we have demonstrated for the first time that a sol–gel zirconia matrix holds promise as a biocompatible scaffold for encapsulation of cells for bioremediation applications. The reusability of encapsulated cells makes it possible to conduct continuous operations in small-scale as well as industrial-scale treatment systems.

Acknowledgments

This manuscript is contribution DiSSPAPA 231.

Supporting Information Available

Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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