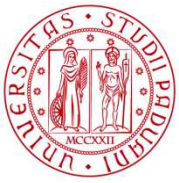

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Corso di Laurea Magistrale in Ingegneria Chimica e dei Processi Industriali

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**Ingegneria Chimica e dei Processi Industriali**

# Hydrolysis and fermentation of microalgal biomass to ethanol production

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*To my family*

## Abstract

This study is focused on the possibility to producing ethanol from microalgal biomass (*Scenedesmus obliquus* and *Chlorella vulgaris*). *Scenedesmus obliquus* was first cultivated in a continuous flat panel photobioreactor and under nutritional stress (nitrogen limitation) to promote carbohydrates accumulation. Acid and enzymatic hydrolysis were optimized and compared (both microalgae - *Scenedesmus* and *Chlorella*). To promote enzymatic hydrolysis a pretreatment based on ultrasonication was applied. Fermentation process was initially studied in a synthetic culture medium to simulate the sugars composition present in these microalgal species. Initial inoculum concentration and consortium by a *Pichia-Saccharomyces* was studied to improve ethanol productivity and yield. Additionally, the effect of salinity on yeasts fermentation was studied. Finally, ethanolic fermentations with microalgal hydrolysate were performed.

# Contents

# Introduction

The global demand for energy is highly dependent on fossil fuels and several studies are trying to improve the availability of renewable energy as a strategy to a more sustainable world where climate change is avoided and control of pollution is ensured. However, the development of new technologies depends on the feasibility, investments/incentives and availability of renewable sources. Biomass is one of them, and can be used to produce biofuels, anyway which ethanol is the most produced one. The demand of bioethanol practically doubled in the last decade reaching a saturation of the first-generation crops exploitation and rising up arable land and food vs fuel issue.

As result of this, investigations on 2$^{nd}$ ethanol generation were stimulated which is based on lignocellulosic material/biomass/waste as raw materials. However, the difficulty to validate this technology at industrial scale due to saccharification problems, severity of the pretreatments, the high chemicals use or higher time of biological pretreatment, cost of enzymes and slower fermentation time and yield opened space to new sources of biomasses such as micro and macroalgae (3$^{rd}$ and 4$^{th}$ generations of bioethanol production).

In particular, microalgae can reach good values of carbohydrate content depending of the environmental/nutritional conditions, have higher growth rate in comparison to higher plants and are not containing lignin, thus they are easier to hydrolyze than lignocellulosics. Often there are no industrial applications of this type of biomass for ethanol production as several process steps need to be developed prior to the expansion of scale, such as hydrolysis and fermentation.

Thus, in this work the acidic and enzymatic hydrolysis of *Scenedesmus obliquus* and *Chlorella vulgaris* biomass were studied and optimized. After that, their hydrolysates were submitted to fermentation with an inoculum composed by a consortium (*Saccharomyces* + *Pichia*) to verify the influence of salinity on ethanol yield.

In chapter 1 the state of art with several information related to ethanol production technologies are reported, while chapter 2 details the material and method used for the experimental procedures of microalgae cultivation and biomass characterization, acidic and enzymatic hydrolysis and ethanolic fermentation.

In chapter 3, the acid and enzymatic hydrolysis of microalgal biomass are studied and discussed. For acidic hydrolysis, the best acid and biomass concentration, temperature and reaction time were determined. During enzymatic hydrolysis, ultrasonication was validated as a pretreatment of the biomass to improve enzyme accessibility and the concentration/type of enzyme was also verified.
In chapter 4, the fermentation process was investigated in order to guarantee a good productivity and ethanol yield. Firstly, the inoculum concentration and species consortium were studied. After, the influence of salinity was performed to understand if the salts concentration in both hydrolysis could affect negatively the fermentation performance. Finally, the microalgal hydrolysates were fermented.

*I would like to thank prof. Alberto Bertucco to give me the opportunity to do this work and a special thanks to MSc. Carlos Eduardo de Farias Silva for help and his precious support.*

# Chapter 1

# Ethanol from microalgae: state of the art

## *1.1 Energetic matrix worldwide*

From the first industrial revolution (1750), humans started to get energy from coal, oil and gas (fossil fuels), and at present, it still represents most of the energy sources and cause serious pollution problems. The combustion of fossil fuels produces gases like carbon dioxide, nitrogen oxide (NOx), sulphur dioxide ($SO_2$), volatile organic (VOCs) and heavy metals compounds. All of these are pollutants that are responsible directly of environmental problems like acid rains, greenhouses effect, ozone depletion, and indirectly the climate change, ecosystem alteration, rising of the sea levels (Mata et al., 2010; Ashokkumar et al., 2015).

A number of several treaties (such as Kyoto, COP 21), have promoted a cooperation in order to change the mix of energy resources with the development of new systems to obtain clean energy (i.e. renewable energy). By definition renewable energy is the energy derived from nature and that can replenish within a human lifespan. This are also call sustainable source of energy became it has a rate of consumption that does not exceed its rate of regeneration (Natural Resources Canada, 2017). At the world level in 2015 the energy derived from fossil fuel was equal to 82% (32% from gas, 42% from oil and 26% from coal), 5% was taken from nuclear fission, 2% from hydropower and 11% from renewable sources which include biomass (Figure 1.1) (World Energy Council, 2016).

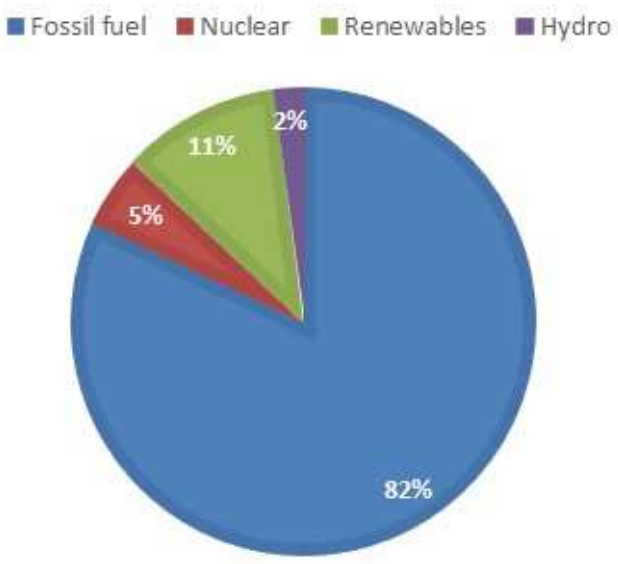


**Figure 1.1:** *Energetic matrix worldwide (WORLD ENERGY COUNCIL, 2016)*

Biomass is a natural product with a high amount of chemical energy stored inside, so that it and it is the raw material of biorefinery system. According to the US congress 2000, biomass is an "organic matter that is available on a renewable or recurring basis (excluding old growth timber), including agricultural food and feed crop residues, dedicated energy crops and trees, wood and wood residues, aquatic plants, animal wastes and other waste materials." The concept of biorefinery is similar to that of petroleum refinery. According to IEA Bioenergy Task 42: "Biorefinering is the sustainable process of biomass into a spectrum of marketable products and energy". This definition includes a great variety of technologies able to transform biomass resources (sugar cane, corn, wood, microalgae…) into building blocks (carbohydrates, triglycerides, proteins…) which can become valuable products, chemicals and biofuels. In brief, biorefinery is a network of facilities that combine biomass conversion process, power, chemicals and operation units to produce biofuels (Figure 1.2) (Cherubini, 2010).

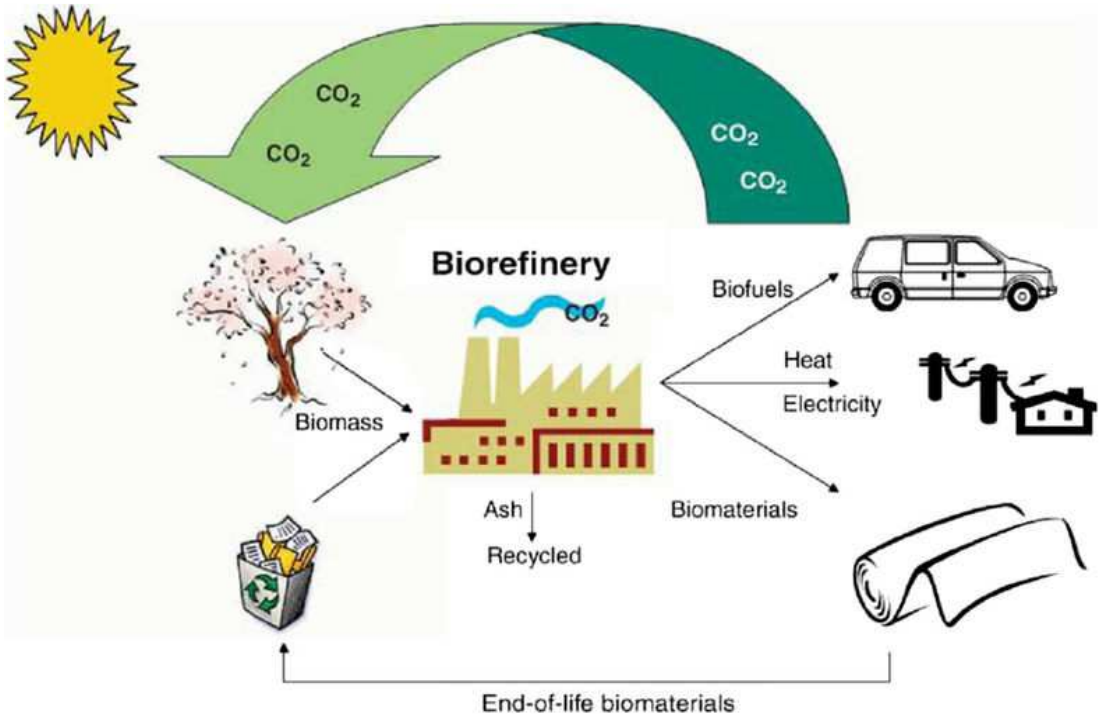


Figure 1.2: *Biorefinery concept as network of facilities source: (Ragauskas et al.2006)*

The principal renewable sources developed are from solar irradiation, wind, biomass and biogas, and represent only 11% of the total energy used.

## *1.2 Ethanol market and generations*

### 1.2.1 Bigger producers

The ethanol production was developed first in Brazil and USA for different reasons such as the economic crisis due to overproduction of sugar, global oil crisis and as alternative to oil derivate molecules. In 1975 the National Alcohol Program (ProAlcool) was started, based on the production of ethanol at the large scale starting from sugar cane as raw material. This activity is nowadays sustained, as in 2015 Brazil's govern approved a law that require a bioethanol content of 20-25% in gasoline (Risoluzione n°6/2009 del Conselho Nacional de Política Energética). In 2016, Brazil was one of the main producer of bioethanol with 7.295 billion gallons out a total of 26.5 billion gallons produced (Table 1.1 and 1.2).

The USA, ethanol industry started from 1980 with the aim to revitalize the farming sector on difficult due to the overproduction. As happened in Brazil, United States

gradually promote the ethanol industries by approving blender fuel made by 85% of bioethanol and 15% of gasoline for vehicles specially designed (Mussatto et al., 2010).

**Table 1.1**: *Share of biofuel production by region (2014 data). Source: World Energy Resource, 2016.*

| Region | Percentage (%) |
|---|---|
| Asia | 10.5 |
| Africa | 1 |
| Middle East | - |
| Europe and Eurasia | 16.5 |
| South and Central America | 28.7 |
| North America | 44.1 |

**Table 1.2**: *Major Bioethanol producer (year 2016). Source: statista.com*

| Region / State | Production (Million gallons) |
|---|---|
| USA | 15,250 |
| Brazil | 7,295 |
| Europe | 1,377 |
| China | 845 |
| Canada | 436 |
| Thailand | 322 |
| Argentina | 264 |
| India | 225 |
| Rest of the world | 490 |
| **Total** | **26,504** |

### 1.2.2 Type of biomass and conversion technology

The bioethanol production from sugars can be summarized in three steps:

1. cultivation and extraction of fermentable sugars;
2. transformation of sugars into ethanol by ethanolic fermentation (usually);
3. ethanol separation and purification (Mussatto et al., 2010).

