



Universidade do Algarve – Faculdade de Ciências e Tecnologias

# Heterotrophic cultivation of *Thraustochytrids* using glycerol and saline medium from a dairy effluent

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Luís André Roque Fortes

**Thesis to obtain the Master Degree in Biological Engineering**

2016

Thesis elaborated under the guidance of Professor Tomáš Brányik and co-supervised by  
Professora Sara Isabel Cacheira Raposo



**INSTITUTE OF  
CHEMICAL TECHNOLOGY  
PRAGUE**

Institute of Chemical Technology – Department of Bioengineering

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Authors and works consulted are properly cited in the text and listed in the  
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Luís Fortes

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### **Acknowledgments**

I would like to thank to my supervisor, Dr. Tomas Branyik, for all the support and the opportunity given to conduct my research under his guidance. I also would like to thank to my co-supervisor Dr<sup>a</sup>. Sara Raposo for all the availability and support demonstrated while I performed research at ICT-Prague and while I was writing my thesis.

I am grateful to all the members of my laboratory for their help and friendship. My special Thanks goes to Tomas Humhal for all the valuable orientation and amazing knowledge provided during my research.

My deepest gratitude goes to my family for their support, patient and love. To my mother, my father and my sister who always encourage me to look further and never give up.

Finally, I want to thank to all my friends who always supported me through this journey, thanks to their advice and teachings I became a better person.

## Abstract

Microalgae are a promising source of biofuels and other valuable chemicals. The reduced economic feasibility of microalgal cultures is due to low cell density and slow growth rate of these cultures. Thus, it is necessary to develop sustainable processes that will be able to increase the productivity, maximize the production yield and reduce production costs. To achieve this goals, it is necessary to improve the understanding of the behavior of microalgal cultures.

A robust method was developed for the growth of *Thraustochytrids* in saline waste medium using glycerol as carbon source. The first study case was the heterotrophic cultivation of *Schizochytrium limacinum* in order to achieve the highest growth and DHA content possible using alternative raw materials. In the second study case, the temperature profiles of *Japanochytrium sp.* were analyzed to identify the optimal temperature for growth and PUFAs production.

The first case study, *Schizochytrium limacinum* was grown in saline waste medium using glycerol as carbon source. After 216 hours of fermentation the values obtained were the following: biomass concentration of 40.4 g.L<sup>-1</sup>, DHA content of 48.5% and a DHA productivity of 424 mg.L<sup>-1</sup>.d<sup>-1</sup>. The results obtained proved that *Schizochytrium limacinum*. is able to grow and produce high levels of DHA using saline waste medium and glycerol as carbon source. Besides, it was made the economic balance of the used media for biomass and DHA production. In standard medium to produce 1 kg of biomass and 1 kg of DHA will cost 105 € and 1.235 €, respectively. While in saline waste medium to produce 1 kg of biomass and 1 kg of DHA will cost 17.16 € and 180.7 €, respectively.

The second case study, *Japanochytrium sp.* was cultivated at different temperatures of 15, 20, 25 and 30 ° C. The highest value of biomass, 22 g.L<sup>-1</sup>, was obtained at 25 °C whereas the lowest value of biomass, 11 g.L<sup>-1</sup>, was obtained at 15 °C. For 20 °C and 30 °C the biomass concentration was 19 g.L<sup>-1</sup> and 16 g.L<sup>-1</sup>, respectively. These

results are in agreement with the literature, where the optimal temperature to the growth of *thraustochytrids* range between 22-28 °C.

Glycerol is the major byproduct of the biodiesel industry and since it is expensive to purify, biodiesel producers must seek alternative methods for its disposal. Hence using glycerol as a carbon source for fermentation is an alternative use for this product.

**Keywords:** Algal fermentation, biodiesel, docosahexaenoic acid, *thraustochytrids*, biomass composition, lipid characterization

## Resumo

Com o decorrer do tempo as microalgas têm vindo a tornar-se uma fonte promissora no ramo dos biocombustíveis e indústrias relacionadas. A baixa viabilidade económica no cultivo de microalgas deve-se a uma fraca densidade celular e baixa taxa de crescimento. Deste modo, é necessário desenvolver processos sustentáveis capazes de aumentar a produtividade, maximizar o rendimento e reduzir os custos de produção. Para atingir estes objetivos é necessário aprofundar conhecimentos para a compreensão do comportamento em culturas de microalgas.

Neste trabalho, foi desenvolvido um meio de cultura para o crescimento de *Thraustochytrids* em meio salino, proveniente do efluente de uma fábrica de laticínios e usando glicerol como fonte de carbono. O primeiro caso de estudo foi o cultivo heterótrófico de *Schizochytrium limacinum* de modo a atingir uma elevada taxa de crescimento desta microalga bem como o conteúdo de ácido docosahexaenoico (ADH) associado usando matérias-primas alternativas. O segundo caso de estudo teve como objectivo identificar os perfis de temperatura da microalga *Japanochytrium sp.* de modo a identificar a temperatura ideal para o seu crescimento e para a produção de ácidos gordos polinsaturados.

A microalga *Schizochytrium limacinum* foi cultivada em meio salino usando glicerol como fonte de carbono. Após 216 horas de fermentação obtiveram-se os seguintes resultados: concentração de biomassa de 40,4 g.L<sup>-1</sup>, conteúdo em ADH de 48,5% e a produtividade de ADH atingiu 424 mg.L<sup>-1</sup>.d<sup>-1</sup>. Os resultados obtidos provaram que a microalga em questão tem uma boa capacidade de crescimento aliado a uma produção elevada de ADH. Foi feito um balanço económico entre o meio salino standard e o meio salino do efluente para a produção de biomassa e ADH. Em meio standard para produzir 1 Kg de biomassa e 1 Kg de ADH custa 105 € e 1.235€, respectivamente.

No segundo caso de estudo, o *Japanochytrium sp.* foi cultivado a diferentes temperaturas: 15, 20, 25 e 30 °C. O valor mais elevado de biomassa, 22 g.L<sup>-1</sup>, foi obtido a 25 °C e o valor mais baixo de biomassa, 11 g.L<sup>-1</sup> foi obtido a 15 °C. Para 20 °C e 30 °C a concentração de biomassa obtida foi, 19 g.L<sup>-1</sup> e 16g.L<sup>-1</sup>, respectivamente. Os resultados obtidos neste estudo

estão de acordo com a literatura, onde a temperatura ótima de crescimento para o crescimento de *thraustochytrids* varia entre 22-28 °C.

O glicerol é o principal subproduto na indústria do biodiesel e uma vez que os custos de purificação para uso na indústria farmacêutica e alimentar são demasiado elevados, os produtores devem procurar métodos alternativos para a sua utilização. Provou-se que utilizando o glicerol como fonte de carbono para fermentações é uma alternativa bastante atrativa para o uso deste produto.

Palavras-Chave: Fermentação, biodiesel, *Schizochytrium sp.*, *Japanochytrium sp.*, ácido docosahexaenoico, *thraustochytrids*, composição da biomassa, caracterização lípidica



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## Nomenclature

ACL	ATP-citrate Lyase
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
BOD	Biochemical Oxygen Demand
DCW	Dried cell weight
DHA	Docosahexaenoic Acid
FAME	Fatty Acid Methyl Ester
FAS	Fatty Acid Synthetase
GHG	Green House Gas
$k_{La}$	Volumetric Mass Transfer Coefficient
NADPH	Nicotinamide Adenosine Dinucleotide Phosphate
OD	Optical Density
PBR	Photobioreactor
PUFA's	Polyunsaturated Fatty Acids
$q_p$	Product Formation
SM	Standard Medium
STR	Stirred Tank Reactor
SWM	Saline Waste Medium
SWW	Saline Wastewater
SCO	Single Cell Oil
TCA	Tricarboxylic Acid Cycle



TFA	Total Fatty Acids
$T_d$	Doubling time
$Y_{x/s}$	Maximal Yield of Cell Mass from Substrate.
$\mu_{\max}$	Maximum specific growth rate

## Chapter 1: Introduction

### Framework of the project

With the current amount of biodiesel produced worldwide over the years, the unrefined glycerol from transesterification of lipids has been increasing. As biodiesel production increases, so does production of the primary co-product, glycerol. Since the existing glycerol supply and demand market was tight, recent increases in glycerol production from biodiesel refining has created a glut in the glycerol market <sup>1</sup>. As result of this the price of glycerol decreased significantly. Since it is expensive to purify glycerol into compounds that can be used in food, pharmaceutical and cosmetic industries it is necessary to develop new strategies to utilize this material to get profit.

Microalgae are a rather underexplored group of microorganisms and considered attractive as natural sources of valuable bioactive molecules since they have the potential to produce a variety of chemical and biological compounds, including long-chain polyunsaturated fatty acids, carotenoids and phycocolloids. The microalgal species which are currently attracting commercial interest grow under heterotrophic conditions and perform efficiently in conventional bioreactors in a similar manner to bacteria or yeast <sup>2</sup>. Thus in the last decades a lot of efforts have been made to explore this organisms to obtain high-value bio products at lower costs <sup>3</sup>.

The microalgae-like marine protists used for this project was *Schizochytrium limacinum* since this specie can use glycerol as energy and carbon source to produce docosahexaenoic acid (DHA) <sup>4</sup> in heterotrophic regime <sup>5</sup>.

From a variety of polyunsaturated fatty acids, docosahexaenoic acid (DHA, 22:6 *n*-3) have attracted increased attention due to its properties in human health. It was shown to have an important role in aging, memory, macular degeneration, neurodegenerative diseases like neuroinflammation, Alzheimer, Parkinson disease and contributes to cardiovascular health too <sup>6,7</sup>.

Currently, the most usual source of omega-3 is fish oil. The efforts to obtain omega-3 polyunsaturated fatty acids from algal biomass bring advantages compared to fish oil. Some of this advantages include solving the problem of overfishing, omega-3 from algal biomass

is odorless compared to the odor from fish oil and its advantageous for the aquaculture industry to use in fish feed to produce fish rich in omega-3<sup>8,9</sup>.

### **Objective of the project**

The main objective of this project is to develop microbial technologies focused on conversion of alternative sources of carbon and energy.

Glycerol and Saline Waste Medium (SWM), provided by dairy industry, will be used as an alternative for *Schizochytrium limacinum* cultivation to obtain biomass with a high biotechnological potential. The production yields of biomass and DHA as well as the production costs of the process using glycerol and saline waste medium against glycerol and standard waste medium will be compared.

In the second case-study, the microalgae used will be *Japanochytrium sp.* The goal was to find the optimal temperature for growth of the model microalgae.

## **Chapter 2: Background and Literature topics**

### **2.1 About Biodiesel**

Biodiesel is a promising renewable energy resource which has the potential to reduce the world's dependence on fossil fuels whilst lessening the environmental impact associated with these.

Thus it is expected that renewable fuels will gradually replace fossil fuels and so mark a historic transition into a sustainable society in which biological feedstock's, processes and products constitute the main pillar of the fuels economy <sup>10</sup>. The important criteria are: the fuel should be effective in propelling a car, should not be harmful to the engine and/or auxiliary parts, should be according to legislation and sustainable, when we talk about sustainability it means that fuel must be environmental friendly with minimum greenhouse gas (GHG) reduction potential, protection of biodiversity and food versus fuel should always be in mind <sup>11,19</sup>.

#### **2.1.1 Environmental Overview**

The combustion of biodiesel does release CO<sub>2</sub> into the atmosphere, however the fuel sources range from wastes to crops and algal products. This means that although carbon dioxide is uptaken during the production, no new CO<sub>2</sub> is being released or the fuel is derived from a source that was going to be disposed of anyway. Nitrogen and sulphur impurities are almost completely removed when compared to normal diesel; this means that there is a decrease of nitrogen dioxide and sulphur dioxide emissions, which reduces instances of acid rain <sup>14, 15</sup>.

In this way biodiesel policy aims to promote the use of fuels made from biomass as well as other renewable fuels. Provide the prospect of new economic opportunities for developing countries, concerns job creation and greater efficiency in the protection of the environment. The use of biodiesel is expected to minimize greenhouse gas emissions, dependence on imported petroleum, and revitalize the economy by increasing demand and prices for agricultural products <sup>16</sup>.

### 2.1.2 Biodiesel production within Portugal

In Portugal biodiesel production started in 2005 with a production of 1000 tons per year and currently are produced about 500 000 tons per year <sup>17</sup>.

There are four main producers of biodiesel in Portugal, Prio (Martifer Group), Iberol, Biovegetal (SGC Group) and Sovenna Group. These groups use waste cooking oil, rapeseeds, soybean seeds and other kind of oilseeds as feedstock's <sup>18,19</sup>. Other important data related to these industries is in the Table 1.

Table 1 – Leading medium-large size biodiesel plants in Portugal with its raw material, production capacity, total investment and the year when the factory started the production. Accessed through Portugal biofuels standing report 2015 <sup>18, 19</sup>

Producers	Raw materials	Capacity (ton/year)	Investment (M€)	Start of Production (Year)
<b>Prio</b>	Palm oil Soybean oil Rapeseed oil	100 000	32	2007
<b>Iberol</b>	Soybean Vegetable oil Palm oil Rapeseed oil	125 000	30	2006
<b>Biovegetal</b>	Palm oil Soybean oil Rapeseed oil	125 000	29	2007
<b>Sovena</b>	Palm oil Soybean oil Rapeseed oil	95 000	23.4	2007
<b>Biopordiesel</b>	Palm oil Soybean oil Rapeseed oil	31 536	-	2011
<b>Enerfuel</b>	Animal fats Used cooking oil	27 000	-	2013
<b>Valourodiesel</b>	Palm oil Soybean oil Rapeseed oil	50 000	-	2011

### **2.1.3 Regulatory Framework**

When the biofuels are used commercially, there is an uncertainty in the economic analysis as the cost of the feedstock will fluctuate and the cost of petroleum will vary to compete with the biofuel. The growth of oil prices in the market, the pressure to reduce atmospheric emissions of CO<sub>2</sub> and the concerns to promote the use in transport of biofuels is reflected in the legal obligation to set up policies by the governments <sup>16, 20</sup>.

### **2.1.4 Global Legislation**

The Kyoto Protocol is a worldwide environmental treaty for developed countries to decrease their GHG emissions. In 2012 GHG emissions were down by at least 18% compared with 1990 levels approaching the headline target to reduce emissions by 20% by 2020 <sup>21</sup>.

### **2.1.5 European Legislation**

Within the European Union (EU), the European Commission (EC) is the entity responsible for setting the legislation and guidelines to treat climate change and promote biofuels use in the EU. In this way the EU issued the *Renewable Energies Directive (2009/28/EC)*, *Biofuel Directive (2003/30/EC)* and *Energy Tax Directive (2003/96/EC)*.

#### *Renewable Energies Directive (RED) (2009/28/EC)*

This is one of the most important EU Directives and it declares that the proportion of renewable energies is to be increased to a mandatory 20% in the EU by the year 2020. In the transport sector, the proportion of renewable energies is to rise to 10% of total fuel consumption over the same. The *Renewable Energies Directive* contains rules on the sustainable production of biofuels as the precondition for official support and for recognition under the EU biofuel targets. With the introduction of sustainability criteria the EU is also ensuring that in future only sustainably produced biofuels are used in the transport sector. It is required that biofuels reduce greenhouse gas emissions by at least 35 %, and from 2017 by as much as 50 %. New biofuel plants constructed after 2017 must achieve greenhouse gas reductions of 60 % <sup>22</sup>.

### 2.1.6 Portuguese Legislation

The *DL nr. 117/2010 of 25<sup>th</sup> October* establishes the framework for biofuels in Portugal until 2020 <sup>23</sup>.

- Transposition of European Directive 2009/28/EC (RED)
- Defines the national mandatory targets for incorporation of biofuels in transport

2011 and 2012 – 5,0%

2013 and 2014 – 5,5%

2015 and 2016 – 7,5%

2017 and 2018 – 9,0%

2019 and 2020 – 10%

- Incorporation of 2,5% in gasoline will start in 2015

### 2.1.7 Biodiesel Standards

To allow the biodiesel to be sold in the European Union it is necessary to meet standard criteria limits. The chart below displays EN 14214 biodiesel standards.