Thanks to its high sugar cane productivity, Brazil developed a 1$^{st}$ generation biorefinery where sugar cane was converted into bioethanol. The process is simple and consist in the milling of the sugar cane for the sugars extraction (hydrolysis is not required). The ethanolic fermentation is done directly after the extraction (Figure 1.3).

As sugarcane needs specific climatic conditions, countries of northern hemisphere used other food crops to develop their ethanol industries (corn in USA and beet in Europe). When corn is used, the process of saccharification requires sugars extraction/depolymerization (hydrolysis) after the milling since starch, a polysaccharide, is the main carbohydrate present. The pretreatment consists in an enzymatic hydrolysis with amyglucosidase and α-amylase of the gelatinized cooked

starch (extracted from biomass). Then the fermentation process can be done. (Figure 1.3).

The main disadvantages of 1st generation ethanol are the geographical limitation, their seasonality and also the ethical problems related to the use of food as raw material to produce energy. This last concern (food vs fuel issue) is really an issue due to the world population growth and led scientist to develop the 2nd generation ethanol biorefinery, where the new raw material is lignocellulosics biomass such agricultural/forest waste and wood. This process, compared to the first generation one is more complex mainly due to the presence of lignin in the biomass cell wall (Figure 1.3 and 1.4). After milling, a pretreatment to improve cellulose accessibility and to remove de-structucture hemicellulose-lignin complex is required. Thus, the main disadvantage is the difficulty to extract sugars from the raw materials. On the other hand, the process is able to use different feedstock even though high capital costs are required.

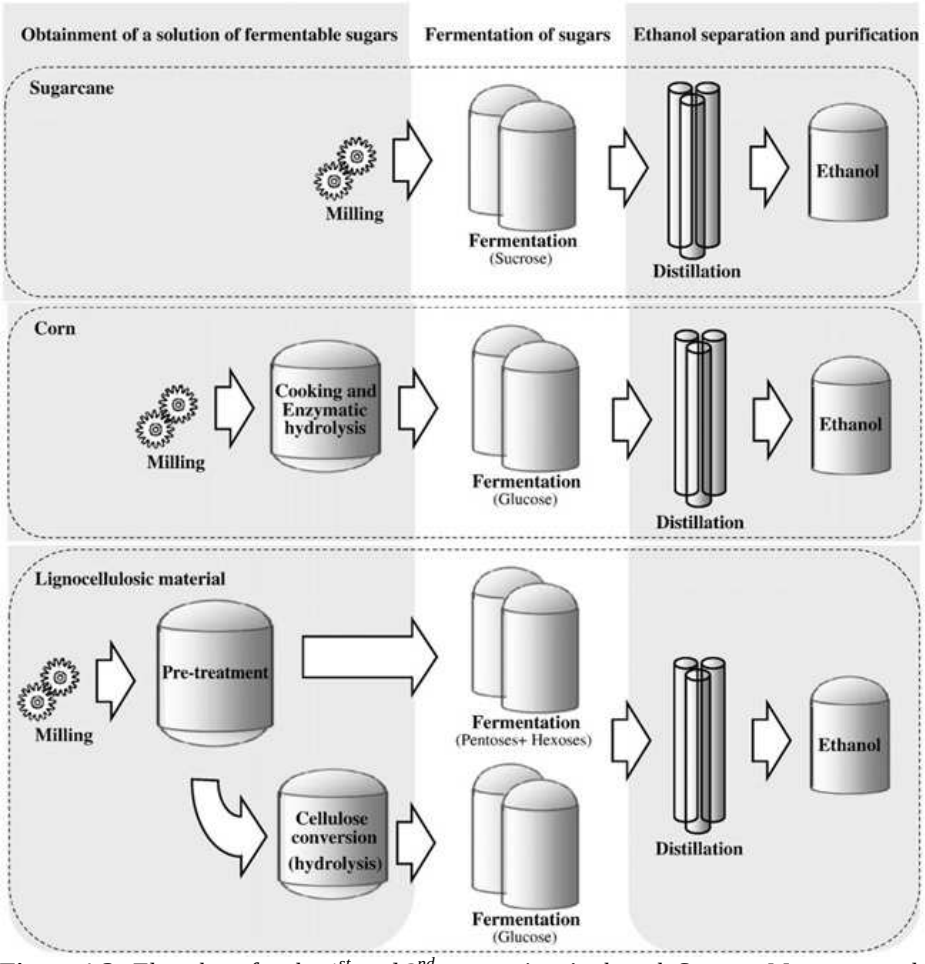


**Figure 1.3:** *Flowchart for the 1st and 2nd generations' ethanol. Source: Mussatto et al., 2010.*

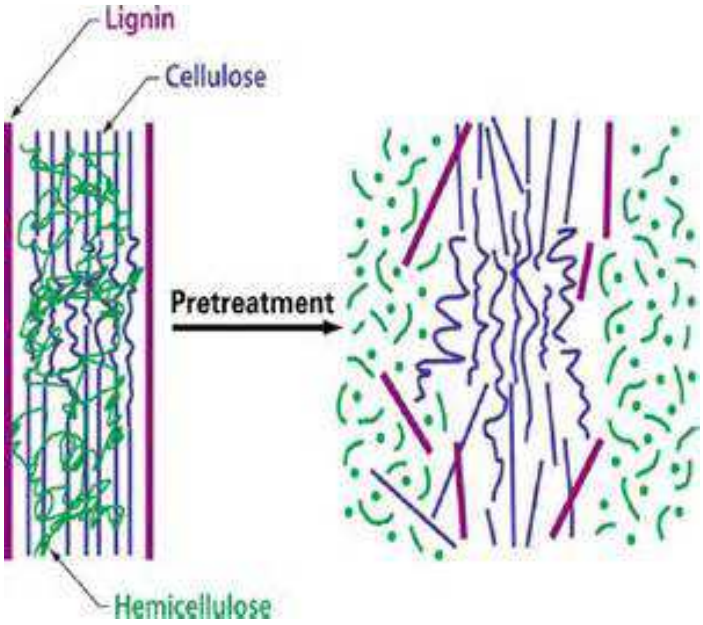


**Figure 1.4** *Effect of the pretreatment on the lignocellulose structure. Source: Bhatia et al., 2012.*

Chemical/physical/biological treatments can be used such as dilute acid, alkaline/organic compounds, steam explosion, impregnation or filamentous fungi (or the combination between them). Each one has their own advantages/disadvantages and the applicability/efficiency depends on the biomass, whose recalcitrance determined by the content of the lignocellulosic fractions and their arrangements (Table 1.3).

**Table 1.3:** *Pretreatment methods of lignocellulosics materials. Source:El-Naggar et al., 2014.*

| **Pretreatment method** | **Processes** | **Advantages** | **Disadvantages** |
|---|---|---|---|
| Physical pretreatment | Milling | Intensive decrystallization<br><br>Increase in accesively surface area and pore size | Energy intensive |
| Physicochemical and chemical pretreatments | Ammonia fiber explosion (AFEX) | Increase accessible area<br><br>Remove lignin and hemicelluloses to an extent<br><br>Does not produce inhibitors | Not efficient for biomass with high lignin content<br><br>Does not significantly solubilize hemicelluloses compared to other pretreatment process |
| Physicochemical and chemical pretreatments | Dilute acid: sulphuric acid | High xylose yields<br><br>Increase the surface area and the pore volume by removing hemicellulose | Equipment corrosion<br><br>Formation of toxic substances<br><br>Relative expensive |
| Physicochemical and chemical pretreatments | Sodium hydroxide | Effective ester removal<br><br>Increase surface area and the porosity | Expensive reagent<br><br>Alkali recovery |
| Physicochemical and chemical pretreatments | Lime | Effective lignin and acetyl removal<br><br>Inexpensive | Less effective due poor solubility of lime |
| Physicochemical and chemical pretreatments | Ammonia | Effective delignification | Alkali recovery<br><br>Relatively expensive |
| Physicochemical and chemical pretreatments | Ozonolysis | Effectively removes lignin<br><br>Does not produce toxic residues<br><br>The reaction is carried out at room temperature and pressure | Large amount of ozone is required, making the process expensive |
| Biological pretreatments | Fungi<br><br>Actinomycetes | Degrades lignin and hemicelluloses<br><br>Mild environmental conditions | Cellulose loss<br><br>Rate of hydrolysis is relatively slow |

After pretreatment, the de-structured biomass can undergo an enzymatic hydrolysis to recover the cellulose fraction, which is less affected by the pretreatments thanks to its strong resistance to thermochemical processes. (Figure 1.4).

Recently, micro/macroalgal biomass has been proposed as a 3rd generation alternative to the ethanol biorefinery. Microalgae are less complex in terms of structure than higher plants, do not depend on arable land, for cultivation, display present higher growth rate and, can consequently increase the productivity per hectare. However, cultivation costs are still high, became acceptable technology able to convert and manage efficiently this type of biomass is lacking (Acién et al., 2012; Slade and Bauen, 2013). Microalgae perform photosynthesis using sunlight energy, water, salts and carbon sources to convert them firstly in sugars and then proteins and lipids. In addition, pretreatment of this biomass is simpler than lignocellulosics because lignin is not present in the cell wall. On the other hand, it is a new technology so that high capital costs are required. More research and development are needed but the advantages will play a key role in the near future.

In parallel to this, a 4th generation biorefinery was the result of the marketing and development of some patents in the USA where genetically modified cyanobacteria are applied to produce ethanol directly from sunlight and nutrients. The advantage of this process is simplification. Thus, the elimination of biomass pretreatments ethanol is obtained by direct distillation of the medium. However, there are not enough studies on that and it is difficult to estimate a real productivity and calculate the capital costs involved (Silva and Bertucco, 2016). The four bioethanol generations technologies are summarized in Table 1.4.

**Table 1.4**: *Biorefinery generations, advantages and disadvantages. Source: Silva and Bertucco, 2016.*

| Generation | Advantages | Disadvantages |
|---|---|---|
| **1st generation** (Sugar cane, corn, beet) | Lower and stable production cost<br>Known technology<br>Can be competitive to fossil fuel | use of edible material<br>Seasonality of raw material<br>Geographical limitation |
| **2nd generation** (Lignocellulosics) | Low geographical limitation<br>Less use of edible material | Pretreatment problems<br>High capital costs<br>Recent technology |
| **3rd generation** (Microalgae, cyanobacteria and macroalgae) | No geographical limitations (water, light, salts and $CO_2$ are needed)<br>No lignin in the cell wall | High cultivation costs<br>High capital costs<br>Recent technology |
| **4th generation** (Genetically modified cyanobacteria) | Composed by two steps: cultivation and distillation | New technology (little information on literature)<br>Use of genetically modified organisms<br>High capital costs |

## *1.3 Microalgae as a promising source for biofuels*

Microalgae/Cyanobacteria are organism typically found in fresh water and marine systems. They are unicellular, prokaryote or eukaryotic (cyanobacteria and microalgae, respectively) and photosynthetic organisms, which live individually or in colonies. There exist more than 50000 species, with a size ranging from 1 to 10 μm (Mata et al., 2010).

### 1.3.1 Advantages of microalgae for biofuels

Using microalgae have many advantages over higher plants in view of producing first and second generation biofuels. Microalgae have a faster growth, they can double their biomass in a short time (≈3.5h hours), they have the ability of growing in harsh condition and they need a lower water amount than terrestrial crops. In addition, microalgae fix more effectively $CO_2$ (1000 g of algal dry biomass utilize about 1.83 kg of $CO_2$); nutrients for cultivation can be obtained by waste water and other valuable byproducts like proteins can be obtained. Unlike terrestrial crops, biomass cultivation does not need pesticide nor herbicide treatments and also the cultivation area requested is lower (Table 1.5) (Mata et al., 2010).

**Table 1.5:** *Ethanol productivity comparison between different biomasses.*

| **Raw material** | **Ethanol productivity (L/(ha year))** | **Reference** |
|---|---|---|
| Corn | 3450 – 4600 | BNDS, 2008 |
| Beet | 5000 – 10000 | BNDS, 2008 |
| Sugarcane | 5400 – 10800 | BNDS, 2008 |
| Lignocellulosic biomass (sugarcane) | 10000 | Santos et al., 2014 |
| Microalgae (20% dry biomass carbohydrate content) | 7093 – 21279 | Acién et al., 2012 |
| Microalgae (35% dry biomass carbohydrate content) | 12413 – 37286 | Acién et al., 2012 |
| Microalgae (50% dry biomass carbohydrate content) | 17733 – 53199 | Acién et al., 2012 |

### 1.3.2 A carbohydrate-rich biomass from microalgae

Microalgae grown in normal condition (excess of nutrients) have the biochemical composition of 20-40% of carbohydrates, 30-50% of proteins and 8-15% of lipids. However, it is possible to increase the carbohydrates and lipid content by properly managing environmental/nutritional conditions the nutrients concentration, light

intensity and residence time as that the carbon assimilation and its metabolism are modified (Chen et al., 2013; Vitovà et al., 2015).

It is important to mention that under nitrogen limitation/starvation microalgae/cyanobacteria can accumulate energetic-reserves of both lipids and carbohydrates depending on the stress condition (Figure 1.5). The main carbohydrate present in microalgae are starch and in cyanobacteria glycogen, (while other polysaccharides such as cellulose, hemicellulose and pectin can be found in the cell wall). All others are glucose based polymers and represent a promising source for fermentation applications such as bioethanol, biobutanol, other alcohols, acetone, methane, and hydrogen (Vitovà et al., 2015).

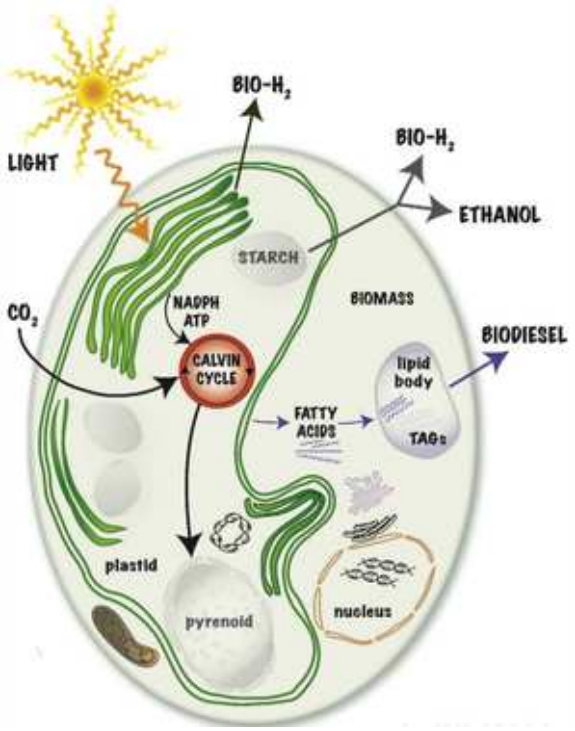


**Figure 1.5**: *Biofuels potentiality from microalgae. Source: Beer et al., 2009*

In Table 1.6 some values of carbohydrate content obtained in microalgae cultivated under nutrient limitation are reported.

**Table 1.6***: Carbohydrate content in some microalgae.* ***Source: Adapted from Silva and Bertucco, 2016.***

| **Microalgae** | **Carbohydrates content (%)** |
|---|---|
| *Chlamydomonas fasciata* Ettl 437 | 43.5 |
| *Chlamydomonas reinhardtii* UTEX 90 | 59.7 |
| *Chlorella vulgaris* P12 | 41 |
| *Chlorella vulgaris* FSP-E | 52 |
| *Chlorella sp.* KR1 | 49.7 |
| *Dunaliella tertiolecta* LB999 | 40.5 |
| *Scenedesmus dimorphus* | 45 – 50 |
| *Scenedesmus obliquus* CNW-N | 51.8 |

These polysaccharides, after hydrolysis, provide mainly glucose (70-80% of dry cell carbohydrates - DCC) and pentose sugars (20-30% - DCC). For example, in *Chlorella sp KR1* 82% glucose and 18% pentose was founded (Lee et al, 2015) and for *Chlorella sorokiniana* 70.8% glucose, 21.5% pentose and 7.7% other sugars (Hernandez et al, 2015). *Scenedesmus obliquus* exhibited a monosaccharide profile of 80% glucose and 20% xylose (Ho et al., 2013) and *Scenedesmus almeriensis* with 52.2% of glucose, 33.4% of xylose, 15.4 of other sugars (Hernandez et al., 2015). In *Nannochloropsis gaditana* was 59% of glucose, 28.8% xylose, 6.5 Ramnose and 5.7% other sugars was founded (Hernandez et al., 2015), for *Chlamydomonas reinhardtii* 88% of glucose, 7% galatose, 4% arabinose and trace of xylose (Nguyen eta al., 2008).

### 1.3.3 Hydrolysis process

Polysaccharides are complex molecules which usually have repetitive structure with monomers or oligomers. Thus, they need to be hydrolyzed to be efficiently fermented by yeast and/or bacteria strains. (Figure 1.6).

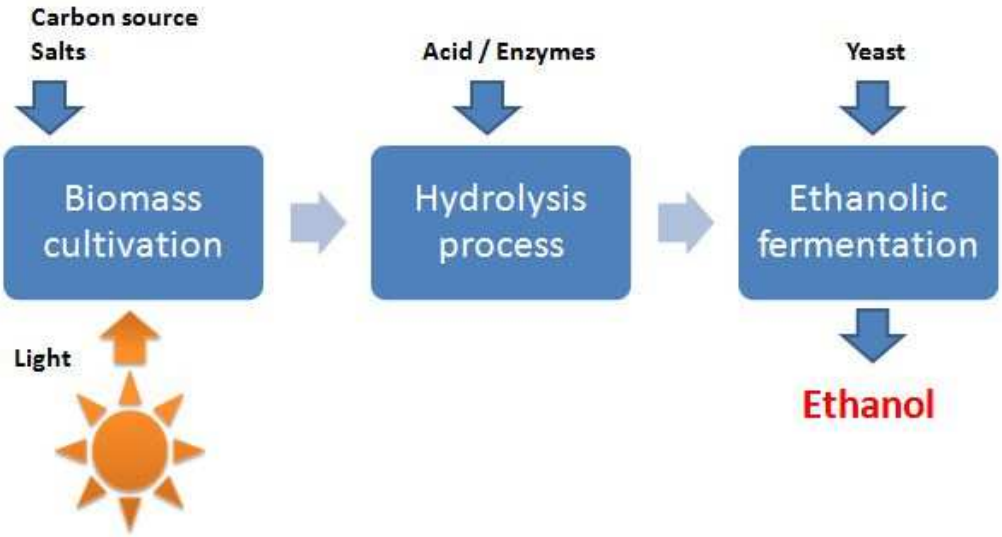


**Figure 1.6:** *Bioethanol production from microalgae biomass block diagram.*

The hydrolysis process allows carbohydrates extraction and their simplification into reduced sugars which are easily fermentable. For microalgae, the main one is glucose, but also xylose and arabinose (pentose) or mannose, galactose can be obtained.

There are several methods of carbohydrate hydrolysis which are basically divided into chemical and biochemical ones. In the literature chemical hydrolysis is generally performed with acids (dilute or concentrated - usually sulphuric acid, chloride acid) at high temperatures, in the range between 110 – 130°C and with times shorter of 15 – 45 minutes. Sugars extraction yields obtained were between 70% and 98%. For example, *Chlamydomonas reinhardtii* with sulphuric acid at 110°C for 30 minutes reached the saccharification yield of 90% (Nguyen et al., 2008). *Scenedesmus bijugatus* biomass at 130 °C and 2% of sulphuric acid yielded around 85% of saccharification efficiency (Ashokkumar et al., 2013). *Scenedesmus obliquus* CNW-N, at 121 °C and with sulphuric acid 2% for 20 min reached 95% of sugars recovery (Ho et al., 2013). *Tribonema* sp. at 121 °C and 3% of sulfuric acid, 30 min reached 90% (Wang et al., 2014). *Chlorella vulgaris* JSC-6, at 121 °C and 3% of sulfuric acid for 20 min achieved a saccharification yield of 90% (Wang et al., 2016). Acidic hydrolysate needs to be neutralized before fermentation causing the formation of high amount of salts which can significantly affect the fermentation yield (Casey et al., 2013).

Biochemical hydrolysis can also be performed with enzymes carefully chosen based on the type of polysaccharide due to their specificity. The environmental conditions must be previous determined and controlled during all the process because, as biologically

active components, they are sensible and inhibition/denaturation can happen (pH, temperature, osmotic pressure, biomass characteristics) (Robinson, 2015).

Amylase, pectinase and cellulase are the most common enzymes used to saccharify microalgal biomass. Amylases are a group of enzymes produced by plants, animals, and microorganisms for starch assimilation. The most important are α -amylase, β –amylase and pullulanase. They have different structure and catalytic mechanism. The best condition for the saccharification were found at pH = 4.5 at temperature between 45 and 55°C (Lee et al., 2015).

Cellulases are produced by fungi, bacteria, protozoans, plants, and animals. These types of enzymes are specialized for cellulose hydrolysis which is achieved through the combination of the catalytic effect of endoglucanases, exoglucanases, cellobiohydrolases and β –glucosidase. According to the literature the best condition for hydrolysis are pH = 5.5 and 55°C (Lee et al., 2015).

Pectinase is an enzyme used for the demolition of pectin (polymer of galacturonic acid), usually contained in the cell wall. The main enzymes of this family are pectolyase, pectozyme, and polygalacturonase. The maximum saccharification yield is found at 45°C and pH = 5.5 (Lee et al., 2015), and Kim et al., (2014) showed a good performance for this type of enzyme at 50°C and pH = 4.8.

In comparison with the chemical method, enzymatic hydrolysis is slower and more complicate because a biomass pretreatment is needed to let the enzyme enter inside the cell and catalyze the reaction. On the other hand, it is more eco-friendly because no-reduced chemicals are used. Some results are reported in Table 1.7 where it is possible to see that a minimal change of the enzyme type or environmental conditions can decrease a saccharification yield from 80 to 20%.

**Table 1.7:** *Enzymatic hydrolysis for some microalgal biomass.*

| Microalgae | Enzyme | Conditions | % Saccharification | Reference |
|---|---|---|---|---|
| *Chlorella vulgaris* | Pectinase | pH=4.8, T=50°C | 41 | Kim et al., 2014 |
| *Chlorella vulgaris* | Pectinase (bead-beating) | pH=4.8, T=50°C | 79 | Kim et al., 2014 |
| *Chlorella vulgaris* | β-glucosidase | pH=4.8, T=50°C | <20 | Kim et al., 2014 |
| *Chlorella vulgaris* | Cellulase | pH=4.8, T=50°C | <20 | Kim et al., 2014 |
| *Chlorella vulgaris* | Amylase | pH=4.8, T=50°C | <20 | Kim et al., 2014 |
| *Chlorella vulgaris* | Chitinase | pH=4.8, T=50°C | <20 | Kim et al., 2014 |
| *Chlorella sp.* KR-1 | Pectinase | pH=5.5, T=45°C | 76.8 | Lee et al., 2015 |
| *Dunaliella tertiolecta* | Amyloglucosidase | pH=5, T=55°C | 42 | Lee et al., 2013 |

### 1.3.4 Ethanolic fermentation

Ethanolic fermentation is one of the best characterized biological processes because is the source of several food and biofuel applications. There are microorganisms having a metabolic activity that is able to transform the sugars contained in the biomass hydrolysate into ethanol. The main reaction of is:

$$C_6H_{12}O_6 \longrightarrow 2CH_2CH_2OH + 2CO_2 \qquad (1.1)$$

It is an anaerobic process and the most common microorganisms used are yeasts (*Saccharomyces* and *Pichia* genus*)* and bacteria (*Zymomonas*). Industrial ethanolic fermentation use glucose or sucrose as the carbon source. However, in the biomass hydrolysate it is common that other mono/oligosaccharides are present, and in some cases, such as pentose or xylose they cannot be fermented by *Saccharomyces* cerevisiae.