Table 2 – Biodiesel Standards EN 14214 <sup>11, 24</sup>

Table B-4. Biodiesel Standard EN 14214 (Europe).				
Property	Test Method	Limits		Units
		min.	max.	
Ester content	EN 14103	96.5		% (m/m)
Density; 15°C	EN ISO 3675 EN ISO 12185	860	900	kg/m <sup>3</sup>
Viscosity; 40°C	EN ISO 3104	3.5	5.0	mm <sup>2</sup> /s
Flash point	EN ISO 2719 EN ISO 3679	101		°C
Sulfur content	EN ISO 20846 EN ISO 20884		10.0	mg/kg
Carbon residue (10% dist. residue)	EN ISO 10370		0.30	% (m/m)
Cetane number	EN ISO 5165	51		
Sulfated ash	ISO 3987		0.02	% (m/m)
Water content	EN ISO 12937		500	mg/kg
Total contamination	EN 12662		24	mg/kg
Copper strip corrosion (3h, 50°C)	EN ISO 2160	1		
Oxidative stability, 110°C	prEN 15751 EN 14112	6.0		h
Acid value	EN 14104		0.50	mg KOH/g
Iodine value	EN 14111		120	g iodine/100g
Linolenic acid content	EN 14103		12	% (m/m)
Content of FAME with 4 or more double bonds			1	% (m/m)
Methanol content	EN 14110		0.20	% (m/m)
Monoglyceride content	EN 14105		0.80	% (m/m)
Diglyceride content	EN 14105		0.20	% (m/m)
Triglyceride content	EN 14105		0.20	% (m/m)
Free glycerine	EN 14105 EN 14106		0.02	% (m/m)
Total glycerine	EN 14105		0.25	% (m/m)
Sodium and Potassium	EN 14108 EN 14109		5.0	mg/kg
Calcium and Magnesium	EN 14538		5.0	mg/kg
Phosphorus content	EN 14107		4.0	mg/kg



### 2.1.8 Pathway of Biodiesel production

Biodiesel is made through transesterification also known as alcoholysis, is the displacement of alcohol from an ester by another alcohol in a process similar to hydrolysis, except than an alcohol is used instead of water. This process has been widely used to reduce the viscosity of triglycerides. This catalyzed chemical reaction (transesterification) is represented by the general equation:



The reaction of a triglyceride with an alcohol (usually methanol) is represented by the general equation in Fig.1

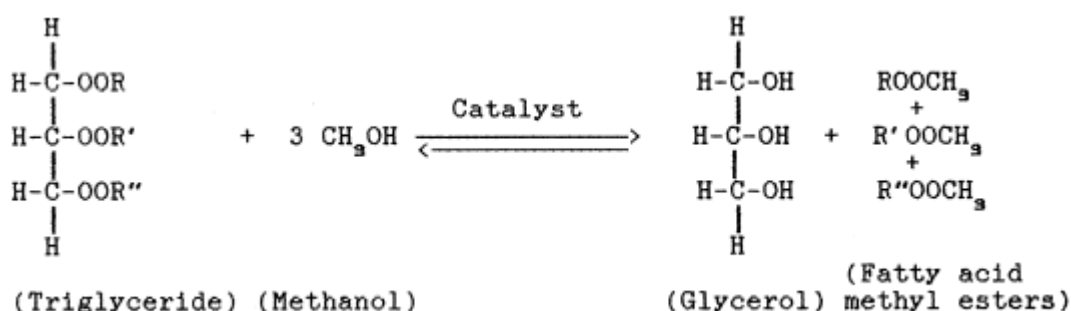


Figure 1 – Transterification reaction to produce biodiesel  
(Adapted from Srivastava *et al.*, 1999)<sup>25</sup>

In this reaction the fatty acids are released from glycerol and react with methanol to form fatty acid methyl esters (FAME) and glycerol. The stoichiometry of the transesterification reaction requires 3 mole of alcohol per mole of triglyceride to yield 3 mole of fatty esters and 1 mole of glycerol. The stepwise reactions are reversible and an excess of alcohol is used to shift the equilibrium towards the products side. When 100% excess methanol is used, the reaction rate is at its highest. A molar ratio of 6:1 is normally used in industrial processes to obtain methyl ester yields higher than 98% by weight. Methanol is usually used because of its low cost, physical and chemical advantages (polar and shortest chain alcohol) and has a great reaction efficiency. It can quickly react with triglycerides and

NaOH is easily dissolved in it. It was also observed that transesterification is faster when catalyzed by alkali and is most often used commercially<sup>25, 26</sup>.

### 2.1.9 Glycerol composition and applications

Glycerol, also known as glycerin is the principal by-product of biodiesel production. Such crude glycerol possesses very low value because of the impurities contained. As the demand and production of biodiesel grows, the quantity of crude glycerol generated will be considerable, and the utilization of it will become an urgent topic. But before the crude glycerol could be considered as value-added utilization, is necessary to characterize his physical and chemical properties. As reported earlier biodiesel producers use excess methanol in transesterification and do not recover all the methanol what makes methanol an impurity present in the glycerol layer ( $\approx 80\%$  methanol), it also contains other elements like magnesium, calcium, phosphorous or sulfur. Thompson *et al.*<sup>27</sup> have characterized glycerol produced from various feedstock like soybean, rapeseed, waste vegetable oil, canola, mustard seeds and crambe seeds. In that study the crude glycerol from those feedstock is between 60% and 70% glycerol, where waste vegetable oil had the highest level of glycerol (76,6%) and mustard seed had the lower level (62%). These authors also investigated the elemental composition of crude glycerol and the elements present are calcium, magnesium, phosphorous, sulphur, sodium, potassium, carbon and nitrogen. Besides being produced by different feedstocks, the elements content is similar. Biodiesel producers with high economic capacity can refine the crude glycerol by filtration and fractional vacuum distillation and move it to food, cosmetic and pharmaceutical industries. Small producers who can't afford the high cost of purification find crude glycerol utilization to be a problem<sup>27</sup>.

It is known as biodiesel production increases, so does the production of the primary co-product, glycerol. Since the existing glycerol supply and demand market was tight, recent increases in glycerol production from biodiesel refining has created a glut in the glycerol market. In this way it's necessary to find processes to convert this glycerol into commercially valued products<sup>28</sup>.

Pagliaro *et al.*<sup>29</sup> describes a variety of catalytic conversion methods for glycerol that will play an important role as raw material in chemical industries. For example by selective oxidation it is possible to obtain glyceric acid, tartronic acid and dihydroxyacetone which

can be used in food and cosmetic industries. By hydrogenolysis it's possible to obtain propylene glycol which is used as antifreeze product, lubricant and food additive. By dehydration it's possible to obtain acrolein a chemical largely employed by the chemical industry for the production of acrylic acid esters, super absorber polymers, and detergents. It is possible to see in the Fig.2 the glycerol as a raw material for functional chemicals.

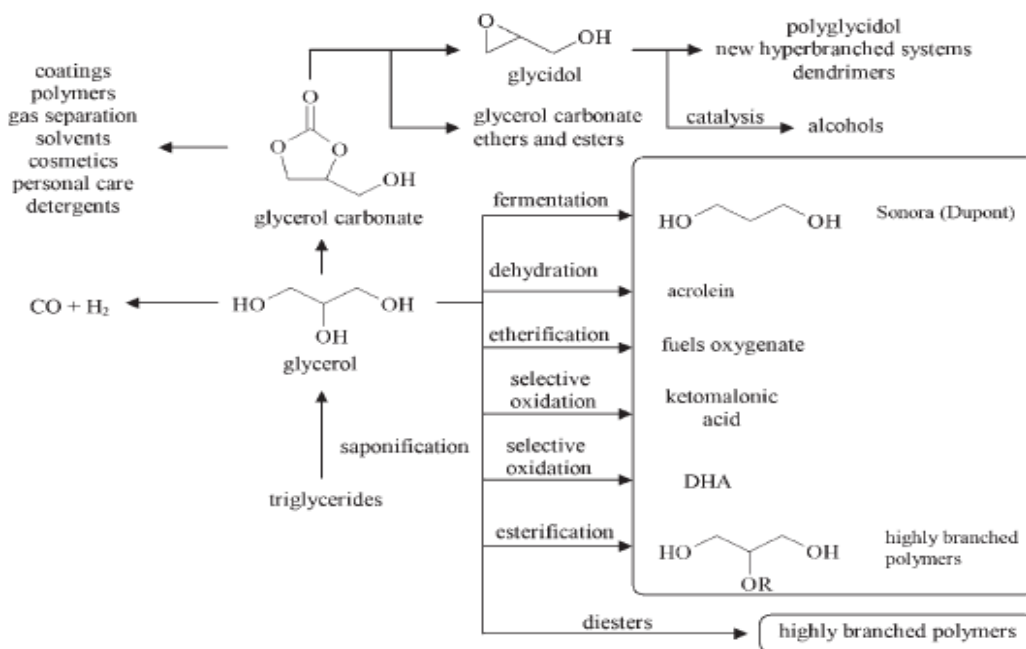


Figure 2 – Glycerol as raw material for chemical compounds production which can be applied in the chemical industry

Furthermore Johnson *et al.*<sup>1</sup> proposed promissory options for biological conversion of glycerol into value products. Yeasts like *Yarrowia sp.*, *Candida sp.*, and *Rhodotorula sp.* has focused on the production of citric acid, sophorolipids, and single cell oil using glycerol as carbon source, but at the moment there are no commercially applications for these products. These authors mention that the most promising option for biological conversion using glycerol is in the fermentative production of 1,3-propanediol a common component used in polymer synthesis, acetic acid, butyric acid, butanol, acetone, ethanol, 2,3-butanediol, lactic acid, succinic acid and format. Lactic acid, butanol and succinic acid are very important in biorefinery platform chemicals, they can be used for the production of new chemical products as well as bio-based alternatives to petroleum chemicals. Butyric acid is commonly used in the food industry as additive.

There have been major efforts developing alternatives strategies for the glycerol uses in chemical and biological processes to create valuable industrial products. With the current increase in biodiesel production and a glut in the market for glycerol this processes should continue to be investigated and commercialized if possible.

## 2.2 Microalgal biotechnology

Algae covers a diverse group of prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure. Ranging in size from giant kelps to the unicellular microalgae (a group of protists which are only a few microns in size) and the prokaryotic cyanobacteria (blue-green algae)<sup>28</sup>. The diversity of algae are illustrated in Fig. 3, referring the taxonomic groups<sup>29</sup>.

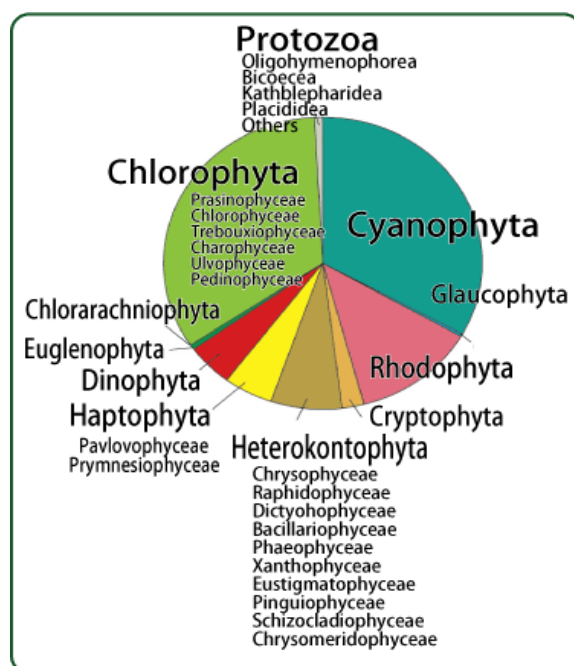


Figure 3 – Proportion of taxonomic groups of algae  
(Adapted from BioResource Newsletter Vol.3 No.10, <http://www.shigen.nig.ac.jp/>)

The interest in these group of organisms lies in their potential to produce biomass for food, feed for aquaculture and chemicals. Microalgae are found all over the world, mainly distributed in the waters, but also in the surface of soils<sup>30</sup>. It is important to mention that even though microalgae are seen as photosynthetic, there are other groups that can be heterotrophs.

### 2.2.1 Applications of microalgae

Microalgae have a great potential as source of biotechnological products due to their adaptability to extreme environmental condition, and flexibility to use different sources of energy and carbon. These microorganisms possess the advantage of a metabolic flexibility. Similarly, their secondary metabolism, which have a huge interest for biotechnology, can be easily triggered applying stress (e.g., lack of a nitrogen source) <sup>31</sup>.

Microalgae are being used for the production of human and animal food, aquaculture feed, pharmaceutical and cosmetic products, biofuels and wastewater treatment <sup>28, 32, 33, 34, 35, 36</sup>.

One of the experiments in this work was focused on the fed-batch cultivation of *Schizochytrium limacinum*, considered a high producer of DHA (Docosahexaenoic acid, C22:6 n-3), on a mixture of saline waste medium from a dairy effluent and glycerol as carbon source. A second case study was developed to analyze the effect of the temperature on the growth of *Japanochytrium sp.* as well as the temperature that gives the higher content of biomass. Both model organisms are microalgae-like marine protists.

### 2.2.2 Microalgae in human and animal nutrition

Microalgae can be used in the production of biofuels, but they are mainly cultivated to obtain highly valuable compounds due to the chemical composition of those species (Table 3) <sup>37</sup>.

Table 3 – Composition of different human food sources and algae  
(% dry matter)  
(Adapted from Spolaore *et al.*<sup>40</sup>)

Commodity	Protein	Carbo- hydrate	Lipid
Bakers' yeast	39	38	1
Meat	43	1	34
Milk	26	38	28
Rice	8	77	2
Soybean	37	30	20
<i>Anabaena cylindrica</i>	43–56	25–30	4–7
<i>Chlamydomonas reinhardtii</i>	48	17	21
<i>Chlorella vulgaris</i>	51–58	12–17	14–22
<i>Dunaliella salina</i>	57	32	6
<i>Porphyridium cruentum</i>	28–39	40–57	9–14
<i>Scenedesmus obliquus</i>	50–56	10–17	12–14
<i>Spirulina maxima</i>	60–71	13–16	6–7
<i>Synechococcus sp.</i>	63	15	11

It should be kept in mind that the figures presented in this table are estimates, since the proportion of individual cell constituents largely depends on environmental parameters.

Microalgae for human nutrition are marketed in different forms like food or food supplement and are commonly sold in tablets, capsules or liquids <sup>37</sup>.

It is relevant to know that the composition from microalgae varies from strain to strain and depends of parameters like pH, temperature, medium content, CO<sub>2</sub> supply etc.

The high protein content is one reason to consider microalgae as an unconventional source of proteins. Humans and animals are limited to the biosynthesis of certain amino acids only, the remaining needs to be given through food. The amino acid profile of microalgae was compared with the proteins in the food and it was reported that there are similarities in the content (Table 4), in this way, since this cells can synthesize all the amino acids, microalgae can be used to provide this essential amino acids to humans and animals <sup>37, 38</sup>.

Carbohydrates in microalgae can be found in the form of starch, cellulose, sugars and other polysaccharides. Their overall digestibility is high, so there is no limitation to use microalgae in foods or feeds.

Table 4 - Amino acid profile of different algae as compared with conventional protein sources and the WHO/FAO reference pattern (g per 100 protein)  
(Adapted from Becker <sup>37</sup>)

Source	Ile	Leu	Val	Lys	Phe	Tyr	Met	Cys	Try	Thr	Ala	Arg	Asp	Glu	Gly	His	Pro	Ser
Egg	6.6	8.8	7.2	5.3	5.8	4.2	3.2	2.3	1.7	5.0	–	6.2	11.0	12.6	4.2	2.4	4.2	6.9
Soybean	5.3	7.7	5.3	6.4	5.0	3.7	1.3	1.9	1.4	4.0	5.0	7.4	1.3	19.0	4.5	2.6	5.3	5.8
<i>C. vulgaris</i>	3.2	9.5	7.0	6.4	5.5	2.8	1.3	–	–	5.3	9.4	6.9	9.3	13.7	6.3	2.0	5.0	5.8
<i>D. bardawil</i>	4.2	11.0	5.8	7.0	5.8	3.7	2.3	1.2	0.7	5.4	7.3	7.3	10.4	12.7	5.5	1.8	3.3	4.6
<i>S. platensis</i>	6.7	9.8	7.1	4.8	5.3	5.3	2.5	0.9	0.3	6.2	9.5	7.3	11.8	10.3	5.7	2.2	4.2	5.1
<i>Aphanizomenon flos-aquae</i>	2.9	5.2	3.2	3.2	2.5	–	0.7	0.2	0.7	3.3	4.7	3.8	4.7	7.8	2.9	0.9	2.9	2.9

The lipid content of microalgae is about 1% to 70% of the dry weight but in certain cases can reach 85-90% of dry weight. The algal lipids are mainly composed of glycerol, sugars or bases esterified to saturated or unsaturated fatty acids. The common fatty acids of particular interest are  $\omega$ 3,  $\omega$ 6,  $\omega$ 9 families.