In this case the use of genetically engineered microorganisms or microorganisms naturally fermenters of pentose and other sugars (such as *Pichia* and *Kleyveromyces*) must be used (Agbogbo et al., 2006; Rouhollah et al., 2007; Rodrussamee et al., 2013).
Values reported in the literature for the fermentation of microalgal hydrolysate show yields between 56% and 90% (Silva and Bertucco, 2016). In details, *Scenedesmus bijugatus* acidic hydrolysate (2% $H_2SO_4$) reached a fermentation yield of 70% using *Saccharomyces cerevisiae* (Ashokkumar et al., 2013). *Chlorella sp. KR-1* pretreated with HCl 0.3N reached a fermentation yield of 80% with *S.cerevisiae* (Lee et al., 2015). *C. vulgaris* after enzymatic hydrolysis with endoglucanase, amylase and β-glucosidase and fermented with the bacteria *Zymomonas mobilis* (ATTC 29191) at 30°C ensured an ethanol yield of 91%.

## *1.4 Aim of the thesis*

This thesis is aimed to study the processes of hydrolysis and fermentation of microalgal biomass to obtain bioethanol as a biofuel. Acidic and enzymatic hydrolysis experiments were performed in order to determine the best conditions to maximize the saccharification yield. Ethanolic fermentation were carried out to understand if it is possible to achieve a fermentation yield similar to the values obtained with traditional crops.
In details:

1) Acidic hydrolysis was optimized acid with respect to biomass concentration, treatment time and temperature;
2) Enzymatic treatment was studied with ultrasonication as pretreatment to increase enzyme accessibility during the hydrolysis process. The effect of enzyme concentration per gram of biomass was evaluated;
3) Fermentation with standard medium was evaluated to study the influence of inoculum concentration, consortium (Saccharomyces and Pichia) and salinity on the ethanol yield and productivity;
4) A validation for the results previously determined was made with the microalgal hydrolysates (acidic and enzymatic).

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# Chapter 2

# Experimental materials and methods

In this chapter the material and methods used to set up the experiments are considered. First some details concerning algal cultivation, such as the continuous growing system and biomass characterization are presented. Then, experimental procedures of acidic and enzymatic hydrolysis are described. Finally, ethanolic fermentation using different yeast strains and how optimizing their performance is discussed.

## *2.1 Algal biomass*

The microalgal chosen for the study were *Scenedesmus Obliquus* and *Chlorella Vulgaris.*

*Chlorella Vulgaris* biomass powder was provided by Neoalgae® (Micro seaweed products B-52501749).

*Scenedesmus Obliquus* 276.7 (SAG- Goettingen) biomass was produced through cultivation in a continuous photobioreactror (PBR) at 23 ± 1 °C, and fed with a modified BG11 with nutrient limitation to promote the accumulation of carbohydrates.

The continuous cultivation was performed in a vertical flat-plate polycarbonate CSTR (continuously stirred tank reactor) PBR with a working volume of 700 mL, a depth of 1.2 cm, and an irradiated surface measuring 30 cm (length) and 19.5 cm (width) (Barbera et al., 2017).

$CO_2$ in excess was provided by a mixture $CO_2$–air (5% v/v) bubbling at the reactor bottom (1L/h of total gas flow rate), which also ensured mixing. Additionally, a magnetic stirrer was used to prevent any deposition of biomass, thus ensuring a good mixing within the reactor. The fresh medium was fed at a constant rate by a peristaltic pump (Watson-Marlow sci400).

The working volume (*Vr*) was controlled by an overflow tube, and the outlet flow rate *Q* (mL/day) was collected in a tank. So, the hydraulic residence time ($\tau$) in the reactor was directly controlled by the peristaltic pump, according to the relationship: $\tau = Vr/Q$.

The inlet flowrate was regulated in order to obtain a residence time $\tau = 2.3 \pm 0.3$ days.

Light was provided by a LED lamp (Photon System Instruments, SN-SL 3500-22). Photon Flux Density (PFD) was measured on both the reactor front and back panels using a photoradiometer (HD 2101.1 from Delta OHM), which quantifies the photosynthetically active radiation (PAR) (Silva, et al., 2017). In Figure 2.1 a picture of the continuous flat panel used for the *Scenedesmus obliquus* biomass cultivation is shown.

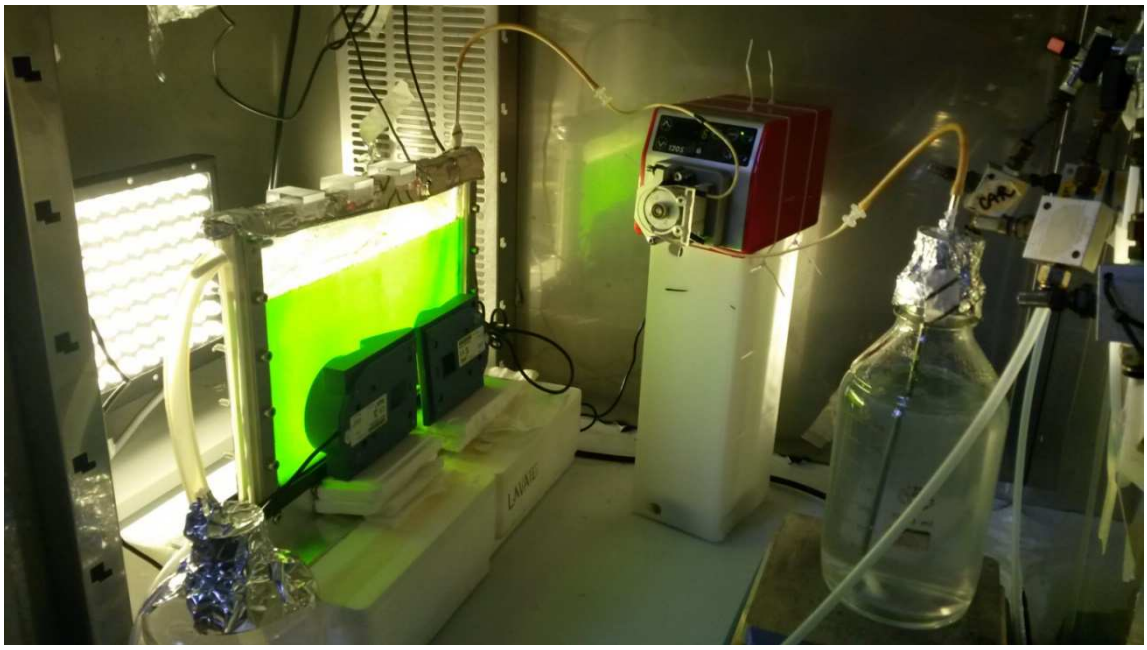

**Figure 2.2:** *Biomass continuous cultivation.*

## *2.2 Microorganisms and Culture Medium*

### 2.2.1 Yeast strains and culture medium

Yeast species chosen for this study were *Saccharomyces cerevisiae* (Cameo S.p.A.) and *Pichia stipitis* ATCC 58785.

The cultures were maintained in YPD-Medium: 10 g/L of yeast extract, 20 g/L of peptone and 20g/L of glucose. The microorganisms also were grown in YPDA (agar plates – 20 g/L) to store them for longer periods at 4°C. For all control fermentations, YPD medium was used with glucose concentrations of 20 (YPD-20) and 50 (YPD-50) g/L.

These sugars content in the medium were depended to study the yeast fermentation with a substrate similar to the ones obtained from 100 or 50 g/L of microalgal biomass after

the hydrolysis process. *Chlorella vulgaris*, a strain with 20-25% of carbohydrate content and *Scenedesmus obliquus* with 45-50% of carbohydrate content, were the sources of their respective carbohydrates which were made available as sugars concentration in the medium.

### 2.2.2 Microalgae strain and Cultivation

As aforementioned *Scenedesmus obliquus* was cultivated in continuous made using BG-11 medium (Table 2.1) (Rippka et al., 1979). To maximize the biomass and carbohydrate production, BG11 medium was modified to provide limitation of nitrogen and to shift towards the accumulation of carbohydrates. In fact, under nutrient limitation (in particular of the nitrate content) microalgae are naturally stimulated to accumulate carbohydrates as a result of the growth limitation and reduced capacity to synthesize proteins (Chen et al., 2013).

The culture medium and all the materials were sterilized in an autoclave at 121 °C for 20 mins in order to prevent any contamination. The pH was kept constant at pH = 8 by using Hepes as buffer.

**Table 2.1:** *Standard and modified composition of BG11 medium.*

| **Component** | **Concentration (mg/L) Rippka et al., 1979** | **Concentration (mg/L) Silva et al., 2017** |
|---|---|---|
| $Na_2Mg$ EDTA | 1 | 1 |
| Ammonium ferric citrate | 6 | 6 |
| Citric acid*$H_2O$ | 6 | 6 |
| $CaCl_2$*$2H_2O$ | 36 | 36 |
| $MgSO_4$ | 75 | 75 |
| $K_2HPO_4$ | 30.5 | 142 |
| $H_3BO_3$ | 2.86 | 2.86 |
| $MnCl_2$*$4H_2O$ | 1.81 | 1.81 |
| $ZnSO_4$*$7H_2O$ | 0.222 | 0.222 |
| $CuSO_4$*$5H_2O$ | 0.70 | 0.70 |
| $COCl_2$*$6H_2O$ | 0.050 | 0.050 |
| $Na_2MoO_4$*$2H_2O$ | 0.391 | 0.391 |
| $Na_2CO_3$ | 20 | 20 |
| $NaNO_3$ | 0.943 | 0.54 |
| HEPES pH 8 | 1M | 1M |

In Table 2.1, the concentration of the medium is summarized. Note that P and N concentration were modified in order to cause a nutritional stress and improve carbohydrate accumulation by *S. obliquus*. P concentration was increased to avoid limitation of this nutrient on cell growth, and nitrogen limitation was the unique condition desired, avoiding growth inhibition due P limitation. On the other hand, reducing nitrogen concentration is the most known method in carbohydrate production from microalgae (Silva and Sforza, 2016; Silva et al., 2017). For *Scenedesmus obliquus*, their respective values were previously determined as 180 mg/L of N and 100 mg/L of P (Silva et al., 2017). Light intensity was equal to 650 µmol $m^{-2}$ $s^{-1}$ and resident time to 2.3 ± 0.3 days.

## *2.3 Analytical Procedures*

### 2.3.1 Growth analysis

For each experiment, the cellular concentration was monitored through the measurement of dry cell weight, optical density (OD) and cellular count.

***Dry cell weight***

Measuring the dry cell weight allows to know the amount of mass per unit volume.

First, a known volume of culture (V) is taken. To separate the aqueous bulk from the biomass, nitrocellulose filters (Whatman®) with pore size of 0.45 µm are used. These filters are first dried up to eliminate the absorbed humidity, then they are weighed to measure the tare (Initial filter weight) on an analytical balance (Atilon Acculab Sartorius Group®, sensibility of 10-4g).

The phase separation is achieved by suction of the liquid volume of culture through the filter, performed by a vacuum flask. After that the filter with the wet biomass, is kept in the oven for 1.5 h at 105°C to eliminate the intracellular water. Then, the final weight of the filter is measured.

The dry weight of the sample is then calculated in the following way:

$$Dry\ weight\ (\frac{g}{L}) = (final\ filter\ weight - initial\ filter\ weight)/sample\ volume \quad (2.1)$$

### *Cell count*

Cell concentration can be directly measured by counting the cells at the optical microscope.

The sample is put in the Bürker® chamber, a glass support containing 2 cells with a depth of 0.1 mm each. The cells are divided into 9 squares with 1 mm sides, which are separated by a triple line. Each of these bigger squares is divided into 16 smaller squares, delimited by a double line.

The measure requires to dilute the sample to have between 20 and 100 cells per bigger square (see Figure 2.2).

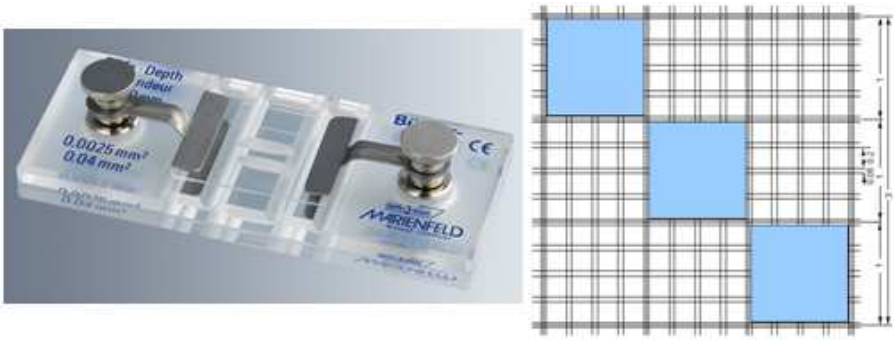


**Figure 2.3:** *Bürker chamber (on the left) and schematic representation (on the right).*

Cell concentration (N) is then calculated in cells/ml:

$$N = n * dil * 10^4 \text{ (cells/mL)} \quad (2.2)$$

where: “n” is the mean number of cells counted in the bigger square (usually only the big diagonal square is counted). “dil” is the sample dilution used. The factor 104 is due to the fact that each bigger square has a volume equal to 0.1 μL.

### *Optical Density (OD)*

This measurement was performed by using a spectrophotometer (Spectronic UV-500® UV-visible). At the wavelength of 750 nm there is a linear relationship for microalgae between absorbance and cell concentration, and it is valid in a range of absorbance between 0.1 and 1. If the sample is too concentrated, it must be diluted to be in the range. For the yeast grow measurement the wavelength of 600 nm was used instead.

Before the measurement, it was necessary to set the zero and removing the medium contribution to the absorption This is done by preparing two cuvettes with the culture

medium as blanks. Then, one of them is used as a reference to the sample during the OD measurement, while the sample is put in the others one.

The final value is given by:

$$OD = abs * dil \tag{2.3}$$

where “abs” is the absorbance (0 to 1) and “dil” is the sample dilution.

### *Growth rate*

From the cellular concentration the cellular growth rate was determined. This is an important parameter that let us know how faster the culture replication is. The growth rate is calculated as:

$$\mu = \frac{1}{[C]}\frac{\partial [C]}{\partial t} \tag{2.4}$$

where k is the growth rate, [C] is the cellular concentration and t is the time. Growth rate is calculated when there is excess of nutrient, i.e., in the exponential growth phase (see Figure 2.3).

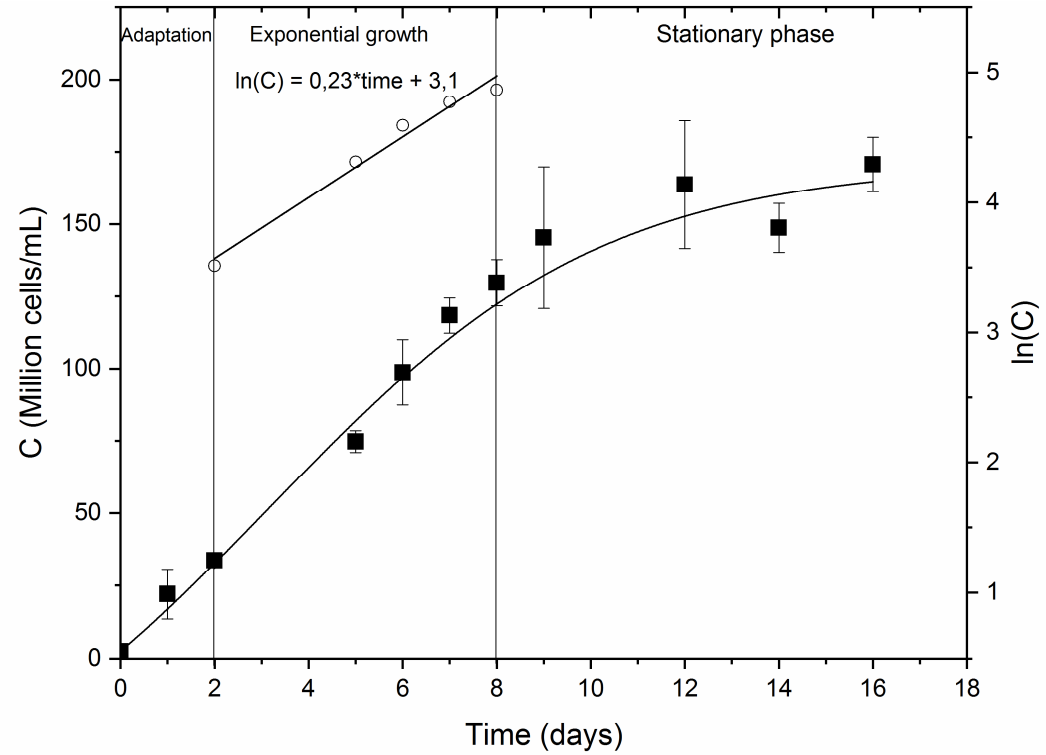


**Figure 2.4:** *Growth curve of Scenedesmus obliquus. (■) Cellular concentration – C (million cells/mL) and (○) ln(C). μ =0.23 $d^{-1}$.*

### 2.3.2 Total Sugars (Anthrone Method) and Reducing Sugars (DNS Method)

Microalgae's biomass total carbohydrate content was monitored with the Anthrone Method. The reactant composition is reported in Table 2.2. The reaction procedure is summarized as follow: 100 µL of sample react with 900 µL of Anthrone reactant in a hot water bath at 100°C for 10 mins. Absorbance measurement is then performed at 625 nm. (Trevelyan and Harrison, 1952). The calibration line (Figure 2.4) was determined by measuring standard solutions at known concentration of glucose. As a colorimetric reaction takes place, the reaction leads to a green/blue color of the sample (Figure 2.5).

**Table 2.2:** *Reactant composition of Anthrone reagent.*

| | |
|---|---|
| $H_2SO_4$ concentrated | 71% v/v |
| $H_2O$ | 29% v/v |
| Anthrone | 2 g/L |

A linear correlation between total carbohydrates and absorbance is given by:

$$[Total\ Carbohydrates]\ (g/L) = 0.2732 * ABS_{625} + 0.0033 \quad (2.5)$$

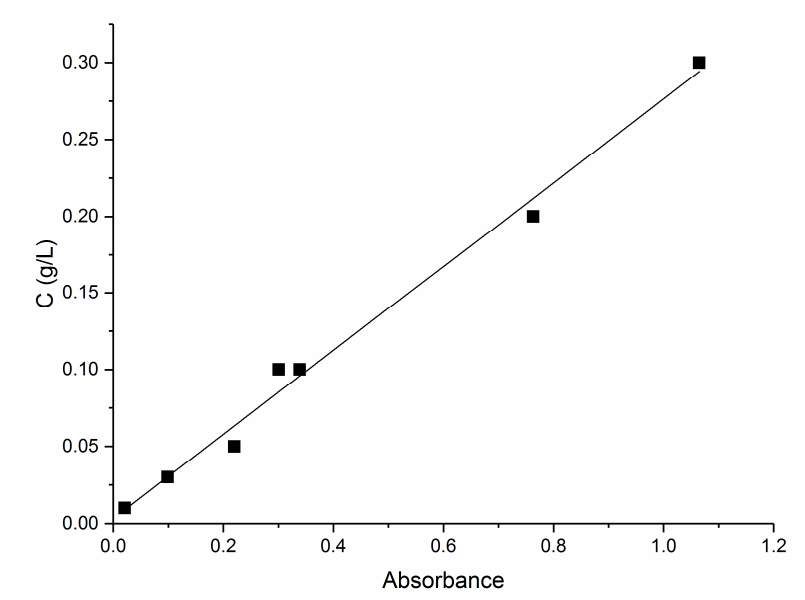


**Figure 2.4:** *Calibration line for Anthrone method. (Regression factor $R^2$=0.991)*

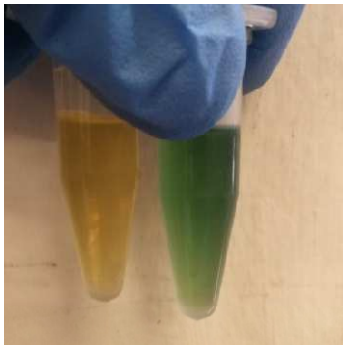

**Figure 2.5:** *Total carbohydrate determination with Anthrone method. On the right there is a sample with a carbohydrate content, on the left the zero sample.*

Reducing Sugars were determined with DNS Method. The reactant is DNS (3,5-Dinitrosalicylic acid) whose composition is reported in Table 2.3. The reaction is done with 500 µL of water, 250 µL of DNS and 250 µL of dluted sample (in order to obtain absorbance between 0 and 1) and takes place in a hot water bath at 100°C for 5mins, then 4 mL of water are added. Absorbance (ABS) measurement is then performed at 540 nm. (Miller, 1959). The calibration line (Figure 2.6) was determined by measuring standard solutions at known concentration of Glucose. The reaction is colorimetric like the previous one, the sample's color lead to red/orange (Figure 2.7).

**Table 2.3:** *Reducing sugars - DNS reagent.*

| | |
|---|---|
| DNS (3,5-Dinitrosalicylic acid) | 1 g |
| NaOH 2N | 20 mL |
| Potassium sodium tartrate | 30 g |
| Complete with $H_2O$ to 100 mL | |

The reducing sugars *[RS]* concentration in g/L are obtained from:

$$[RS](g/L) = 3.8315 * ABS_{540} + 0.2088 \quad (2.6)$$

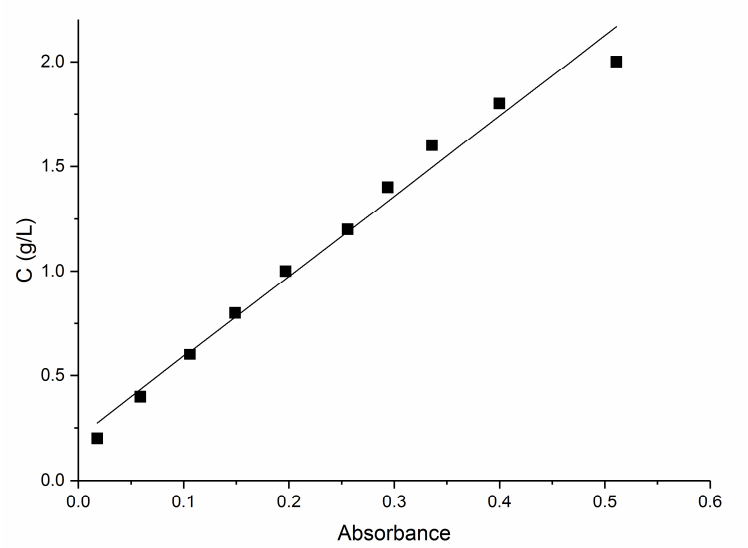


**Figure 2.6:** *Calibration line of DNS method for reducing sugars determination. (Regression factor $R^2$=0.9832)*

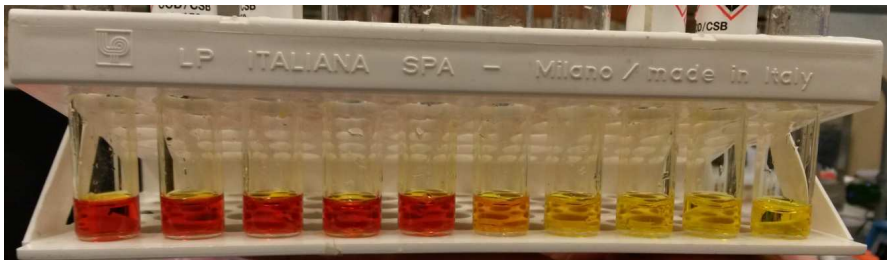


**Figure 2.7:** *Reducing sugars with DNS method. On the right there are samples with lower concentration of sugars (Yellow). On the right there are samples with high concentration of sugars (red - orange).*

### 2.3.3 Ethanol determination

Ethanol was by a mixture of sulphuric acid and potassium dichromate (Table 2.4) determined through a chemical method based on the oxidation of organic substances, such as alcohols. The methodology consisted in the distillation of 5 mL of sample diluted in 20 mL of water for the extraction of ethanol. The extract solution is stored in a falcon and summed with water for a final volume of 25 mL. After that 1 mL of sample is taken react with 1 mL of reactant.