Another important constituent of microalgae are the vitamins. They represent a source of almost all essential vitamins (Table 5) what makes them more attractive to use as nutritional supplement <sup>39, 40</sup>.

Microalgae are also rich in pigments like chlorophyll (0.5 – 1.5% of dry weight), carotenoids (0.1 – 0.2% of dry weight) and phycobiliproteins.

All this compounds has a wide range of commercial applications, however, before being commercialized this algal material must be subjected to analysis in order to detect the presence of toxic compounds <sup>37</sup>.

Table 5 - Vitamin content of different algae in comparison with common foodstuffs (values in mg/kg dry matter)  
(Adapted from Becker <sup>37</sup>)

Source	Vit A	Vit B <sub>1</sub>	Vit B <sub>2</sub>	Vit B <sub>6</sub>	Vit B <sub>12</sub> *	Vit C	Vit E	Nicotinate	Biotin	Folic acid	Pantothenic acid
RDI (mg/d)	1.7	1.5	2.0	2.5	0.005	50.0	30.0	18.0	–	0.6	8.0
Liver	60.0	3.0	29.0	7.0	0.65	310.0	10.0	136.0	1.0	2.9	73.0
Spinach	130.0	0.9	1.8	1.8	–	470.0	–	5.5	0.007	0.7	2.8
Baker's yeast	trace	7.1	16.5	21.0	–	trace	112.0	4.0	5.0	53.0	
<i>S. platensis</i>	840.0	44.0	37.0	3.0	7.0	80	120.0	–	0.3	0.4	13.0
<i>Aphanizomenon flos-aquae</i>		4.8	57.3	11.1	8.0	0.7	–	0.1	0.3	1.0	6.8
<i>C. pyrenoidosa</i>	480.0	10.0	36.0	23.0	–	–	–	240.0	0.15	–	20.0
<i>S. quadricauda</i>	554.0	11.5	27.0	–	1.1	396.0	–	108.0	–	–	46.0

### 2.2.3 Microalgae in Pharmaceutical and Cosmetic industries

Many desirable chemicals are the product of secondary metabolism triggered under certain conditions. In this way, microalgae have become extremely important microorganisms because once a chemical is discovered and characterized it might be produced synthetically and the biochemical pathway of the chemical can be transferred to a easily cultivable organism, in this way the microalgae potential could attract the attention of biopharmaceutical companies.

Guedes *et al.*<sup>36</sup> showed in their research that microalgae are great producers of bio-compounds with beneficial properties to human health like antioxidants, anti-inflammatory, antimicrobial, antiviral and antitumoral compounds.

Plaza *et al.*<sup>41</sup> reported that *Chlorella vulgaris* produce astaxanthin, *Dunaliella salina* produces  $\beta$ -carotene and *Haematococcus pluvialis* produce lutein, all of them with antioxidant effects where astaxanthin has anti-inflammatory and antitumoral effects too.

Eicosapentaenoic acid,  $\alpha$ -Linolenic acid, Goniodomin A produced by *Phaeodactylum tricornutum*, *Chlorococcum* HS-101, and *Goniodoma pseudogoniaulax*, respectively, are known to have antimicrobial actions <sup>36</sup>.

Compounds with antiviral actions are p-KG03 exopolysaccharide produced by *Gyrodinium impudicum* and allophycocyanin produced by *Cryptomonads* <sup>36, 42</sup>.

In the cosmetics field, microalgal can be mainly found in face and skin care products like anti-aging cream, algal clay masks, algal beauty oil, anti-irritant products, sun protection and hair protection with the main specie being *Chlorella*. *Chlorella vulgaris* extract is commercialized as a skin conditioning agent and protecting agent that can be used in anti-aging products to prevent the breakdown the skin's collagen and elasticity.<sup>40</sup>

#### 2.2.4 Environmental applications of microalgae

Besides the uses in human health and animal feed industry, microalgae had a strong impact in the environmental area. For example, the company Mera Pharmaceuticals studied the utilization of microalgae for carbon sequestration. The objective was combining the CO<sub>2</sub> from the fossil fuel combustion system and nutrients in a photobioreactor (PBR) where microalgae photosynthetically convert the CO<sub>2</sub> into compounds of high value in this way it is possible to lower the effective cost of sequestration and generate valuable byproducts<sup>43</sup>. Schematic diagram of the process is shown on Figure 5.

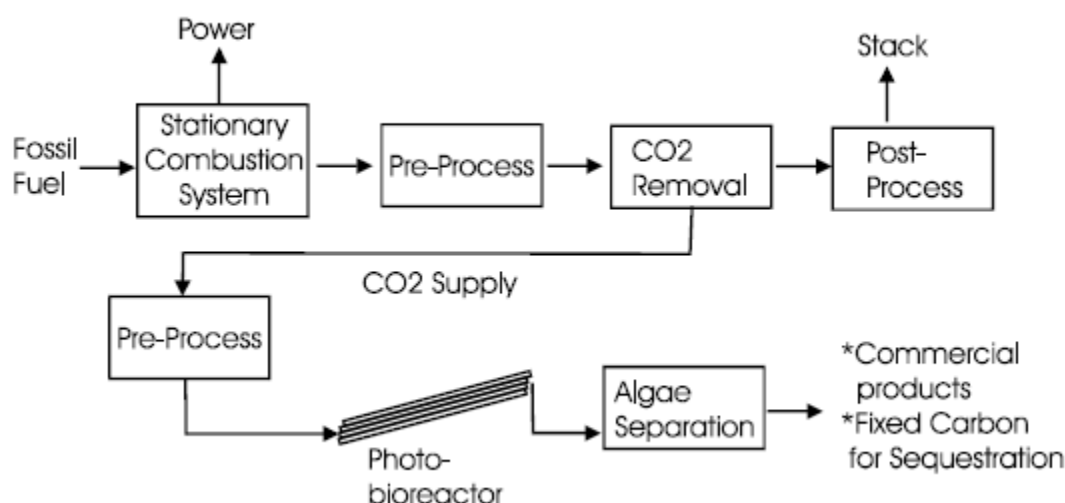


Figure 4 – Diagram for a microalgal-based carbon capture and sequestration scheme developed by Mera Pharmaceuticals  
(Adapted from Olaizola<sup>43</sup>)

Abdel-R. *et al.*<sup>44</sup> showed that microalgae can also be used in the wastewater treatment. In this process it is intended to decrease biochemical oxygen demand (BOD), suspended solids, ions, coliform bacteria and toxicity in order to obtain the higher level of wastewater



purification. It is necessary a combination of successive steps to accomplish these objectives. The problem is the associated cost with each successive step, in this way it is possible to introduce photobioreactors where microalgae can replace some of these steps since this microorganisms have the possibility to incorporate nutrients, they have photosynthetic capabilities and are able to remove toxic compounds without leading to pollution <sup>44</sup>.

Among the 42.000 species <sup>45</sup> only a few are investigated and cultivated in industrial quantities. The development of microalgae biotechnology has not the attention of larges companies, this may be due to the shortage of investment in this area or the lack of success at the industrial production so far. Future research aimed at improvement of cultivation methods and appeal to genetic engineering in order to use genetically modified strains can lead to an interesting development in this field <sup>40, 43</sup>.

As stated before, microalgae have high flexibility, they are photoautotrophic but can perform heterotrophy and mixotrophy. In this way it is possible to use a wide arrangement of cultivation systems. The microalgal production system will be presented in the section 2.7.

### **2.3 Microalgal growth**

It is relevant to understand the factors that determine algal growth rate and product yield. The different cultivation systems plays an important role in the algal growth rate and product yield as well the carbon and nitrogen metabolism, oxygen and others macro- and micronutrients.

#### **2.3.1 Nutritional routes**

There are a variety of possible nutritional routes for algae being autotrophy, heterotrophy and various combinations are possible between these two major forms of nutrition.

Autotrophic organisms capture the energy trough absorption of light energy and reduction of CO<sub>2</sub> by the oxidation of substrates (e.g. water). Autotrophic organisms can be chemoautotrophs too if they utilize electrons donors as a source of energy, from organic or inorganic sources.

Heterotrophic organisms can't fix carbon so they obtain their energy from organic compounds, (e.g. glucose, glycerol). Since there are algal species that can grow on organic substrates, heterotrophic regime has become a viable option in bioreactors systems, always under specific growth conditions. Photoheterotrophic organisms require light as energy source to use organic compounds as nutrients.

For the last, mixotrophic organisms can use a mix of different sources of energy and carbon, some of them have incomplete Calvin cycles so they can't fix carbon dioxide and need to use an organic carbon source<sup>46, 47, 48</sup>.

Several research studies were made to understand which nutritional mode could give the highest biomass growth, cell density and lipid productivity. For example, Ratha *et al.*<sup>48</sup> studied the biomass and lipid productivity under photoautotrophic, heterotrophic and mixotrophic routes. Under photoautotrophic conditions lipid productivity ranged from 2 - 13 %, under mixotrophic 1.7 - 32% and 0.9 - 20% under heterotrophic conditions. Furthermore, Santos *et al.*<sup>49</sup> compared heterotrophic, autotrophic and mixotrophic using *Chlorella protothecoides* for lipid production, they achieved the highest biomass productivity and lipid content (>22%) under heterotrophic conditions. Li *et al.*<sup>108</sup> compared the effects of autotrophic and mixotrophic growth regime on the cell growth and lipid productivity for biodiesel production. They achieved a biomass production 14x higher and a lipid productivity 5.6x higher under mixotrophic than autotrophic conditions. Despite that, under mixotrophic conditions the culture assimilated 99.7% more nitrogen and 75.9% phosphorous from piggy wastewater reducing the nutritional requirements for culture, hence the associated costs.

Despite mixotrophic and heterotrophic conditions showed to be the most attractive way for cultivation, it should be taken into account that the cultivation of microorganisms depends from several factors like the product desired, type of microorganisms, microorganism strains, nutrients, fermentation conditions and systems for microalgal cultures.

### **2.3.2 Systems for mass microalgal cultures**

#### **Open ponds**

Open ponds are the oldest and simplest systems where algae are cultivated under conditions similar to the external environment. The biggest advantage of these ponds is their simplicity, resulting in low production costs and low operation costs. However, it has some drawbacks owing to the fact that the contaminations are not under control, and the biomass productivity is low due to light limitation.

#### **Photobioreactors**

Photobioreactors were developed to overcome the problems encountered in open ponds. These reactors possess artificial illumination (optional), CO<sub>2</sub> addition, stirring and cooling facilities. They offer many advantages over open ponds such as a better control of culture, large surface-to-volume ration, reduced fouling and better protection from outside contamination. However there are disadvantages associated with PBRs, such as, capital and production cost are very high, light limitation cannot be totally overcome since light penetration is inversely proportional to the cell concentration and the accumulation of oxygen is still another unsolved problem.

#### **Heterotrophic systems**

Sugars or organic acids are used as carbon and energy sources instead of CO<sub>2</sub> and light. In this way, microalgae species need to be isolated in the presence of organic carbon substrates and from sources rich in organic materials<sup>50</sup>. Heterotrophic systems offer the possibility of greatly increase cell density and productivity since they eliminates light dependence and the culture conditions can be controlled. A heterotrophic culture process may be further intensified by using cultures techniques such as fed-batch leading to high cell densities<sup>2, 34, 51, 54</sup>.

Chen *et al.*<sup>51</sup> listed the required initial characteristics that microalgae species must fulfill to be used in heterotrophic cultivation: (a) Ability to grow in culture media with easy-

to-sterile organic substances where energy required for heterotrophic growth must be supplied by oxidation of part of the organic substrate. (b) Cell division and active metabolisms in absence of light. (c) Ability to adapt to fast environmental changes. (d) Capacity to resist hydromechanical stress inside the fermenters. In addition to these parameters for heterotrophic cultivation, the main practical key issues in large-scale for heterotrophic cultures of microalgae are: a) Good survival of the strain during cultivation, b) Overall low cultivation costs reflected as the ability of the strain to efficiently use inexpensive carbon sources, tolerate environmental changes and generate economically valuable metabolites and d) At industrial level, the strain must be easy to handle and should produce high density biomass<sup>51, 52</sup>.

### 2.3.3 Composition of culture medium and microalgal biomass

The culture media for growing microalgae have been developed using the stoichiometric composition of the microbial biomass grown under regular physiological conditions<sup>53</sup>. The information about the composition of microbial biomass in relation to the formation of a product depend on the microalgal specie and culture conditions.

A suggested molar ratio of C<sub>106</sub>N<sub>16</sub>P<sub>1</sub> was described by Falkowski<sup>109</sup>. Recently has been extended to include other important elements such as K<sup>+</sup>, Mg<sup>2+</sup>, Na<sup>+</sup>, Ca<sup>2+</sup>, SO<sub>4</sub><sup>-</sup> and Cl<sup>-</sup><sup>46, 54</sup>. For a recipe to algal cultivation it is also important to consider the total salt content, which is determined by the habitat from where the algae originates, nitrogen sources, like nitrate, ammonia and urea, carbon source either CO<sub>2</sub> or HCO<sub>3</sub><sup>-</sup>, pH, vitamins and chelating agents (EDTA). It's important to realize the purpose for which the algae will be cultivated, this means, they can be kept in culture collection, can be grown for optimal biomass yields or can be grown in stress conditions for the biosynthesis of value biocompounds<sup>46</sup>.

In medium for heterotrophic cultures that support optimal growth, all of the constituents are supplied in stoichiometric excess to the organic carbon source<sup>54</sup>.

In Fig.7 it is shown the biomass composition and macro and micro-elements of one of the most known species capable of heterotrophic growth, *Chlorella* sp.

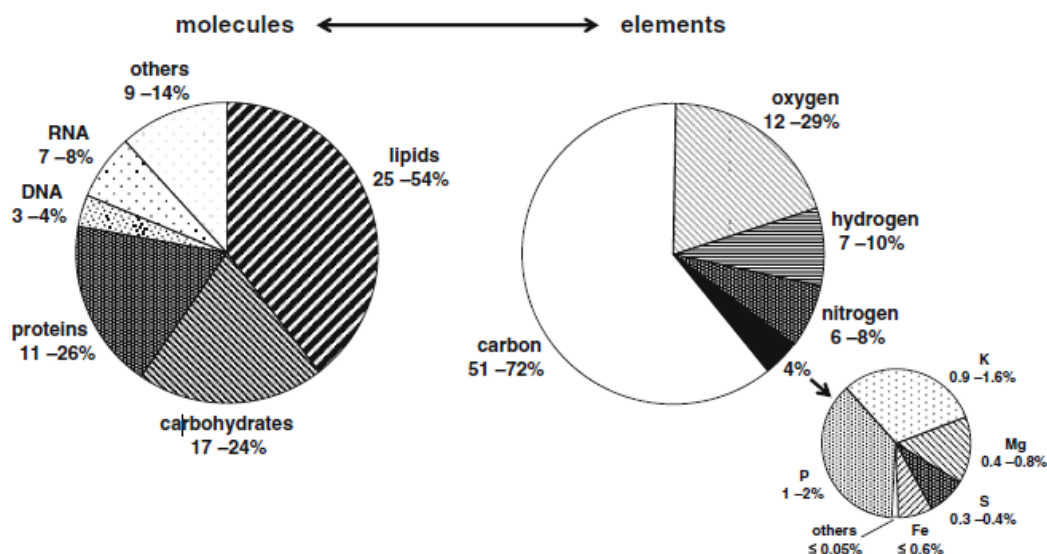


Figure 5 – Molecular composition of *Chlorella* sp. biomass on the left and macro and micro elements on the right. The lower and upper limits were determined at low and high nitrogen availability (Adapted from Oh-Hama and Miyachi 1988)

### 2.3.4 Enhancing biomass and product formation

The development of appropriate strategies for enhancing biomass or product formation is exploring the flexibility of biomass composition within its upper and lower limits as defined by different culture conditions or with the supply of chemical elements in the culture medium.