**Table 2.4:** *Composition for 1L of reactant.*

| | |
|---|---|
| sulphuric acid (98 % p/p) | 325 mL |
| potassium dichromate | 33.68 g |

Absorbance measurement was then performed at 600 nm. The calibration line (Figure 2.8) was determined by measuring standard solutions at known concentration of ethanol. Reaction takes place in a hot water bath for 30 min, and is a colorimetric reaction where sample's color leads to brown (Figure 2.9).

The ethanol linear correlation is given by:

$$[Ethanol](g/L) = 53.063 * ABS_{600} - 0.3871 \quad (2.7)$$

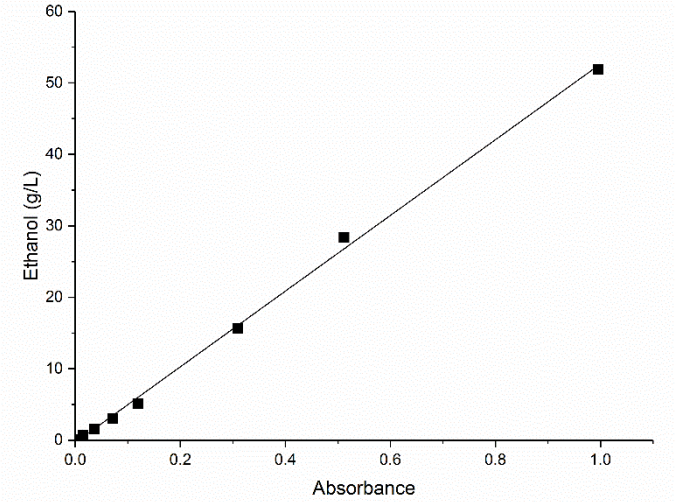


**Figure 2.8:** *Calibration curve of ethanol. (Regression factor $R^2$=0.9983)*

**Figure 2.9:** *Ethanol determination, on the left there is a sample without ethanol (zero), on the right there is a sample with a concentration of 20 g/L of ethanol.*

## *2.4 Biochemical characterization*

Biochemical characterization of microalgal biomass included the determination of moisture (AOAC official method 934.01), ash (AOAC official method 942.05), protein (AOAC official method 2001.11), lipid content (AOAC official method 2003.05), carbohydrates and monomers (HPLC) (AOAC, 2002).

## *2.5 Hydrolysis*

### 2.5.1 Acidic Hydrolysis

Acidic hydrolysis was performed with 5-10% of solids load (microalgal biomass), in autoclave (Autoclave vapour-lineeco VWR), using temperatures between 100-130 °C (P ~ 1 atm), and changing the concentration of catalyst (H2SO4 – 98% or HCl – 37% - Sigma ®, at 0, 1, 3 and 5% v v-1 respectively) and the reaction time (0-60 min).

### 2.5.2 Enzymatic Hydrolysis

Enzymatic hydrolysis was performed using citrate buffer 50 mM, pH 5.0 at 50 °C. The enzyme mix was composed by:

Viscozyme® L (Novozymes cellulases mixture with ≥ 100 FBGU/g – betaglucanase units);

AMG 300 L (amyglucosidase from Aspergillus niger with 260 U/mL);

Pectinex Ultra SP-L (pectinase from Aspergillus aculeans with ≥ 3,800 U/mL.

All of them were produced by Novozymes® and purchased at Sigma-Aldrich ®.

The amount of enzyme per gram of biomass was fixed, because the experiments must validate the effect of ultrasonication on extraction and saccharification of microalgal sugars.

The concentrations were:

Viscozyme L® – 20U/gbiomass;

AMG 300 L® – 100U/gbiomass;

PectineX Ultra SP-L® – 1000U/gbiomass.

All experimental conditions were based on published papers (Danquah, Harun, 2011; Asada et al., 2012; McMillan et al.,2013; Kim et al., 2014; Lee et al., 2015), any environmental conditions able to permit each enzyme to work with a sufficient activity to perform the hydrolysis adequately. Sodium azide was used at concentration of 0.02% (w/v) to prevent contamination.

### *2.5.2.1 Ultrasonication*

Ultrasonication was done by using an Ultrasonic generator (AA–WG1–800W – SN 154, Aktive Arc Sarl, Switzerland) with different amplitude/offset and time options. The parameters were set to 50% of amplitude and 25% of offset for 40 min, resulting in an energy consumption of 30 W. These parameter values were based on previous works (Asada et al., 2012; McMillan et al., 2013; Lee et al., 2015). To study the effect of ultrasonication a statistical analysis was done. The variable studied were the *pretreatment time*, the *biomass concentration* and the *sonication intensity* (*amplitude*). For these experiments *Scenedesmus obliquus* biomass was used.

Ultrasonication assays were carried out as a factorial experimental design $2^3$ with three central point, totalizing 11 experiments. The variables studied were time (min), intensity of sonication (amplitude/offset) and biomass concentration (g/L). The levels of the experimental design are summarized in Table 2.5.

**Table 2.5:** *Levels of the factorial experimental design*

| Variable | -1 | 0 | +1 |
|---|---|---|---|
| Time (min) | 5 | 15 | 25 |
| Amplitude/Offset (%)* | 50/-40 | 60/-10 | 70/25 |
| Biomass Concentration (g/L) | 10 | 55 | 100 |

All statistical analysis was performed by the software Statistica® for the factorial design analysis considering $p < 0.05$ (95% of significance), for the variables and their linear interactions.

The efficiency of the process was compared also with respect to the energy consumed per gram of biomass to verify if it is the intensity and/or the energy applied by ultrasonication that provides higher accessibility of biomass to the hydrolysis process.

#### *2.5.2.2 Enzymatic mix*

The experiments were performed with 100 g/L of microalgal biomass. For the following runs the enzymatic mix in the concentrations of 1x, 1/2x and 1/10x were used; where x = Viscozyme: 20 FPU cellulose/$g_{biomass}$, AMG: 100U/$g_{biomass,}$ Pectinex: 1000U/$g_{biomass}$. The saccharification extent (%) was measured by DNS method since enzymatic saccharification is very specific and must lead to reducing sugars at the end of hydrolysis process.

### 2.5.3 Process parameters

Hydrolysis experiments were validated by the solubilization of biomass, extracted sugars (total sugars) and hydrolyzed sugars (reducing sugars).

After the hydrolysis, the mass yield (MY) of the process was evaluated on a dry weight basis by gravimetry using cellulose acetate filters of 0.45 μm (Whatman®) at 105°C and 2 hours. Filters were pre-dried for 10 min at 105 °C in order to remove any moisture. The relation between solubilized biomass and mass yield is given by:

$$[Solubilized\ Biomass](\%) = \frac{Initial\ Biomass\ \left(\frac{g}{L}\right) - Mass\ yield\ \left(\frac{g}{L}\right)}{Initial\ biomass\ Load\ \left(\frac{g}{L}\right)} * 100 \qquad (2.8)$$

The amount of total extracted sugars (TS) was determined by the Anthrone method and reducing sugars (monomers, RS) using the DNS method. The % of sugars extracted/hydrolyzed were calculated by:

$$[Sugar](\%) = \frac{Sugar\ concentration\ in\ the\ liquor\ \left(\frac{g}{L}\right)}{Initial\ biomass\ load\ \left(\frac{g}{L}\right) * Carbohydrates\ content} * 100 \qquad (2.9)$$

where the carbohydrates content was that obtained with the Anthrone method, the initial biomass load was measured with dry weight initially and the sugar concentration was determined with Anthrone and DNS method.

## *2.5 Ethanolic fermentation*

Fermentation experiments were performed with two different yeasts: *Saccharomyces cerevisiae* (Cameo S.p.A.) and *Pichia stipitis* (ATCC 58785). Inoculums were stored in liquid and solid YPD medium. All the experiments were carried out at 30±1°C. Reducing sugars were measured by DNS method and cellular growth by dry weight and

cell count (§2.3.1). Ethanol was determined by chemical method (described in §2.3.3). Conversion factors were calculated by:

$$Y_{X/S} = \frac{\Delta X}{\Delta Sugars} \quad (2.10)$$

$$Y_{E/S} = \frac{\Delta Ethanol}{\Delta Sugars} \quad (2.11)$$

$$\mathrm{Y_{X/E}} = \frac{\Delta \mathrm{X}}{\Delta \mathrm{Ethanol}} \quad (2.12)$$

where: *ΔX* is the difference between the initial dry weight and the final dry weight, *ΔSugars* is the difference between the initial reducing sugars and the final reducing sugars, *ΔEthanol* is the difference between the initial concentration of ethanol and the final concentration of ethanol.

Process and fermentation (biochemical) yield (dimensionless) are evaluated as:

$$Process\ Yield\ (\%) = \frac{Ethanol\ produced}{0.511 * Initial\ Sugars} 100 \quad (2.13)$$

$$Biochemical\ Yield\ (\%) = \frac{Ethanol\ produced}{0.511 * \Delta Sugars} 100 \quad (2.14)$$

where 0.511 is the glucose-ethanol conversion factor according to the stoichiometry of Gay-Lussac, and *ΔSugars* is the difference between the initial reducing sugars and the final reducing sugars.

The ethanol productivity is determined as:

$$Productivity\ \left(\frac{g}{L\ h}\right) = \frac{\Delta Ethanol}{\Delta t} \quad (2.15)$$

where *ΔEthanol* is the difference between the initial concentration of ethanol and the final concentration of ethanol, and *Δt* is the time required to reach the maximum concentration value of ethanol.

Four sets of experiments were carried out:

- First, the efficiency of each strain (*S.cerevisiae* and *P. stipitis*) and inoculum concentration (0.1, 0.5, 2.5 and 12.5 g/L) were validated in terms of ethanol productivity; The strains were cultivated in YPD-20 (20 g/L of glucose, 20 g/L Peptone and 10 g/L of Yeast extract) and YPD-50 (50 g/L of glucose, 20 g/L Peptone and 10 g/L of Yeast extract).
- Then, the presence of xylose (20% of sugars present in the medium – generally the presence of pentose in microalgal biomass), and the best consortium combination (*Saccharomyces cerevisiae* and *Pichia stipitis* in different percentages) were validated. Using a consortium *Saccharomyces* + *Pichia* can increase the fermentation productivity since *Pichia* has the ability to ferment pentose (*Saccharomyces* not). The modified culture medium has the following composition:
    - YPD-20: (16 g/L of glucose, 4 g/L of xylose, 20 g/L Peptone and 10 g/L of Yeast extract)
    - YPD-50: (40 g/L of glucose, 10 g/L of xylose, 20 g/L Peptone and 10 g/L of Yeast extract).
- The third step evaluated the influence of salinity in the fermentation process. During the acidic hydrolysis after neutralization of the broth, salts are formed in a concentration range of 10-50 g/L which can influence significantly the productivity. The strains used were the same as in the previous point with process addition of a certain concentration of NaCl or $Na_2SO_4$.
- Finally, acidic and enzymatic hydrolysates were fermented with the best conditions of inoculum concentration and consortium as determined in the preliminary experiments, and a comparison between the standard medium and microalgal broth was made.

The sets of experiments considered are summarized in Figure 2.10.