The objective for any microalgal process it is to achieve the highest product concentration in the shortest possible time. Maintaining the growth at the maximum specific growth rate ( $\mu$ , in  $\text{h}^{-1}$ ) does not often correlate with the highest attainable rate of specific product formation ( $q_p$ , in  $\text{g g}^{-1} \text{h}^{-1}$ ). When product formation is not correlated with maximal growth, i.e. more cells doesn't mean more product, it is advantageous to have control over the specific growth rate and this can usually be achieved in fed-batch cultivation. The culture can be increasing the biomass production at the expense of a decrease in product formation. In fed-batch it is possible to control the substrate concentration, with this particularity we can control the specific growth rate. Different species showed to have different product rates. For example there are species where we can achieve higher productivities with higher substrate concentration and lower productivities with lower substrate concentrations.

Fed-batch cultivation it is believed to be the most effective technique to achieve the highest product concentration in the shortest time controlling the addition rate of the organic carbon and energy source and where osmotic or toxic effects due to high substrate concentrations can be avoided <sup>54</sup>.

Pulsed and continuous addition of organic carbon source is a frequently applied strategy in fed-batch cultivation. Pulsed fed-batch strategies are appropriate to species where growth is inhibited by very high substrate concentrations but where residual substrate concentrations can be tolerated. Combined production strategies can be applied to systems where rapidly built biomass is essential during the first process, prior to a subsequent differently controlled phase that promotes product formation <sup>55</sup>.

### **2.3.5 Carbon and Nitrogen sources**

All organisms, including microalgae, use the same metabolic pathways for respiration. However, it is hard to predict which specific substrates can be used or preferred by any given microalgae <sup>56</sup>.

Glucose is the most commonly used carbon source for heterotrophic cultures. Glucose promote physiological changes in certain species of microalgae, which strongly affects the metabolic pathways of carbon assimilation, size of the cells, volume densities of storage materials, such as starch, lipids, protein, chlorophyll and vitamin contents <sup>59</sup>. However, pure glucose would be very expensive at commercial production scale.

Glycerol is another promising carbon source for heterotrophic cultivation, it's an economical carbon source for energy supply and carbon requirements and it's a very compatible solute for enzymes and membranes, with almost no toxic effects even at high concentrations <sup>57</sup>. Several species can assimilate glycerol from the medium increasing their growth rate and inducing specific biochemical and structural changes in their photosynthetic systems, such as, reduction of cell phycoerythrin content, degree of thylakoid, packing, number of thylakoids per cell, and the size of Photo System II particles, suggesting that in glycerol-growth there's an enhancement of heterotrophic potential <sup>60</sup>. The conversion threshold of glycerol, ( $0.10 \pm 0.02$  g/g), into single cell oil is generally lower than that of

glucose, presumably due to poor regulation of the enzymes involved in the primary metabolic steps of glycerol assimilation, such as glycerol kinase and 3-p-glycerol dehydrogenase<sup>67, 102</sup>.

Nowadays one of the most promising alternative organic carbon substrates is wastewater derived from industrial sources due to the dual advantage of pre-treating an existing waste stream while providing free organic carbon source for microalgae growth. For example, acetate can be used as a glucose alternative. Acetate can be metabolized by microalgae cells via acetyl-coA in the glyoxylate cycle and converted to ATP in the Krebs cycle<sup>110</sup>. Perez *et al.*<sup>57</sup> investigate nutrient removal from sterilized municipal wastewater by *Chlorella vulgaris* and it was found that sodium acetate resulted in the greatest cell growth as well as ammonia removal<sup>57</sup>. Acid acetic has also been investigated as a carbon source and it showed to have positive results. De Swaaf *et al.*<sup>111</sup> obtained biomass yields of 109 g L<sup>-1</sup> with a lipid content of 55 % in the heterotrophic cultivation of *Cryptocodinium cohnii* using acid acetic.

Monosaccharides like fructose, galactose, mannose, xylose and even some disaccharides (sucrose, lactose) have been investigated as organic carbon sources for the cultivation of microorganisms. There are several studies regarding to these carbon sources. Sharma *et al.*<sup>112</sup> studied the effect of sucrose over glucose and glycerol on *Chlorella* sp. growth and lipid production. Sucrose showed to have a biomass productivity of 69.38±9.2 mg L<sup>-1</sup> day<sup>-1</sup> and lipid yield of 109.04±10.8 mg.L<sup>-1</sup> however the values obtained for glucose and glycerol were slightly higher. Vidotti *et al.*<sup>113</sup> used low cost carbon sources for the cultivation of two different strains of microalgae, *Chlorella vulgaris* and *Scenedesmus bijugus*. The carbon sources used were cassava wastewater, sugarcane molasses, glycerol, sucrose, fructose, glucose and sodium acetate. Glucose produced the higher biomass growth rates in both *Chlorella vulgaris* and *Scenedesmus bijugus* cultivation. The lowest growth rates were associated when fructose was used as carbon source. However, the cultivation of *Chlorella vulgaris* with fructose resulted in cell death, indicating that this strain was unable to consume fructose. Velu *et al.*<sup>114</sup> studied the effect glucose, fructose, sucrose, lactose and galactose in three different species of marine microalgae, *Nannochloropsis salina*, *Dunaliella tertiolecta* and *Tetraselmis suecica*. Among the carbon sources tested these species showed a higher growth and higher lipid production in the order of glucose, sucrose, fructose,

galactose and lactose treated culture. It should be noted that the carbon sources behave differently depending on the type of microorganism utilized due to the enzymatic complexes.

Following carbon, nitrogen is the most important nutrient contributing to the growth of biomass. The nitrogen content of biomass can range from 1% to 10% and may vary between different groups and species. Carbon and nitrogen metabolism are linked in microalgae because they share the carbon supplied directly from respiration of fixed CO<sub>2</sub> (autotrophic growth) or assimilated organic carbon (heterotrophic growth) and the energy generated in the TCA cycle and in the mitochondrial electron transport chain <sup>46, 57</sup>.

A variety of organic N compounds like ammonia (NH<sub>4</sub><sup>+</sup>), nitrate (NO<sub>3</sub><sup>-</sup>), urea, yeast extract, peptone and amino acids are utilized by algae <sup>46, 51, 55</sup>. Ammonia is often the preferred N-source for algae since it requires less energy for its uptake, what makes the more energetically efficient source <sup>46, 61, 62</sup>.

Typical responses to nitrogen limitation is discoloration of the cells (decrease in chlorophylls and increase in the carotenoids) and accumulation of organic carbon compounds such as polysaccharides and lipids. The metabolic pathways involved in carbon assimilation for glucose and glycerol, and in nitrogen assimilation for ammonium are annexed depicted.

Growth rates are enhanced by higher levels of aeration what makes oxygen a key factor in heterotrophic cultivation <sup>57</sup>.

For instance, Wu *et al.*<sup>58</sup> made a study where the limitation of oxygen in a culture may reduce the specific growth rate of *Chlorella sp.* and consequently lower the productivity of biomass at high cell density.

The requirement of oxygen depends on the culture mode, i.e., in photoautotrophic conditions in which CO<sub>2</sub> is used as carbon source, oxygen is toxic for the cells and should be removed out of the culture system. While in heterotrophic conditions, oxygen is crucial for cell growth. Oxygen is also important to the PUFAs formation in microalgae because the enzymes involved in desaturation and elongation of PUFAs are oxygen dependent.

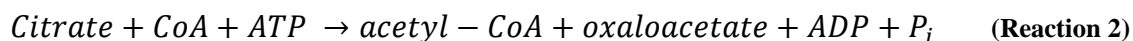


## 2.4 Lipid Accumulation

Several species of microalgae can be induced to overproduce particular fatty acids through simple manipulations of the physical and chemical properties of the culture medium. This makes microalgae a valuable source of lipids and fatty acids <sup>63</sup>.

The accumulation mechanism of lipids in the microalgae and oleaginous microorganisms depends on diverse factors like growth temperature, pH, nutrient intake (carbon, nitrogen and other components), nutritional mode (autotrophic, heterotrophic or mixotrophic), the age of the culture and the microorganism strain. However, it is also important to realize that not every parameter will affect fatty acid content in every algal species. The extent to which fatty acid content responds to a particular parameter can be known only through practical experimentation. However, the most popular strategy for lipid accumulation is nitrogen limitation <sup>4, 63, 64, 65</sup>.

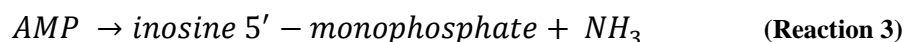
Under the same conditions (nitrogen-limiting) non-oleaginous microorganisms tend to cease cell proliferation or, if they continue to assimilate the available substrate, it will lead to the formation of polysaccharides. In view of the foregoing it can be concluded that the ability of an organism to accumulate oil does not depend of the lipid biosynthesis mechanism as this biosynthetic machinery is common to all microorganisms. So, the reasons for oleaginicinity could come from two pathways (a) supply of acetyl-CoA in the cytosol of the cell that works as precursor for fatty acid synthetase (FAS) and, (b) large supply of NADPH as reductant used in fatty acid biosynthesis. Formation of acetyl-CoA comes from the action of ATP-citrate lyase (ACL) (Reaction 2).



Citric acid in cytosol is important for efficient synthesis of fatty acids. This component is synthesized in tricarboxylic acid cycle (TCA) <sup>66</sup>.

Oleaginous microorganisms accumulate citric acid, when the intra-mitochondrial citric acid concentration reaches a critical value and citrate enters the cytoplasm <sup>67</sup>. Citric acid accumulation comes from the activity of isocitrate dehydrogenase that is part of the TCA

cycle and is dependent on the presence of adenosine monophosphate (AMP). AMP concentration is regulated by the activity of AMP deaminase (reaction 3).



Nitrogen exhaustion induces a reaction chain leading to the formation of acetyl-CoA. At the beginning of nitrogen privation occurs an increase of AMP deaminase activity about five times higher than in cells before nitrogen privation. The increased activity of AMP diaminase will contribute to a fall in the cellular content of AMP. Lower content of AMP stops isocitrate dehydrogenase activity. Once isocitrate cannot be metabolized, it accumulates and is then equilibrated with citric acid by enzyme aconitase. Subsequently citrate will be transferred to cytosol. In cytosol citrate is cleaved by ACL and form acetyl-CoA and oxaloacetate (Reaction 2). Acetyl-CoA is used for fatty acid biosynthesis and oxaloacetate is integrated in the citrate/malate cycle. This cycle consists in recycling the oxaloacetate that is converted via malate dehydrogenase to malate and then malate via citrate synthase is converted to citrate<sup>66,67</sup>. A summary and a scheme of lipid biosynthesis is shown in Fig. 6 and Fig. 7 respectively.

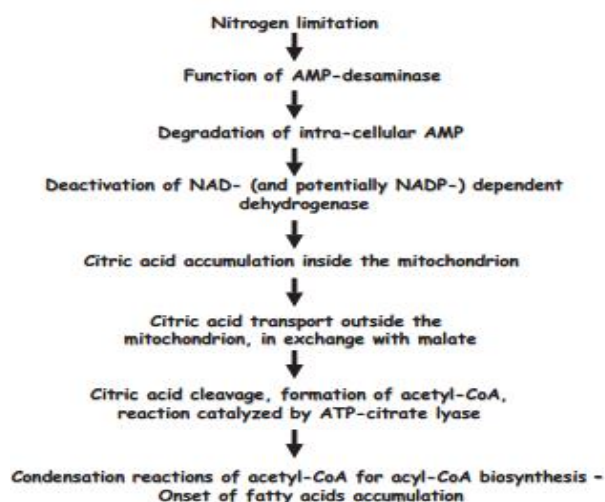


Figure 6 – Summary steps of lipid biosynthesis in oleaginous microorganisms under nitrogen exhaustion (Adapted from Papanikolaou *et al.*<sup>67</sup>)

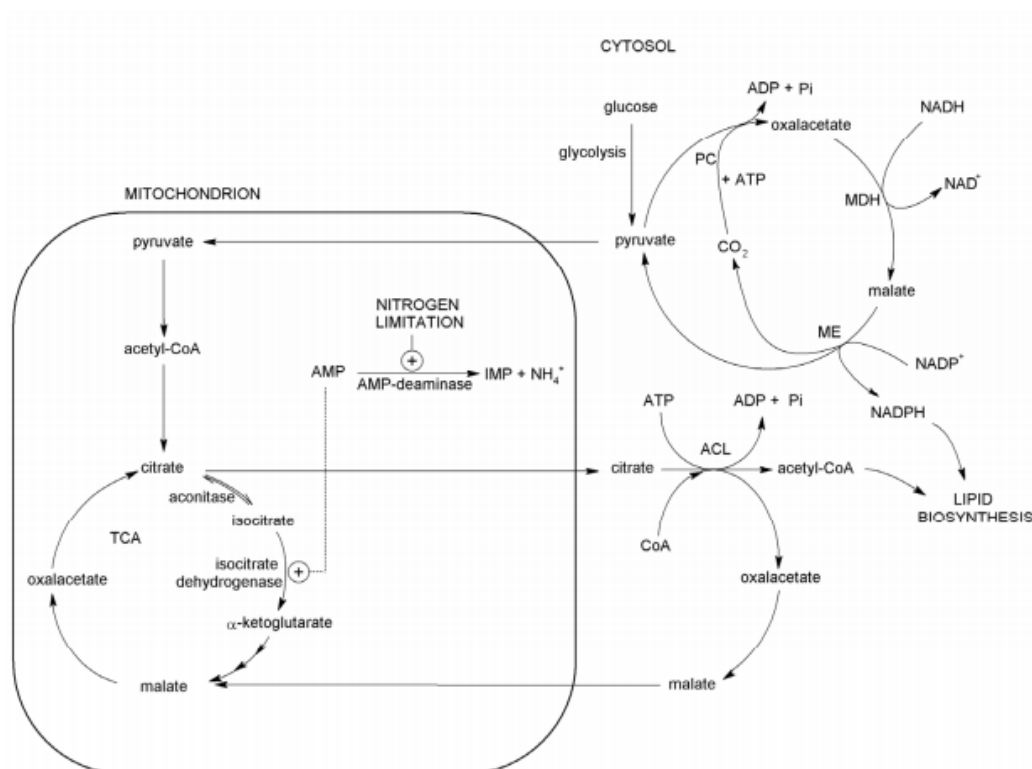
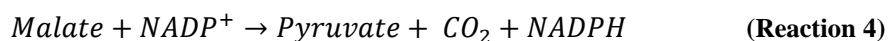


Figure 7 - Scheme to show how the citrate/malate cycle and the cytosolic transhydrogenase cycle could provide sufficient precursors of acetyl-CoA and NADPH for lipogenesis in oleaginous microorganisms. PC – pyruvate carboxylase, MDH – malate dehydrogenase, ME – malic enzyme  
(Adapted from Ratledge <sup>66</sup>)

Once fatty acids are highly reduced materials, to achieve their synthesis other enzymes can be required to achieve lipid accumulation. Malic enzyme (malate dehydrogenase) catalyze the decarboxylation reaction of malate to pyruvate with the formation of NADPH that is essential in fatty acids synthesis. So the major supplier of NADPH for fatty acid biosynthesis is considered to be malic enzyme (Reaction 4) <sup>64, 66, 67</sup>.



## 2.5 Omega-3 Polyunsaturated fatty acids (PUFA'S)

Omega-3 polyunsaturated fatty acids are fatty acids containing two or more double bonds, with the last double bond located at the 3<sup>rd</sup> carbon atom from the methyl end of the fatty acid chain. PUFAs are the important components of all cell membranes and precursors of eicosanoids that are essential bioregulators of many cellular processes <sup>68</sup>.  $\alpha$ -Linolenic acid

(18:3), eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) are the most common omega-3 fatty acids and the interest in these PUFA's has increased significantly due to their recognition as being beneficial for human health<sup>68, 69</sup>.