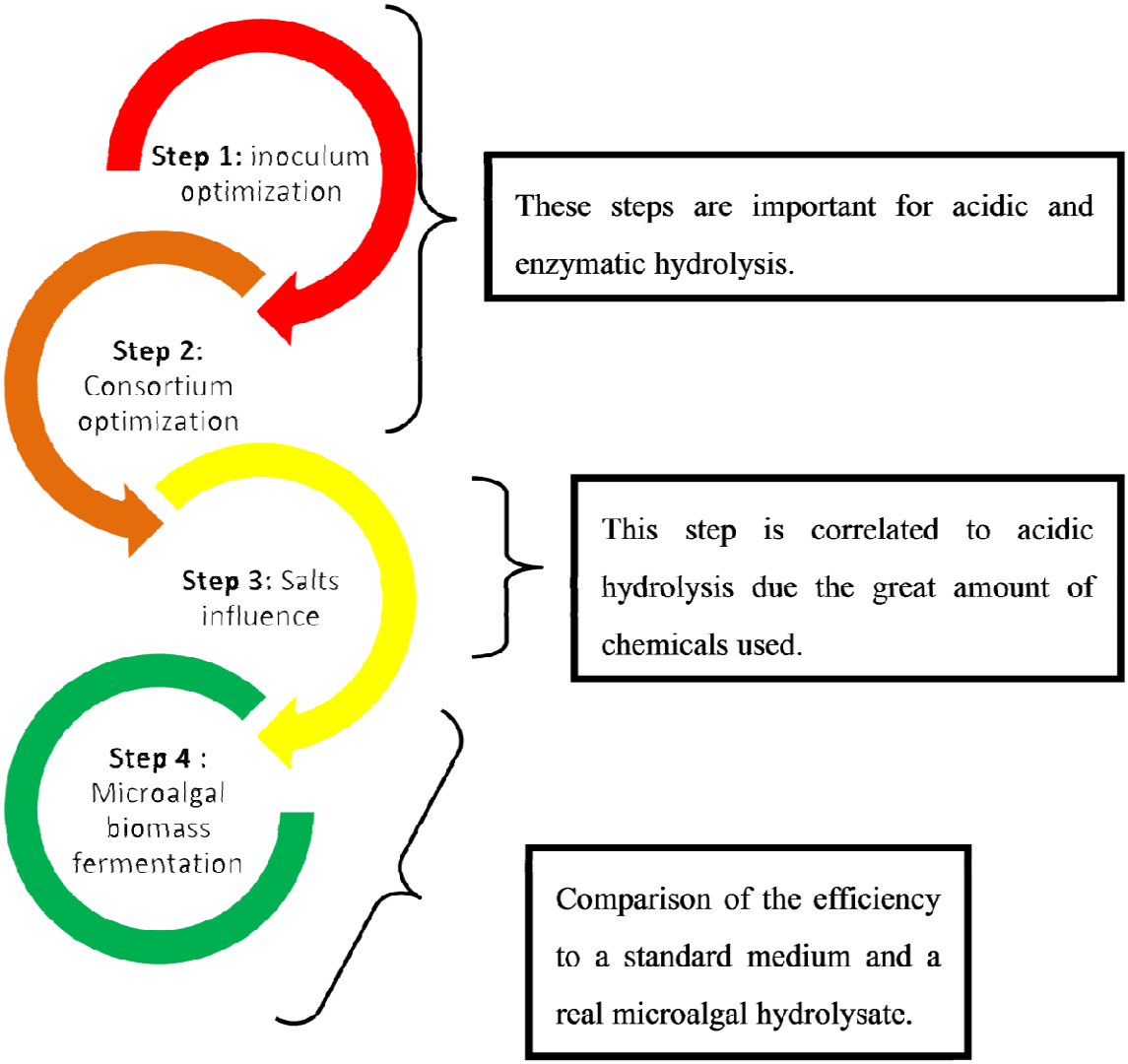


**Figure 2.10:** *Flowchart of the fermentation experiments.*

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# Chapter 3

# Microalgal hydrolysis

In this chapter besides the characteristics of the *Chlorella vulgaris* and *Scenedesmus obliquus* biomass (cultivation system and biochemical composition) used in the experiments, the experiments of hydrolysis (acidic and enzymatic) are described. During the acidic hydrolysis reaction time, temperature, acid and biomass concentration were varied and optimized. On the other hand, to perform enzymatic hydrolysis efficiently, a pretreatment step for *Scenedesmus obliquus* was required and ultrasonication was the method chosen.

## *3.1 Algal biomass*

*Scenedesmus obliquus* and *Chlorella vulgaris* are very common and studied as feedstock for the bioethanol production (Silva and Bertucco, 2016). As aforementioned, *Chlorella vulgaris* biomass powder was purchased from *Neoalgae®*. *Scenedemus obliquus* was cultivated in a continuous stirred flat panel photobioreactor (PBR). The cultivation conditions were reported in the chapter 2, §2.2.2.

In Figures 3.1 and 3.2 it is shown the dry cell weight and carbohydrate content measured during the biomass cultivation at steady-state which was operated for more than a hundred days, guarantying physiological and biochemical stability of the biomass produced. Growth and productivity parameters are reported in Table 3.1.

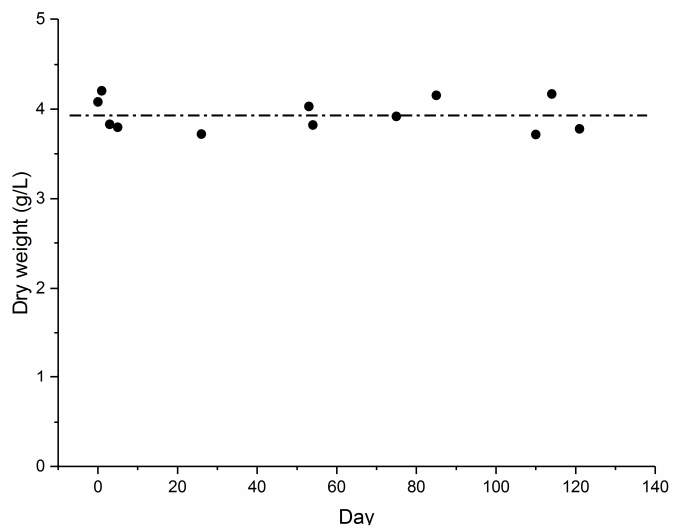


**Figure 3.1:** *Continuous biomass cultivation of Scenedesmus obliquus, dry weight, along with cultivation time.*

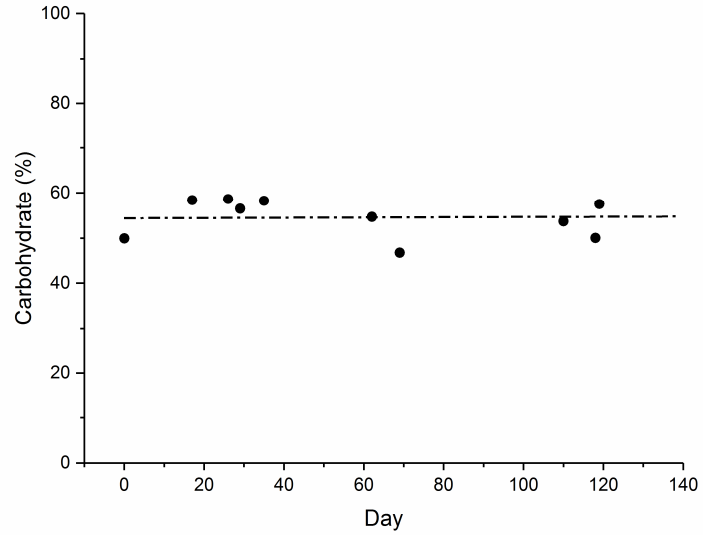


**Figure 3.2:** *Carbohydrate content during the continuous cultivation of Scenedesmus obliquus biomass.*

The results obtained in the biomass cultivation are summarized in Table 3.1. The carbohydrate and biomass productivity are higher than the values found in literature because the culture medium was optimized in order to provide nitrogen limitation/starvation in combination with light intensity and residence time (Silva et al., 2017). The operating conditions are reported in §2.2.2.

**Table 3.1:** *Steady state conditions of continuous biomass cultivation (Scenedesmus obliquus).*

| | |
|---|---|
| Dry weight (g/L) | 3.94 ± 0.18 |
| Optical Density (750 nm) | 19.3 ± 2.21 |
| Carbohydrate content (%) | 54.4 ± 4.1 |
| Biomass productivity (g $L^{-1}day^{-1}$) | 1.70 ± 0.11 |
| Carbohydrate productivity (g $L^{-1}day^{-1}$) | 0.93 ± 0.09 |

In literature, *Scenedesmus obliquus CNW-N* cultivated in glass vessel at 28°C, pH 6.2, 2.5% $CO_2$-air at 210-230 µmol $m^{-2}$ $s^{-1}$ and under nitrogen starvation achieved a biomass and carbohydrate productivity of 0.5 g $L^{-1}$ $day^{-1}$ and 0.26 g $L^{-1}$ $day^{-1}$, respectively (52% carbohydrate content) (Ho et al., 2013). Wang et al., (2013) with *Scenedesmus dimorphus* cultivated in a photobioreactor at 38°C, with 2% $CO_2$ air supply and outdoors conditions obtained a biomass productivity of 0.6 g $L^{-1}day^{-1}$ and a carbohydrate productivity of 0.24 g $L^{-1}day^{-1}$ (40% of carbohydrate content).

## *3.2 Biochemical characterization*

The biochemical composition of *Chlorella vulgaris* and *Scenedesmus obliquus* are shown in Table 3.2. These species presented quite a different composition in terms of protein, carbohydrate and lipid content. It is important to remember that microalgae display a biochemical plasticity able to change their composition according to the nutritional and environmental factors, and with a relatively fast dynamic. Specifically, for these microalgae, nitrogen availability, residence time and light intensity allow to accumulate more or less carbohydrate in *Scenedesmus obliquus* (Silva et al., 2017), while *Chlorella vulgaris* probably was cultivated in excess of nutrients, which shifted the biochemical synthesis towards the production of proteins (Chen et al., 2013).

**Table3.2:** *Macrocomponents and sugars profile in Chlorella vulgaris and Scenedesmus obliquus*

| **Components** | ***Chlorella vulgaris*** | ***Scenedesmus obliquus*** |
|---|---|---|
| | **% of dry cell weight** | |
| Protein | 49.5 ±0.29 | 33.63 ±4.04 |
| Lipid | 6.3±0.15 | 25.34±0.64 |
| Carbohydrates | 23.0±2.0 | 45.9±4.5 |
| **Glucose*** | **70.15** | **79.78** |
| **Xylose*** | **10.65** | **16.14** |
| **Arabinose*** | **10.91** | **-** |
| **Rhamnose*** | **5.73** | **-** |
| Other* | 2.56 | 4.08 |
| Ash | 7.18±0.01 | 6.83±0.01 |
| Moisture | 5.41±0.05 | 7.05±0.01 |

*% respect to the carbohydrate content.

Carbohydrates in microalgae are present as cell wall components (generally cellulose and soluble hemicellulose) and plastids (mainly in the form of starch) (Chen et al., 2013). Glucose was found as the predominant monosaccharide in the biomass and accounts for more than 70% of total sugars, together with xylose (10.65% for *Chlorella*, 16.14% for *Scenedesmus*), arabinose (10.91% for *Chlorella*) and rhamnose (5.73%, *Chlorella*).

Similar compositions were found in the literature for these species. *Chlorella sp KR1* (36.1% of carbohydrate content where 82% glucose, 18% pentose) (Lee et al, 2015) and *Chlorella sorokiniana* (18.2% of carbohydrate content where 70.8% glucose, 21.5% pentose and 7.7% other) (Hernandez et al, 2015). *Scenedesmus obliquus* with 50% of carbohydrate content exhibited a monosaccharide profile composed by 80% glucose and 20% xylose (Ho et al., 2013). *Scenedesmus almeriensis* with 14.5% of carbohydrate content exhibited a sugars composition of 52.2% of glucose, 33.4% of xylose, 15.4 others (Hernandez et al., 2015).

## *3.3 Acid hydrolysis*

During this part of the study, the efficiency of the process was based on biomass' carbohydrate extraction and its conversion into reducing sugars (monomers). These experiments were designed to study the influence of different acid concentration (HCl and $H_2SO_4$, 0-8% v/v), reaction times (0-60 min) and biomass concentration (50-100 g/L) at 120 °C.

### 3.3.1 Acid hydrolysis of *Chlorella vulgaris*

Several experiments already showed 120°C is the best temperature (Silva and Bertucco, 2017). As shown in Figure 3.3, higher acid concentration and reaction time provided a higher sugars recovery. Sugar extracted (total Sugars) were saccharified into reducing sugars with a high efficiency.