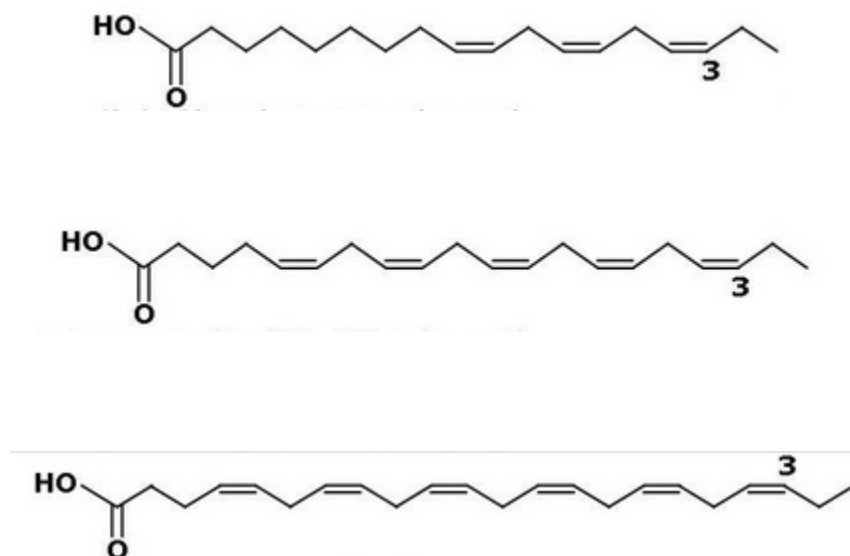


Figure 8 – Chemical structure of  $\alpha$ -linolenic acid (18:3), EPA (20:5) and DHA (22:6), respectively

Omega-3 fatty acids have proven beneficial effects in prevention of cancers, Alzheimer, cardiovascular diseases, infant brain and vision development<sup>68, 70, 71</sup>. Once human body cannot synthesize PUFAs, they must be obtained from the diet to sustain homeostasis<sup>68, 70</sup>.

Omega-3 fatty acids have beneficial effects on cardiovascular system. Simopoulos<sup>72</sup> reported that increasing the consumption of omega-3 could decrease the risk of suffering from diseases such as diabetes, obesity and asthma. Mozaffarian *et al.*<sup>71</sup> and Saravanan *et al.*<sup>73</sup> stated that omega-3 PUFAs consumption lower the resting heart rate, blood pressure, lower inflammation, improve vascular function and experimental studies demonstrate direct anti-arrhythmic effects. Regulatory entities assume that the recommendations for general population consumption to maintain a healthy heart should be at least 250mg/day of omega-3 PUFAs<sup>71, 73</sup>.

Omega-3 fatty acids have been shown to possess anti-carcinogenic properties. The number of studies from the use of omega-3 fatty acids in cancer has increased. The

mechanisms are not fully understood, but great efforts have been undertaken to further explore the beneficial effects on the treatment of various cancers<sup>74, 75</sup>.

Sharma *et al.*<sup>76</sup> showed that Omega-3 fatty acids had anti-proliferative effect on epithelial ovarian cancer cells. This effects can be associated to p53 gene<sup>76</sup>.

Lou Y-R *et al.*<sup>77</sup> studied the effects of high-fat diets rich in either omega-3 on UVB-induced skin carcinogenesis in SKH-1 mice. Their results showed that omega-3 fatty acids in diet of the mice increased latency for the development of UVB-induced skin tumors, decreased the formation of papilloma, keratoacanthoma and carcinoma by 64, 52 and 46% respectively and decrease the size of papilloma, keratoacanthoma and carcinoma by 98, 80 and 83%, respectively. Summarizing, the results indicate that omega-3 fatty acid diet have beneficial effects against UVB-skin carcinogenesis and these effects may be associated with an inhibition on UVB-induced inflammatory response.

Hardman W.<sup>78</sup> has shown that mice implanted with human breast tumor consumed canola oil (high content in  $\alpha$ -linolenic acid), resulting in slower growth of cancer cells.

Besides having positive effects on cardiovascular diseases and cancer, studies have also shown beneficial effects on the treatment of schizophrenia<sup>79-82</sup> and Alzheimer<sup>84, 85</sup>. DHA is a major structural component of neuronal membranes, and changing the fatty acid composition of neuronal membranes leads to functional changes in the activity of receptors and other proteins embedded in the membrane phospholipid<sup>81, 85</sup>. It has also be shown that schizophrenia patients have reduced cell membranes levels of PUFA's particularly DHA<sup>80, 82, 83</sup>. This leads the researchers to believe that the mechanism behind these beneficial effects can be due to changes in brain membrane fluidity, more exactly due to alteration in the fatty acid composition of brain membranes.

### 2.5.1 Biosynthesis of Omega-3 Fatty Acids

The biosynthetic pathway of omega-3 Fatty Acids is divided in two stages. The first stage is the *de novo* synthesis of oleic acid from acetate. The second stage comprehend a series of desaturation and elongation reactions from oleic acid to form a family of longer chain polyunsaturated fatty acids ( $\omega$ -9,  $\omega$ -6,  $\omega$ -3 PUFAs)<sup>86</sup>. In Fig. 9 are shown the two stages of PUFAs synthesis.

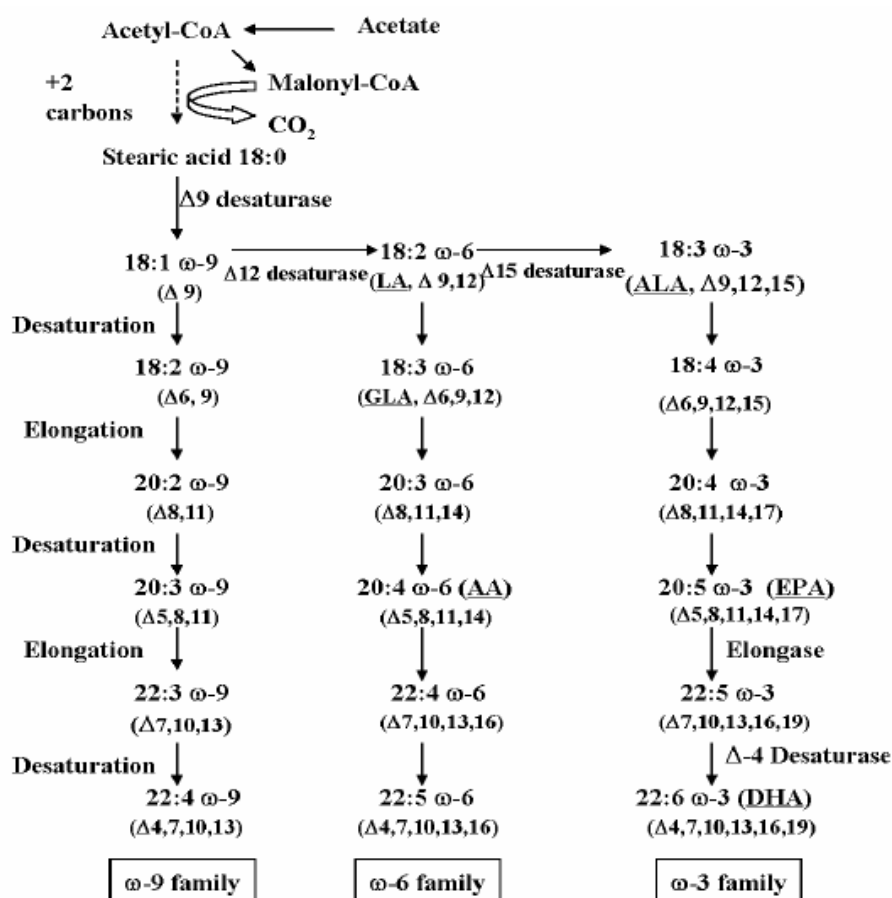


Figure 9 – Biosynthetic pathway for formation of PUFAs. Enzymes Δ9 Δ12 Δ15 are fatty acid desaturases. (Adapted from Wen<sup>86</sup>)

Once oleic acid (C 18:1 ω-9) was synthesized it was further desaturated by Δ12 and Δ15 fatty acid desaturases to form linoleic acid (LA C18:2 ω-6) and α-linolenic acid (ALA C18:3 ω-3), respectively. After the three fatty acids are formed (oleic acid, LA, ALA), they will compete for Δ6 desaturase, for which generally ALA has the highest affinity<sup>86</sup>. Several microorganisms like algae, fungi, bacteria and insects possess these enzymes required to the synthesis of PUFAs. However, humans do have the ability to produce EPA and DHA, but the conversion rates are very low<sup>87</sup>. Thus it is of extremely importance to include in human diet Omega-3 through the dietary sources.

## **2.5.2 Sources of Omega-3 Fatty Acids**

It is clearly understood that omega-3 fatty acids are of extreme importance to human health, so it necessary to elucidate and develop new strategies and economical sources to obtain omega-3 fatty acids.

### **2.5.2.1 Traditional Sources**

The main source of omega-3 fatty acids is mostly from marine fish oil. Blue fish, white fish, cephalopods and crustaceans are a natural source of EPA and DHA and can provide 100% of the daily requirements of Omega-3 to humans. It is believed that fish do not synthesize this fatty acids themselves, they acquire them from the phytoplankton that they eat <sup>88</sup>.

The annual global production of fish oil is around one million and seventy thousand tonnes and it is expected to reach around two million tonnes in 2022 <sup>89</sup>. Significant problems of using this traditional sources may include variation of fish oil due to different species, climatic and seasonal change, which can affect the quality of the fish oil, complex and expensive purification methods for raw fish oil, peculiar taste and odor and another concern is the sustainability of this natural source, since the global fish oil demand continues to grow year after year <sup>90</sup>.

Hereupon it's clearly that PUFA production from traditional sources it can become a problem in the future. So to supply the growing PUFA market it is necessary to seek for alternative sources to compete in cost with current sources.

### **2.5.2.2 Alternative Sources**

The problems stated above lead to an extensive research of potential alternative sources of PUFAs such as microalgae, fungi, bacteria and mosses (Table 6).

Fish do not have the *de novo* mechanism to produce omega-3. Microorganisms such as algae and fungi have the necessary biosynthetic pathway to produce PUFAs. This microorganisms have been extensively studied due to this particularity what makes them potential sources of PUFAs.

Table 6 - Current and alternatives sources of PUFA (Adapted from Alonso and Maroto <sup>90</sup>)

PUFA	Current sources	Alternative sources
AA	Animal viscera (brain)	Fungi: <i>Mortierella alpina</i> Microalgae: <i>Porphyridium cruentum</i>
EPA	Oil fish: <i>Sardina</i> , <i>Engraulis</i> , etc.	Fungi: <i>M. alpina</i> , <i>Saprolegnia</i> sp. Microalgae: <i>Phaeodactylum tricornutum</i> , <i>Monodus subterraneus</i> Mosses: <i>Phytium irregulare</i> Bacteria: <i>Shewanella</i> sp.
DHA	Oil fish: <i>Sardina</i> , <i>Engraulis</i> , etc.	Fungi: Traustochitrids Microalgae: Dynophyceae

In a range of microorganisms producers of omega-3 fatty acids, microalgae, fungi and bacteria stand-out.

Certain species of microalgae are capable of producing EPA and DHA. Tan *et al.* <sup>91</sup> reported *Nitzschia laevis* as an EPA producer under heterotrophic conditions <sup>86,91,92</sup>. Among DHA production, thraustochytrids as *Schizochytrium* sp. and *Ulkenia* sp. are capable of producing high levels of DHA under heterotrophic conditions <sup>2,4,55,93</sup>. The Swiss company Lonza produces and commercialize microencapsulated DHA-rich oil from the microalgae *Ulkenia* sp. and from *Schizochytrium* sp. <sup>94</sup>. *Cryptocodinium cohnii* is another efficient DHA producer and is the microorganism used by DSM company to produce DHA <sup>69</sup>.

Fungi are another group capable of producing high levels of omega-3 fatty acids. *Mortierella alpina* has been studied as a producer of AA under normal growth conditions and DHA and EPA producer under stress conditions <sup>95</sup>. Other fungal species like *Trichoderma* sp. and *Aspergillus niger* has been identified as omega-3 producers where *Trichoderma* sp. is preferred since it produced considerable amounts of DHA ( $\approx 7,47$  mg/g) in comparison with *Aspergillus niger* ( $\approx 0,136$  mg/g) <sup>96</sup>.

Over the time it was thought that PUFAs were absent in bacterial membranes because several well studied species like *Escherichia coli* was found to have no PUFAs. Later studies suggest that several bacterial species were able to produce PUFAs. For example, Yazawa <sup>97</sup> isolated new species of marine bacterium close to *Shewanella putrefaciens* that was able to produce EPA <sup>97</sup>. Despite several studies made with bacteria, the production yields were found to be low and the growth condition are very sensitive considering bacteria an inefficient way for commercial production.



## 2.6 *Schizochytrium limacinum*

*Thraustochytrids* like *Schizochytrium limacinum* sp. are heterotrophic marine protists that inhabit in tropical areas, which are often referred to as microalga-like organisms. They are associated with mangrove swamps, seawater and seaweeds<sup>98</sup>. These microorganisms show a high content of DHA, making it a promising specie for this purpose<sup>4, 55, 93, 98</sup>.

This microorganism was first isolated from sea water from a mangrove area of the Yap Islands of Micronesia in 1994. *Schizochytrium limacinum* differs from other *Schizochytrium* spp. in its limaciform amoeboid cells, the size of zoospores and assimilation of carbon<sup>98, 99</sup>.

### 2.6.1 Culture conditions

Once *Schizochytrium limacinum* is considered a prolific producer of DHA, this led the researchers to study and develop culture conditions in order to optimize the production yields of DHA. The usual parameters like carbon source, nitrogen source, temperature and salinity have been investigated<sup>4, 100</sup>.

In heterotrophic cultures, glucose has conventionally been used as carbon source<sup>101</sup>. However, several alternative carbon and nitrogen sources have been tested for *S. limacinum* growth in order to decrease the production costs. Yokochi *et al.*<sup>99</sup> found that glucose, glycerol and fructose supported the best cell growth and DHA yield over sucrose, linseed oil, maltose and lactose. The most viable nitrogen sources, corn steep liquor and yeast extract were the ones that led to the highest DHA production. Ammonium acetate was also tested as inorganic nitrogen source and led to positive results, namely, increased cell growth with the increase of ammonium acetate amount and the DHA content was not affected by this increase.

In terms of temperature and culture salinity, the optimum temperature for grow *S. limacinum* range between 20°C and 30°C and the maximum dry weight achieved ranged between 50% and 200% of seawater<sup>4</sup>.

The cost of turning sugar into oil is never going to be economically feasible, as the cost of sugar is never less than a quarter of the price of plant oils<sup>5</sup>. In order to reduce the production cost of algal DHA the cheap carbon sources as alternatives should be investigated.

Glycerol is the major byproduct of the biodiesel industry and since there's a glut in the market, using the glycerol as carbon source for *Schizochytrium limacinum* cultivation to obtain high valuable compounds attracted the attention of researchers.

## 2.7 Types of Fermentation processes

### 2.7.1 Submerged cultivation

Submerged cultivation are the most common fermentation processes since they provide a controlled environment for the efficient production of high-quality products and to achieve optimum yields and productivity<sup>101</sup>. The most common “modus operandi” of bioreactors are batch, fed-batch or continuous mode depending on the type of microorganism that will be utilized and the final product that is desired to obtain. In the following sections the different types of submerged cultivation will be briefly discussed.

### 2.7.2 Batch cultivation

Batch culture is represented by a closed system which the medium, nutrients and inoculum are added to the bioreactor at the beginning of cultivation, always under aseptic condition. The volume of the culture in the bioreactor is theoretically constant during cultivation (there will be some deviations in culture volume due to feed of acid/base solutions to keep the pH, by sampling or introducing air/gas in the culture)<sup>101</sup>.

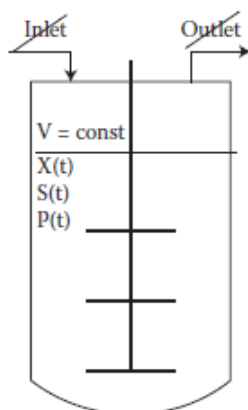


Figure 10 – Schematic representation of a batch cultivation  
(Adapted from Paulová *et al.*<sup>101</sup>)

At the beginning of batch cultivation the inoculum is added into the bioreactor that is already filled with sterilized medium with the required nutrients. After the inoculation, the

cell culture follow the typical growth curve described by Monod kinetics. Divided into 4 phases, the *lag phase* occurs immediately after inoculation and is a period of adaptation of cells to a new environment. Depending on the composition of nutrients, new enzymes are synthesized, the synthesis of some other enzymes is repressed, and the internal machinery of cells is adapted to the new environmental conditions. The length of the lag phase is influenced mainly by the concentration of cells in the inoculum and their physiological state, the composition of the inoculation and cultivation medium (source of carbon and energy, pH and temperature), the size of the inoculum, ( $\approx 5\%$  to  $10\%$  by volume), and the age of the inoculum. To minimize the lag phase, cells should be adapted to the growth medium and conditions before inoculation. The *exponential phase* (logarithmic growth), the cells have adjusted to their new environment. This phase is characterized by rapid cell proliferation (biomass concentration is an exponential function of the time), constant specific growth rate, which is equal to the maximum specific growth rate of the culture under conditions of absence of growth limitation (growth rate is not limited because all nutrients are present in excess, while also not attaining growth-inhibiting concentrations), fast consumption of the carbon and energy source, and a high rate of primary metabolite production. The depletion of nutrients by the end of the exponential phase (in case of aerobically grown cultures are signaled by a rapid increase in dissolved oxygen concentration) causes a progressive reduction in the specific growth rate and a transition to the *stationary phase*, characterized by the stagnation of growth (growth rate = 0) or when the growth rate is equal to the death rate. Even though the net growth is zero during the stationary phase, cells are still metabolically active and produce secondary metabolites. Primary metabolites are growth-related products and secondary metabolites are non growth-related (e.g., antibiotics, hormones). During the course of the stationary phase, one or more of the following phenomena may take place: a) total cell mass concentration may stay constant, but the number of viable cells may decrease; b) cell lysis may occur and viable cell mass may drop. For the last, the death phase follows the stationary phase. Some cell death may start during the stationary phase, so it become difficult to distinguish between this two phases. This phase occurs because of either nutrient depletion or toxic product accumulation<sup>102</sup>.

Most of the industrial reactors are operated in batch mode due to the relative simplicity of this process. The steps in batch operation are: medium formulation, filling the

bioreactor, sterilization in place (SIP), inoculation, cultivation, product harvesting and bioreactor cleaning in place (CIP). While using batch bioreactor it is important to minimize all nonproductive steps (all listed above except cultivation), achieve a high rate of product synthesis, optimize productivity, and maximize the yield of the end product. An extension of exponential growth is advantageous for the efficient production of biomass or primary metabolites, whereas in case of secondary metabolite production, the exponential phase is shortened (by the limitation of one nutrient, usually nitrogen) and the stationary phase is prolonged to achieve the maximum yield of the product <sup>101</sup>.

### 2.7.3 Fed-batch cultivation

Fed-batch mode is represented as a semi-continuous system in which one or more nutrients are aseptically added to the bioreactor while the product is retained inside, in this case, the volume of the culture in the bioreactor will increase with time.

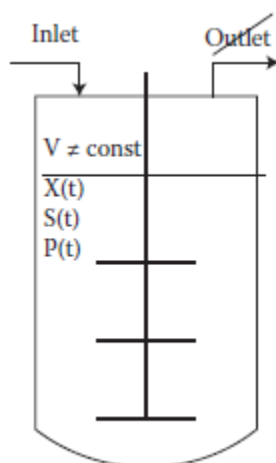


Figure 11 – Schematic representation of fed-batch cultivation  
(Adapted from Paulová *et al.* <sup>101</sup>)

The advantages of fed-batch over the batch cultures are the following: the possibility to prolong product synthesis, the ability to achieve higher cell densities and increase the amount of product, the capacity to enhance yield or productivity by controlled sequential addition of nutrients.

Fed-batch is used in processes where substrate or catabolic repression is expected; where a high cell density is required, where a high production rate should be achieved, for the least where a high viscosity of culture is expected. The common feeding strategies to

enhance the culture performance are the following: discontinuous feeding, achieved by regular or irregular pulses of substrates and regular continuous feeding of nutrients designed based on online measured variables (pH, dissolved oxygen, CO<sub>2</sub> and biomass concentration)<sup>101</sup>.

#### 2.7.4 Continuous cultivation

Continuous culture is represented as system in which nutrients are aseptically and continuously added to the bioreactor, and the culture broth is removed at the same time. This means that microbial growth takes place under steady-state conditions hence the volume of the culture is constant due to a constant feed-in and feed-out rate<sup>103</sup>.

Continuous culture can be used as synonym for a chemostat, represented by a constant specific growth rate of cells, which is equal to the dilution rate and is controlled by the availability of the limiting nutrient<sup>101</sup>.

Factors as pH value, concentrations of nutrients, metabolic products and oxygen, which inevitably change during the growth cycle of a batch culture, are all maintained constant in a continuous culture.

It is an ideal way to study the basic physiological behaviors of algal cells because the kinetic parameters such as specific growth rate, cell density and productivity can be kept constant under condition of steady state.

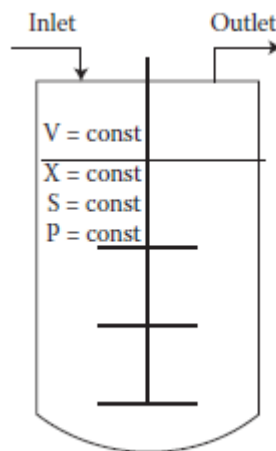


Figure 12 – Schematic representation of fed-batch cultivation  
(Adapted from Paulová *et al.*<sup>101</sup>)

## 2.8 Objective

This work was conducted during my stay at the Institute of Chemical Technology at Bioengineering Department. The main objective of this project was the cultivation of algae-like microorganisms, in particular, microorganisms of the genus *Thraustochytriales*, and use of waste brine solution from a dairy effluent as a component of the cultivation medium and glycerol as carbon source for the purpose of biomass production. The produced biomass was analyzed for its PUFA content. Two specific objectives were made during this work:

- Achieve the DHA content on the biomass, produced by *Schizochytrium limacinum*, using saline waste medium and glycerol as raw materials.
- Determine the best temperature to achieve the highest growth of *Japanochytrium*, using saline waste medium and glycerol as raw materials.

## **Chapter 3: DHA production by *Schizochytrium limacinum* in dairy saline waste medium using glycerol as carbon source**

### **3.1 Materials and methods**

#### **3.1.1 Microalgae strains and medium pre-treatment**

The microalgae *Schizochytrium limacinum* used in this work was provided by EcoFuel Labs., a Czech biotechnology company.

The cells were maintained in culture plates with GPY, (Glucose Peptone Yeast Extract), medium containing 20 g/L of glucose, 10 g/L of peptone, 5 g/L of yeast extract, 20 g/L of sodium chloride and 20 g/L of agar<sup>99</sup>.

The dairy saline wastewater (SWW) (Table 7) used as fermentation medium and inocula was pre-treated by adding NaOH till a pH of 7 (EUTECH instruments pH 510), heated ( $\approx 100^{\circ}\text{C}$ ) for precipitation of undesirable proteins and then centrifuged (Sorvall Evolution RC, SLC – 6000) at 6500 rpm during 7 minutes.

Erlenmeyer flasks used for inoculation of the cells were sterilized by autoclaving at  $110^{\circ}\text{C}$  for 30 minutes.

#### **3.1.2 Preparation of inoculum**

Under aseptic conditions the cells of *Schizochytrium limacinum* were inoculated in 500 mL Erlenmeyer flasks containing 200 mL of saline waste medium (SWM) consisting of pre-treated SWW (Table 7), 30 g/L of glycerol, 10 g/L of yeast extract, and then, the Erlenmeyer flasks were placed on a rotary shaker at room temperature for 7 days. This procedure was made in triplicate.

#### **3.1.3 Cultivation in Stirred Tank Bioreactor (STR)**

Fed-batch fermentations were made in a 2.7 L stirred tank bioreactor (Infor HT Labfor 4, Switzerland) with a working volume of 2 L. These bioreactors are equipped with a panel with rotary navigation knob for single or multi bioreactors systems, with four peristaltic

pumps, a gas control system that can be configured for a wide range of options e.g. gas mix, mass flow and online measurement of antifoam level, pH and temperature. The vessel was equipped with two Rushton impellers containing six blades each, probes for antifoam levels, pH and temperature.

The inoculum volume was 10% v/v of the bioreactor volume. The startup saline waste medium (SWM) includes 90 g/L of glycerol, 10 g/L of yeast extract and 0.5 % (v/v) ammonia. Pulsed addition of ammonium with a concentration of 7% v/v was added (0.15 L) at 95 and 150 hours of bioreactor operation. (Fig.14)

The culture conditions were kept at a temperature of 23°C, pH was controlled at 7 with H<sub>2</sub>SO<sub>4</sub> (15% v/v) and KOH (20% v/v). Airflow, agitation and k<sub>L</sub>a were set at 0,18 m<sup>3</sup>/h, 100 rpm and 72 h<sup>-1</sup> respectively.

Samples were taken from culture broth twice a day at a defined time during the cultivation process. The samples were subject to monitoring of optical density by spectrophotometry, biomass concentration by gravimetric analysis, residual glycerol concentration by HPLC and the lipid content was measured by gas chromatography. Sterility tests of the collected sample were made through observation under optical microscope (Nikon Eclipse E100) and by inoculation on Petri dishes with salty agar. The biomass was collected before the nitrogen limiting step and after limiting step. The samples were freeze dried (HGTO Power Dry LL3000, Thermo Electron Corporation) and the DHA content was compared.

Table 7 – Composition of dairy saline wastewater used for fermentation (in g.Kg<sup>-1</sup>)

<b>Dry matter</b>	16.80	<b>Sulfate</b>	0.38
<b>Ash</b>	11.30	<b>Calcium</b>	0.79
<b>Nitrogen</b>	0.20	<b>Magnesium</b>	0.15
<b>Lactose</b>	3.00	<b>Potassium</b>	3.32
<b>Chlorine</b>	3.49	<b>Sodium</b>	0.96
<b>Phosphorous</b>	0.64	<b>Nitrate</b>	0.20



## **3.2 Analytical Methods**

The analytical methods were carried out in triplicate and the data given here are the average of the measurements.

### **3.2.1 Optical Density (O.D)**

Optical density was one of the two methods used to follow cellular growth. The OD readings of the samples collected from the bioreactor were analyzed in a spectrophotometer (Spekol 1300 Analytik Jena) at 578nm using distilled water as blank. If necessary, samples were diluted to avoid the linearity loss with Lambert-Beer Law.

### **3.2.2 Gravimetric Analysis**

The growth of biomass was determined by gravimetric analysis. 2 ml of sample were transferred to pre-weighed microtubes and centrifuged (Mikro 120 Hettich Zentrifugen) at 14000 rpm during 5 minutes. The cell pellet was separated from supernatant and the supernatant was transferred to a new microtube and was freeze for later residual glycerol concentration analysis by HPLC. Cell pellet was washed using 2 ml of distilled water to remove the salt. The microtubes were placed again in the centrifuge at the same conditions. The microtubes containing the cell pellet were placed into a dryer at 105 °C during 24 hours. After the 24 hours the microtubes were cooled in a desiccator and weighted with analytical balance (Kern 770).

### **3.2.3 Glycerol Analysis by HPLC**

Glycerol was quantified by High-Performance Liquid Chromatography (HPLC) using the supernatant from gravimetry. The supernatant was filtered using a 0.22 µm syringe filter in order to remove residual cells. A polymer IEX Ca form 8 µm column was used as stationary phase, diluted sulfuric acid (9mM) as mobile phase and a refractive index detector. The quantification conditions were kept at a temperature of 25°C and a flow of 0.5 mL/min.

Glycerol standards with concentrations ranging from 10 to 50 g/L were prepared and analyzed by HPLC. This allowed comparing the peak area of the standards with the samples, using a linear regression curve.

### 3.2.4 Lipid Extraction

Cellular lipid content was determined following a procedure described by Liang *et al.*<sup>104</sup> Briefly, 0.5 g dried cell pellet was transferred to a 7 mL chamber of a bead-beater (Bio-Spec Products, Bartlesville, OK, USA). This chamber was filled with 0.5 mm zirconium beads to approximately 5 mL. Methanol was then added to fill the rest of the chamber. After cells were disrupted by bead-beating for 2 min, the entire content was transferred to a 50 mL glass centrifuge tube. The chamber was washed twice using methanol to collect the algae residue. Chloroform was then added to the tube to make the chloroform/methanol (2:1, v/v). The tube was vortexed for 5 min and was allowed to stand for 24 h. After that, the tube was centrifuged at 4000g for 15 min to remove the zirconium beads and algal solids. The supernatant was collected and the solvent was vaporized using a rotary evaporator. The amount of lipids extracted (g) is equal to the difference between the weight of the flask with lipids and weight of the flask. Lipid percentage (p/p) was achieved with the following equation:

$$\%lipids = \frac{lipid\ mass\ extracted\ (g)}{cell\ mass\ utilized\ (g)} \times 100 \quad \text{(Equation 1)}$$

### 3.2.5 Determination of Total Fatty Acid profiles

The total fatty acid profiles were analyzed by following the procedure described by Liang *et al.*<sup>105</sup>

Lipid samples were subjected to acid-catalyzed transmethylation performed overnight at 50 °C. The resultant FAME were separated using gas chromatography (Hewlett-Packard HP 4890) equipped with a flame ionization detector (FID) fitted with a permanently bonded polyethylene glycol, fused silica capillary column. The injection volume was 1.0 µL, helium was the carrier gas (30 cm/s, 205 °C), and the injector temperature was 250 °C. A

split-less injection technique (100:1) was used, and the temperature program was as follows: 50 °C held for 2 min, increased to 220 °C at 4 °C/min, and held at 220 °C for 15 min. Individual FAME was identified by reference to external standards (Supelco 37 Component FAME Mix, PUFA-1, and PUFA-3; Supelco, Bellefonte, PA, USA).

### 3.3 Growth kinetic

The model employs equations for biomass ( $X$ ), DHA production ( $P$ ) and glycerol ( $S$ ) to describe the fermentation process.

#### 3.3.1 Maximum specific growth rate ( $\mu_{\max}$ )

To calculate the maximum specific growth rate, the following formula was used in the exponential growth phase.

$$\mu_{\max} = \ln \frac{x_1}{x_0} \times \frac{1}{t_1 - t_0} \quad (\text{Equation 2})$$

#### 3.3.2 Doubling Time ( $T_d$ )

The doubling time, expressed in (h), was calculated using the equation 3.

$$T_d = \frac{\ln 2}{\mu} \quad (\text{Equation 3})$$

### 3.4 Yields and Productivities

#### 3.4.1 Biomass yield as a function of substrate

The biomass yield produced as a function of substrate consumed ( $S$ ) corresponds to the slope calculated by linear regression of the values of biomass produced ( $X$ ) in relation to the glycerol consumed.  $Y_{x/s}$  is expressed in g of biomass / g of glycerol.

### **3.4.2 Biomass productivity**

The biomass productivity was calculated by dividing the final concentration of biomass with the fermentation time (216 h).

### **3.4.3 Lipid concentration**

The lipid concentration was achieved multiplying the biomass concentration (g/L) by the lipid content (% DCW, w/w)

### **3.4.4 DHA content and DHA concentration**

DHA concentration was achieved by multiplying the DHA (% of DCW) by the biomass concentration (g/L). The percentage of DHA was directly obtained by the program that performed the lipid quantification.

### **3.4.5 DHA productivity**

The DHA productivity was calculated by dividing the final concentration of DHA with fermentation time (216 h).

### **3.5 Economic Balance**

It was made an economic balance between the standard medium and saline waste medium as well as the quantities of the raw materials used in each work according the data on table 12. Note that the fermentation time course wasn't taken into account. The considered variables were the variables on table 12.

#### **3.5.1 Medium Cost**

The medium cost was calculated multiplying the raw material cost (€/Kg) for the quantity of the raw material used (Kg/L).

#### **3.5.2 Biomass production cost**

The biomass production cost was calculated by dividing the medium cost (€/L) with the biomass concentration (g/L)

#### **3.5.3 DHA production cost**

The DHA production cost was calculated by dividing the medium cost (€/L) with the DHA concentration (g/L)

### 3.6 Results and Discussion

Several studies shows the growth of *Thraustochytrids* as a very attractive alternative for high-value products. However the use of expensive raw materials leaves this processes far behind for industrial production.

In order to work around this problem a robust cultivation process was developed using cheaper and efficient alternative raw materials, such as dairy SWW and glycerol, for the growth and production of DHA by *Thraustochytrids*.

#### 3.6.1 Medium selection and cell growth in fed-batch bioreactor

A first experiment to grow the microalgae *Schizochytrium limacinum* was conducted in 500 mL Erlenmeyer flasks using 30 g/L of glycerol as a carbon source in standard medium (yeast extract, artificial seawater and glycerol)<sup>5</sup> and for comparison also in saline waste medium, that consists of 200 mL of pre-treated SWW, 30 g/L of glycerol, 10 g/L of yeast extract. The Erlenmeyer flasks were placed on rotary shaker at room temperature during 7 days. As shown in Figure 13, the cells grew very well and in a similar way in both culture media. Between 240-350h it was even possible to achieve a higher growth using SWM. Consequently SWM was selected for the subsequent fermentations in bioreactors.

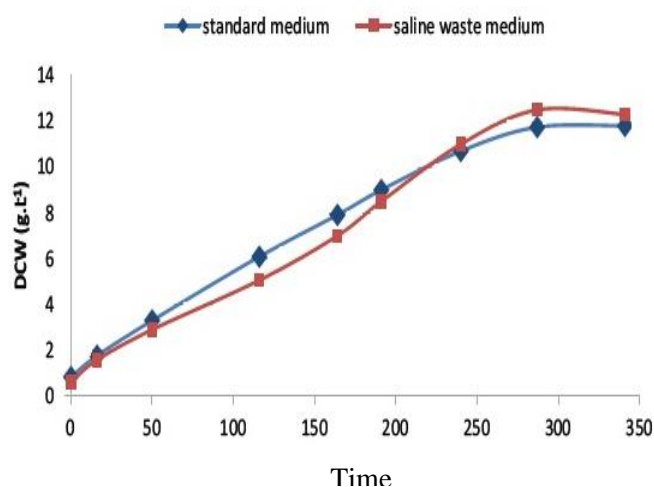


Figure 13 – Comparison of *Schizochytrium limacinum* biomass growth in standard medium and saline waste medium in Erlenmeyer flask

Microalgae are highly nutritional dependent. The microalga *Schizochytrium limacinum* was grown in SWM, glycerol (90 g/L), yeast extract (10 g/L) and ammonia at 23 °C during 216 h. A typical plot of the nutrient consumption and cell growth of *S. limacinum* is given in Fig. 14. The specific growth rate ( $\mu$ ) and maximum cell dry weight (DCW) of *S. limacinum* are summarized in table 8. In this work a doubling time of 15 h was obtained which is in agreement within the range of doubling times (7 - 15h) reported in other studies for *S. limacinum*<sup>54</sup>. The exponential phase took about 100 h, and was always followed by the consumption of the carbon source, glycerol. The interval used to calculate the specific growth rate was between 44 h and 91,5 h. After 216 h of fed-batch fermentation the growth yield coefficient,  $Y_{x/s}$ , based on glycerol was 0.43 g.g<sup>-1</sup>.

*Table 8 – Kinetic parameters of fed-batch growth of the microalgae Schizochytrium limacinum under heterotrophic conditions. The microalga was grown with SWM medium, during 216 h in a 2.7 L STR at 23 °C, 0.18 m<sup>3</sup>/h and 100 rpm.*

Parameters <sup>a</sup>		
DCW	(g.L <sup>-1</sup> )	40.4
$\mu$	(d <sup>-1</sup> )	0.045
$Y_{x/s}$	(g.g <sup>-1</sup> )	0.43

<sup>a</sup> DCW, cell dry weight;  $\mu$ , specific growth rate;  $Y_{x/s}$ , cell yield based on glycerol

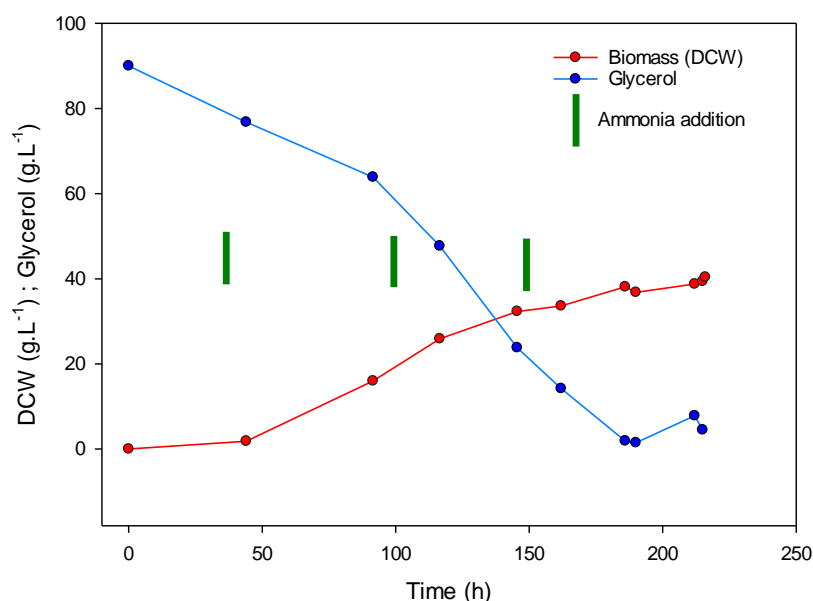


Figure 14 – Time course fermentation profiles of *Schizochytrium limacinum* in dairy saline waste medium. Cell growth (red) and glycerol consumption (blue). The microalga was grown during 216 h in a 2.7 L STR under Fed-batch mode at 23 °C, 0.18 m<sup>3</sup>/h and 100 rpm. The green bars represent pulsed ammonia addition (50 mL each) at different stages of fermentation under the form of ammonium hydroxide.

In fed-batch culture ammonia was used as nitrogen source. As shown in Fig. 14 the green bars represent the addition of ammonia at different times. From the plot it can be seen that those ammonia additions had a positive effect on the biomass growth. This can be explained by the fact that ammonia is the main product of protein metabolism in aquatic organisms and furthermore heterotrophic organisms can absorb ammonia as a precursor for cellular protein synthesis and contribute to biomass growth.

As shown in Fig.14 around 200 h of fermentation it is possible to notice a decline in the growth but suddenly there is an increase in the glycerol, thus a slow but noticeable increase in the growth. A reason that might explain this is the possibility of some residual concentration of glycerol in the bioreactor that led to this behavior.



### 3.6.2 Fatty Acid composition and DHA yields

The fatty acid composition of *S. limacinum* is shown in Table 9. The oil has a simple profile of fatty acids where C16:0 (palmitic acid), C22:5 DPA (docosapentaenoic acid) and C22:6 DHA (docosahexaenoic acid) were the major fatty acids in the cells accounting for about 88% of the total fatty acids. The high content of fatty acids might be due to the fact that more storage lipids were accumulating resulting from carbon abundance.

Table 9 – Fatty acid composition (g.kg<sup>-1</sup>) of *Schizochytrium limacinum*. The microalga was grown during 216 h in a 2.7 L STR under Fed-batch mode at 23 °C, 0.18 m<sup>3</sup>/h and 100 rpm.

<i>Fatty Acids</i>		
<b>Pentadecylic Acid</b>	<b>(C15:0)</b>	6.58
<b>Palmitic Acid</b>	<b>(C16:0)</b>	46.5
<b>Stearic Acid</b>	<b>(C18:0)</b>	2.90
<b>Oleic Acid</b>	<b>(C18:1)</b>	0.62
<b>Linoleic Acid</b>	<b>(C18:2)</b>	-
<b>Docosapentaenoic Acid (DPA)</b>	<b>(C22:5)</b>	25.5
<b>Docosahexaenoic Acid (DHA)</b>	<b>(C22:6)</b>	102
<b>Others</b>		12.4
<b>Total Fatty Acid</b>		<b>196</b>

At the end of the fed-batch fermentation the biomass concentration was 40.4 g.L<sup>-1</sup> which had a content of 9.5 % w/w of DHA and 19.6 % w/w of TFA. The productivity of DHA and the DHA content obtained were 424 mg.L<sup>-1</sup>.d<sup>-1</sup> and 48.5%, respectively (Table 10).

Table 10 – Lipids and DHA productivity by *Schizochytrium limacinum*. The microalga was grown during 216 h in a 2.7 L STR under fed-batch mode at 23 °C, 0.18 m<sup>3</sup>/h and 100 rpm.

<b>TFA <sup>a</sup></b>	<b>(% of DCW, w.w<sup>-1</sup>)</b>	19.6
<b>Lipid Concentration</b>	<b>(g.L<sup>-1</sup>)</b>	7.90
<b>DHA</b>	<b>(% of TFA)</b>	48.5
<b>DHA <sup>b</sup></b>	<b>(% of DCW, w.w<sup>-1</sup>)</b>	9.5
<b>DHA Concentration</b>	<b>(g.L<sup>-1</sup>)</b>	3.80
<b>DHA Productivity</b>	<b>(mg.L<sup>-1</sup>.d<sup>-1</sup>)</b>	424

<sup>a</sup> Percentage of Total fatty acids in biomass, <sup>b</sup> Percentage of DHA in biomass

The results were compared with other studies using *Thraustochytrids* and others microalgae-like marine protist producers of DHA to determine if the microalga *S.limacinum* it is a viable DHA producer under the previously highlighted culture conditions.

Ren *et al.*<sup>107</sup> obtained biomass with a TFA content of 20.1% and 60% of DHA. The TFA content is similar to our experiment, however the DHA content is slightly higher. Ren *et al.*<sup>107</sup> used malic acid as feed in the lipid accumulation phase, reinforcing the NADPH supply and leading to an increase of the DHA content which can explain the differences between both experiments <sup>107</sup>.

Accordingly to Swaaf *et al.*<sup>69</sup> and Jiang *et al.*<sup>106</sup> the heterotrophic cultivation with *Crypthecodinium choynii* on glucose shown a DHA productivity of 422 mg.L<sup>-1</sup>.d<sup>-1</sup> and DHA contents in a range of 38-45% of TFA. The commercial production of DHA under this study is currently owned by the company DSM.

Chang *et al.*<sup>5</sup> achieved after 72 hours of fed-batch culture the values summarized on table 11. The data from this study were chosen to be compared with our results because it is a recent work and besides that the procedure followed by these authors was similar to our procedure.

Table 11 – Comparison of DHA production by *Schizochytrium limacinum* in fed-batch with a  $k_{La}$  of  $143\text{ h}^{-1}$  and  $72\text{ h}^{-1}$  for Chang *et al.*, 2013 and for the present work , respectively.

	Chang <i>et al.</i> , 2013	Present work
<b>DCW</b> (g.L <sup>-1</sup> )	26.6	40.4
<b>TFA</b> (% of DCW, w.w <sup>-1</sup> )	41.1	19.6
<b>Lipid concentration</b> (g.L <sup>-1</sup> )	10.9	7.90
<b>DHA</b> (% of TFA)	22.9	48.5
<b>DHA Concentration</b> (g.L <sup>-1</sup> )	2.28	3.80
<b>DHA Productivity</b> (mg.L <sup>-1</sup> .d <sup>-1</sup> )	768	424
<b>Culture Time</b> (h)	<b>72</b>	<b>216</b>

When it comes to microalgae growth and DHA production,  $k_{La}$  is a key parameter. From table 11 we can assume that higher values of  $k_{La}$  enhance cell proliferation and lipid accumulation. Moreover, higher  $k_{La}$  enhances oxygen transfer, which induces higher metabolic activity and the rate of glycerol assimilation to produce more acetyl-CoA and NADPH for lipogenesis, consequently increasing the DHA productivity. The DHA productivity, total fatty acids and lipid concentration was lower in our study, a reason can be due to the lower  $k_{La}$  achieved ( $72\text{ h}^{-1}$ ), the prolonged culture duration and a lower biomass concentration.

Nitrogen limitation induced the accumulation of lipids, because nitrogen depletion inhibits TCA cycle and thus the citric acid concentration inside the mitochondria will increase and be in excess. As a consequence the citric acid that is in excess will be transported outside the mitochondria in exchange with malate. As a result, citrate acid is cleavage by ATP-citrate lyase in cytoplasm to produce acetyl-CoA and malate by malic enzyme to produce NADPH. This will result in a continuous supply of acetyl-CoA and NADPH, which are the prerequisites for oleaginous microorganisms to accumulate lipids <sup>66</sup>.

From table 11 it can be seen that the values for DCW, DHA (% TFA) and DHA concentration were higher in our study than those reported by Chang *et al.*<sup>5</sup>, however it should be taken in consideration the culture time between both studies.

To strengthen the viability of this study it was made an economic balance of the used media. The results obtained using SWM, were compared with the results obtained by Chang *et al.*<sup>5</sup> using SM (Table 12). Comparing the costs of the two media it can be stated that

the SM costs are around 2.8€, while the SWM costs are 0.7€ per liter. Consequently, to produce 1 kg of biomass it costs 17€ and 105€ using SWM and SM, respectively. Fermentation time was not taken into account in the economic study, so further investigation can be required to have a more accurate economic balance.

Fed-Batch cultivation of *Schizochytrium limacinum* on glycerol and SWM showed efficient and economical results for DHA production. The method proved to be efficient for high cell density culture, and thus it would be viable for industrial production of DHA from glycerol and SWM. However, the oxygen transfer rate ( $k_{La}$ ) at industrial scale will require further investigation.

Table 12 – Economic balance using different media (Saline waste medium and standard medium) for biomass and DHA production

	Raw material costs €Kg <sup>-1</sup>	Quantity of raw material Kg.L <sup>-1</sup>		Costs of medium €L <sup>-1</sup>		Costs per biomass €Kg <sup>-1</sup>		Costs per DHA €Kg <sup>-1</sup>	
		SWM	SM	SWM	SM	SWM	SM	SWM	SM
<b>Glycerol</b>	3.1	0.09	0.12	0.27	0.35	6.72	13.5	70.79	152.23
<b>Yeast Extract</b>	34.9	0.01	0.05	0.35	1.74	8.63	65.55	90.88	764.15
<b>Ammonium</b>	20.0	0.004	-	0.07	-	1.80	-	18.99	-
<b>Artificial seawater</b>	0,72	-	1	-	0.73	-	27.34	-	318.98
<b>Saline waste medium</b>	0	1	-	0	-	0	-	0	-
<b>Total</b>	-	-	-	0.69	2.82	17.16	105.94	180.7	1235.4

The data relating to standard medium (SM) was collected from Chang *et al.*, 2013

### 3.7 Conclusions

This case study showed the potential of using alternative raw materials to produce DHA from microalgae. *Schizochytrium limacinum* was cultivated in a 2.7 L stirred tank bioreactor in saline waste medium and the optimal glycerol content was 90 g.L<sup>-1</sup>. Previously studies showed that the optimal temperature was 23 °C, aeration 0.18 m<sup>3</sup>.h<sup>-1</sup> and agitation 100 rpm for the fermentation process. Under the optimal culture conditions, DHA content in the biomass was 9.5 % with a yield of 424 mg.L<sup>-1</sup>.day<sup>-1</sup>. Both ammonia and pH control also had an important role in the microalgae biomass growth and in the lipid content. Ammonia was added at three different stages of the fermentation, each successive addition led to an increase of the biomass growth. Overall, the results suggest that standard medium can be replaced by a cheaper saline waste medium from a dairy effluent. Using the alternative saline waste medium it was possible to reduce the biomass and DHA production costs in comparison with standard medium. This means that there is a potential for industrial use, however, the oxygen transfer rate ( $k_{La}$ ) should be taken into account in the scale up, since it is expected to increase the productivity.

## Chapter 4: The optimal temperature for the growth of *Japanochytrium*

### 4.1 Materials and methods

#### 4.1.1 Microalgae strains, medium and culture conditions

The microalgae *Japanochytrium sp.* used in this work was provided by EcoFuel Labs., a Czech biotechnology company. This microorganism is included in the category of thraustochytrids and it is a heterotrophic marine protist, like *Schizochytrium limacinum*. The effect of temperature on growth of *Japanochytrium sp.* was analyzed and the optimum temperature was determined.

The procedure for culture medium and inoculum medium is similar to the procedure reported above for *Schizochytrium limacinum*.

The *Japanochytrium sp.* cells were maintained in culture plates with GPY, (Glucose Peptone Yeast Extract), medium such as the cells of *Schizochytrium*.

The SWW (Table 7) was pre-treated by adding NaOH till a pH of 7 (EUTECH instruments pH 510), heated ( $\approx 100^{\circ}\text{C}$ ) for precipitation of undesirable proteins and then centrifuged (Sorvall Evolution RC, SLC – 6000) at 6500 rpm during 7 minutes.

#### 4.1.2 Preparation of inoculum

The cells of *Japanochytrium* were inoculated in 500 ml Erlenmeyer flasks with 200 mL containing SWW (Table 7), 30 g/L of glycerol, 10 g/L of yeast extract, always under aseptic conditions. Then, Erlenmeyer flasks were placed on a rotary shaker at room temperature for 7 days. This procedure was made in triplicate. Note that, before the inoculation of the cells, Erlenmeyer flasks were sterilized by autoclaving at  $110^{\circ}\text{C}$  for 30 minutes.

### 4.1.3 Cultivation in Stirred Tank Bioreactor (STR)

To determine the effect of temperature on growth of *Japanochytrium*, four parallel fed-batch cultivations were carried out in a 0.5 L stirred-tank bioreactors (Infors HT Multifors, Switzerland) (Fig. 16) at 15, 20, 25 and 30 °C, during 67 hours. The components of initial fermentation were 40 g/L of glycerol, 20 g/L of yeast extract in SWW (Table 7) and the inoculum volume was 10% (v/v) of initial fermentation medium. During the fermentation process pH was maintained at 7 with H<sub>2</sub>SO<sub>4</sub> (15 % w/v) and KOH (20 % w/v). The agitation speed and air flow rates were set at 180 rpm and 0.12 m<sup>3</sup>/h, respectively.

The analytical methods used for the measurement of optical density and biomass of *Japanochytrium* were the same as those used in the previous case study (chapter 3).

## 4.2 Analytical methods

### 4.2.1 Optical Density (O.D)

Optical density was one of the two methods used to follow cellular growth. The OD readings of the samples collected from the bioreactor were analyzed in a spectrophotometer (Spekol 1300 Analytik Jena) at 578nm using distilled water as blank. If necessary, samples were diluted to avoid the linearity loss with Lambert-Beer Law.

### 4.2.2 Gravimetric Analysis

The growth of biomass was determined by gravimetric analysis. 2 ml of sample were transferred to pre-weighed microtubes and centrifuged (Mikro 120 Hettich Zentrifugen) at 14000 rpm during 5 minutes. The cell pellet was separated from supernatant. Cell pellet was washed using 2 ml of distilled water to remove the salt. The microtubes were placed again in the centrifuge at the same conditions. The microtubes containing the cell pellet were placed into a dryer at 105 °C during 24 hours. After the 24 hours the microtubes were cooled in a desiccator and weighted with analytical balance (Kern 770).



### 4.3 Results and Discussion

In figure 15 plot (a) are represented the values of OD measured at 578nm during the fermentation course. Although OD is a convenient measure it should be taken into account that it is an indirect method, consequently we are measuring a combination of light dispersal by the particles (algae cells) and absorbance by the pigments. So things like cell shape, size and pigment content can affect the values. In this case using DCW over OD would be the most accurate choice in order to obtain more realistic results.

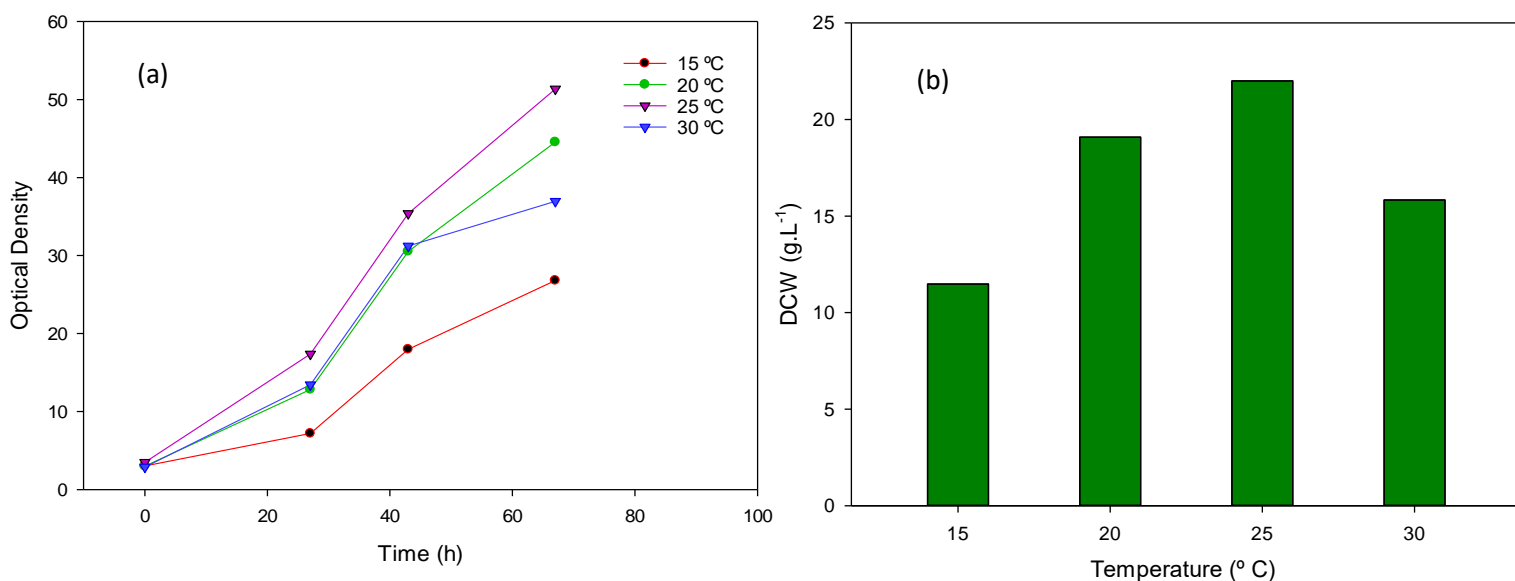


Figure 15 – Time course fermentation profiles of *Japanochytrium* in dairy saline waste medium. Each temperature is represented by a different colour (a). The microalga was grown during 67 h in four, 0.5 L, parallel fed-batch stirred-tank bioreactors (Infors HT Multifors) at 15, 20, 25 and 30 °C, 0.12 m<sup>3</sup>/h and 180 rpm.

(a) Display of *Japanochytrium* growth. Be aware, growth can never start at OD = 0 (which would mean that there are no cells to begin with).  
 (b) This plot shows the biomass values obtained for each temperature

The range of four studied temperatures, (Figure 15), shows that the best temperature for the growth of *Japanochytrium* is at 25 °C. Since the beginning of the fermentation the growth at 25 °C was always the highest. The results obtained are in accord with the literature, where the optimal temperatures for the growth of *Thraustochytrids* range from 22-28 °C.

The maximum specific growth rates ( $\mu_{\max}$ ) were calculated for the time interval between 0 and 43 h. The results obtained at 15, 20, 25 and 30 °C are 0.041, 0.053, 0.057 and

0.054 h<sup>-1</sup>, respectively. The values of the maximum obtained biomass at 15, 20, 25 and 30 °C are 12, 19, 22 and 16 g/L, respectively. This data reinforced that 25 °C was the best temperature for the growth of *Japanochytrium*.

## Chapter 5: Final conclusions and future prospects

Cultivation of microalgae under heterotrophic conditions for the production of economical and useful products has shown to have a great potential to be applied at industrial scale.

The work on chapter 3 has shown that the alga *Schizochytrium limacinum* is capable of using dairy effluent and glycerol for DHA production. Ammonia additions also had a strong effect since it induces the growth of biomass. The economical balance, has shown that using SWM over SM it was possible to reduce the production costs of DHA from 1235€ to 180€. At the optimal culture conditions a DHA yield of 3.80 g/L with 40.4 g/L biomass concentration was obtained.

On chapter 4, *Japanochytrium* was able to utilize dairy effluent and glycerol, however it was only possible to determine the optimal temperature for the growth of this microorganism. The results of this study has shown that the optimal temperature to grow this alga is at 25°C. A continuation of this study is necessary in a similar way of the *Schizochytrium limacinum* in order to determine if *Japanochytrium* is an omega-3 fatty acids producer.

Further work needs to be implemented in order to develop optimized culture protocols. Adding ammonium source at the beginning of the *Schizochytrium* fermentation might prevent the large lag phase during the first 50 hours. This could lead to a reduction of fermentation time and thus increasing the productivity and reducing the production costs.

After lipid extraction, lipids that are not targets of interest could be reused in other processes. For example, palmitic acid could be used on esterification for biodiesel production. Also an improvement of oil extraction methods might be interesting to investigate.

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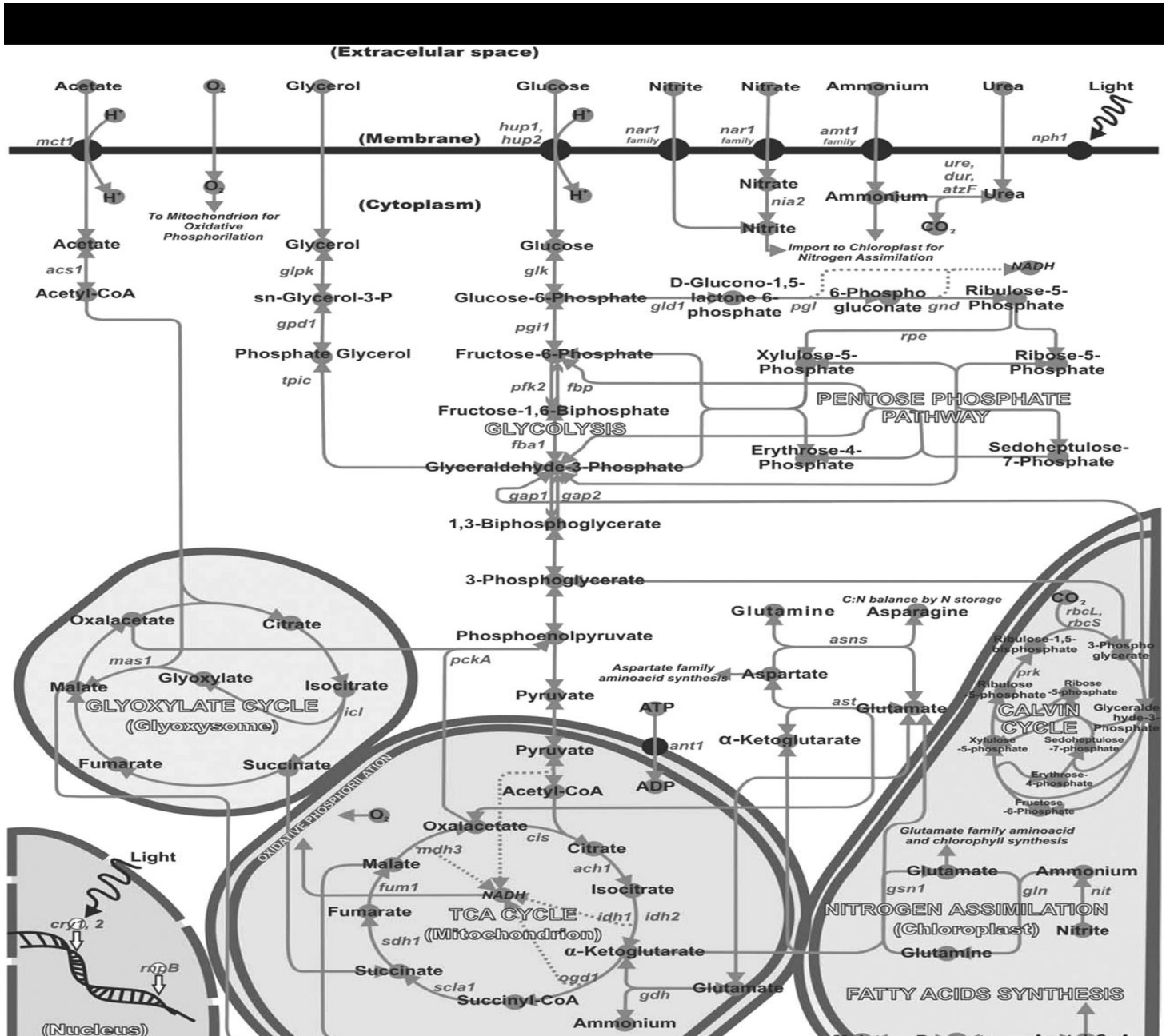
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## Attachments

1. Metabolic pathways involved in carbon assimilation for glucose and glycerol, and in nitrogen assimilation for ammonium



2. Quantitative analysis of sugars (glucose, fructose, maltose, saccharose and maltotriose) and alcohols (ethanol and glycerol) using HPLC (high-performance liquid chromatography).

**Techniques by separation mechanism:**

Ion exchange chromatography (IEC)

**Conditions:**

- Stationary phase: Sulfonated styrene-divinylbenzene sorbent in different ionic forms H form (hydrogen) is suitable for organic acids, alcohols and some sugars (Column: Polymer IEX H Form 8  $\mu\text{m}$ )
- Mobile Phase: Diluted sulphuric acid (9mM)
- Flow: 0,5mL/min (pressure 4,5 MPa)
- Detection: refractometric

**Preparation of mobile phase:**

Preparation of demineralized water (demi) from distilled water using demineralizing machine. Mix the demi water with sulphuric acid (95%) and create diluted sulphuric acid (9mM). Degassing of demi using an ultrasonic cleaner (30 min).

**Preparation of the samples:**

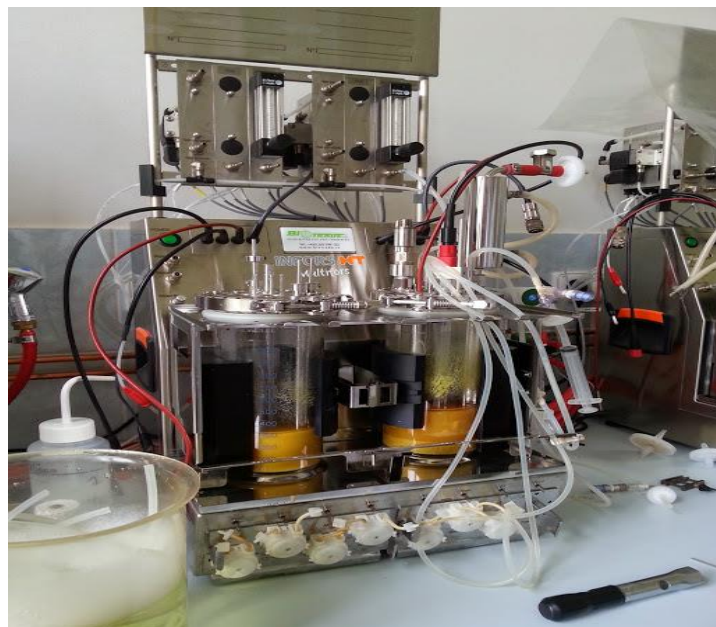
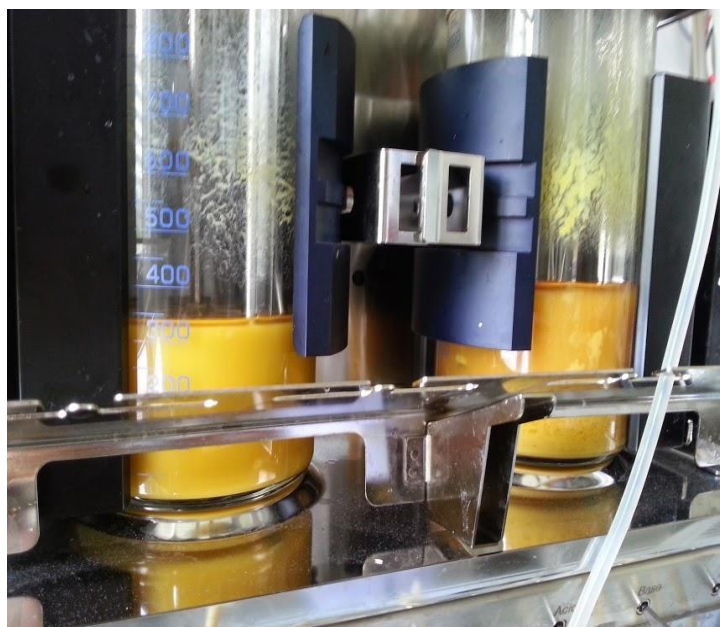
The supernatant was filtered using a 0.22  $\mu\text{m}$  syringe filter in order to remove residual cells.



### 3. Workstation



Three parallel stirred tank bioreactors, (Infor HT Labfor 4, Switzerland), used for *Schizochytrium limacinum* cultivation. The picture on the right side shows a portion of the biomass produced by *Schizochytrium limacinum*.



Stirred tank bioreactors, (Infor HT Multifors, Switzerland), used for the cultivation of *Japanochytrium*.