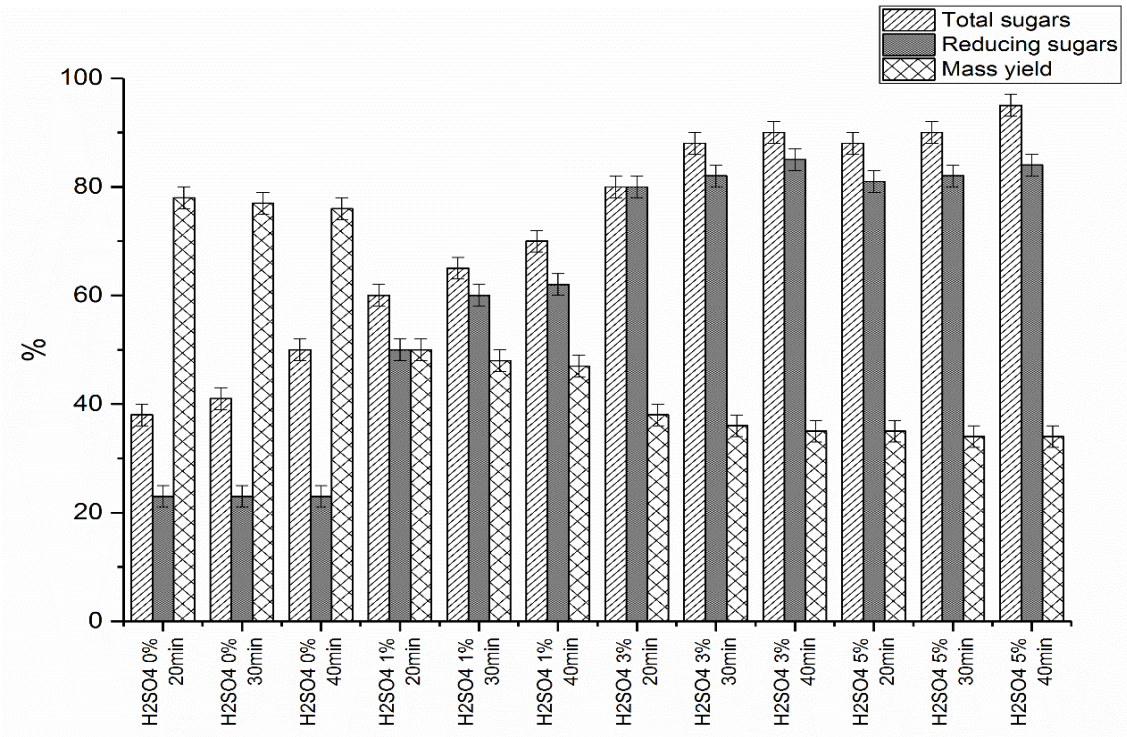


**Figure 3.3:** *Acid hydrolysis for Chlorella performed at T =120°C and 100 g/L of biomass load.*

At 120 °C, more than 90% of reducing sugars were obtained when 3% $H_2SO_4$ and 30 min of reaction time were used. This was considered as the best condition in the range of the experiments performed. In fact, according to literature, 90% of biomass has been hydrolyzed when 50 g/L of *Tribonema* sp. was submitted at 121 °C and 3% of sulfuric acid for 30 min (Wang et al., 2014). *Chlorella vulgaris* JSC-6 (120 g/L), at 121 °C and 3% of sulfuric acid for 20 min reached a saccharification yield of 90% (Wang et al., 2016). *Dunaliella tertiolecta* LB999 (50 g/L) at 121 °C and 3.73% of sulfuric acid for 15 min achieved a hydrolysis yield of 44.31%, but here time was probably limiting (Lee et al., 2013).

As seen in Figure 3.4, $H_2SO_4$ gives better carbohydrate conversion into reducing sugars than HCl, confirming as best condition 3% of acid concentration and 30 min of reaction time at 120°C. This check was necessary since Lee et al. (2015) found better performance for *Chlorella* using HCl 0.5N with 15 minutes of treatment at 121°C and 50 g/L of biomass concentration, reaching 98% in comparison with sulphuric acid (0.5N, 121°C, 15min) which yielded 80% as maximum sugars recovery. With the conditions used in Figure 3.4 was proved that using sulphuric acid 3%v/v is better because it needed a lower concentration of the reactant, i.e. a possible economical advantage.

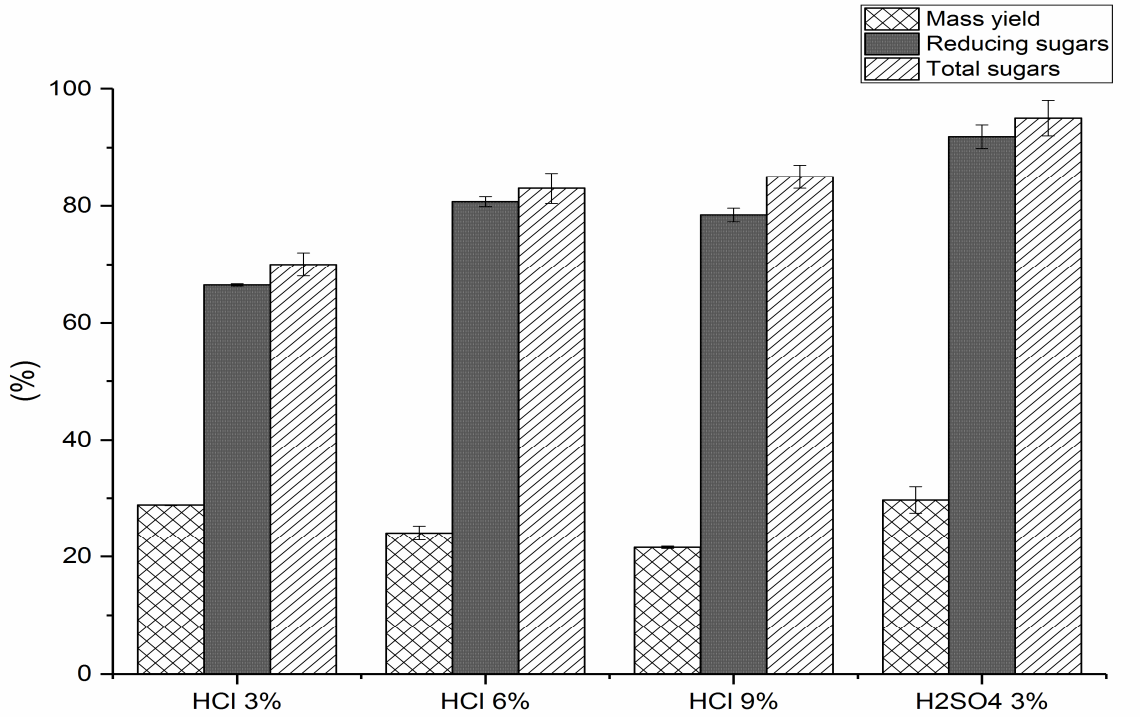


**Figure 3.4:** *Comparison between the performance with HCl and $H_2SO_4$. Chlorella vulgaris (100 g/L) with 30 min at 120 °C.*

### 3.3.2 Acid hydrolysis of *Scenedesmus Obliquus*

These experiments were done with 50 g/L of biomass at 120°C and 3% of acid concentration. Initially, reaction time was 30 min, according to the best condition found for *Chlorella vulgaris*. But this was not sufficiently for *Scenedesmus obliquus* which reached 90% of sugars extraction and 64% of saccharification yield (reducing sugars – monomer) as visualized in Figure 3.5.

The results of Figure 3.5 are in agreement with literature. *Scenedesmus obliquus* hydrolyzed (50 g/L) at 120 °C and $H_2SO_4$ 5% for 30 min, provided 90% of saccharification yield (Miranda et al., 2012). Ashokkumar et al. (2013) hydrolyzed 20 g/L of *Scenedesmus bijugatus* biomass at 130 °C and 2% of acid obtained around 85% of saccharification. Ho et al., (2013) with *Scenedesmus obliquus* CNW-N, 10–40 g/L, at 121 °C and with different concentrations of $H_2SO_4$ (1.5–2%) for 20 min reached 95% of sugars recovery.

With $H_2SO_4$ 5% v/v it was visualized a significant sugars degradation from 87 to 70%, exhibiting sugars degradation. The sugars thermal degradation from sugar cane broth was evidenced by Nolasco and Massaguer (2006). It was demonstrated that sugars concentration decreases when increasing the temperature and the time of treatment

(ranges: 110-140°C and 0-500 hours). A similar study was accomplished by Woo et al., (2015) for glucose and maltose (range studied was 110 to 150°C). In addition, it was observed that under low pH (2.89 – 3.76) the thermal degradation increased due the formation of organic acids. Accordingly, the sugars degradation process is highly catalyzed by acid concentration. When 3% of acid was used, final pH was less than 1, extremely acid, and it can justify the fast reduction of sugars concentration in solution.

Sugars degradation was already evidenced for *Tribonema sp*. with $H_2SO_4$ 3%v/v, biomass concentration of 70 g/L at 121°C in the treatment time range of 15-90 minutes. It was found a maximum saccharification of 85% at 35 minutes, after that the sugars yield decreased fast enough: at 60 minutes the saccharification was 65%, at 75 minutes 40% (Wang et al., 2014).

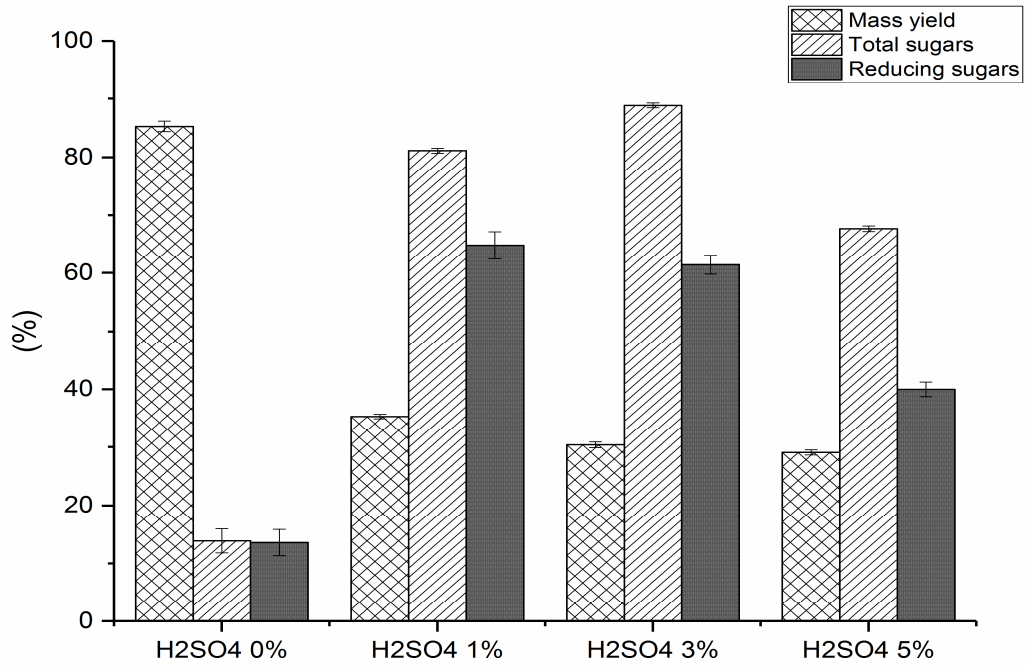


**Figure 3.5:** *Acid hydrolysis for Scenedesmus obliquus (50 g/L), influence of different $H_2SO_4$ concentration.*

Miranda et al., (2012) with *Scenedesmus obliquus* studied the effects of $H_2SO_4$ concentration (0.05-10N) performed at 120°C for 30 minutes. The maximum yield obtained was 30 % (g $eq_{glu}/g_{dry\ biomass}$) with $H_2SO_4$ 2N. For acid concentration > 2N the sugars yield decreased faster. Additionally, Ho et al., (2013) performed a study for *Scenedesmus obliquus* where the $H_2SO_4$ concentration from 0.5% to 3% (10 g/L of biomass, 120°C and 20 minutes) was changed and it was found an optimal acid concentration of 2% achieving almost 100% of sugars recovery, being equal to when

3% of acid concentration was used. In conclusion, comparing the results of the two different microalgae, *Scenedesmus obliquus* is more sensitive to thermal degradation and acid concentration than *Chlorella vulgaris*.

As a slight increase of acid concentration caused degradation of the sugars, it was decided to study the effect of the reaction time with $H_2SO_4$ varying between 0-45min at 120°C and 3% of acid (Figure 3.6). Increasing the reaction time (35 and 45 min) a linear degradation process of the sugars was noted, showing high sensitivity; as well, none advantages were obtained.

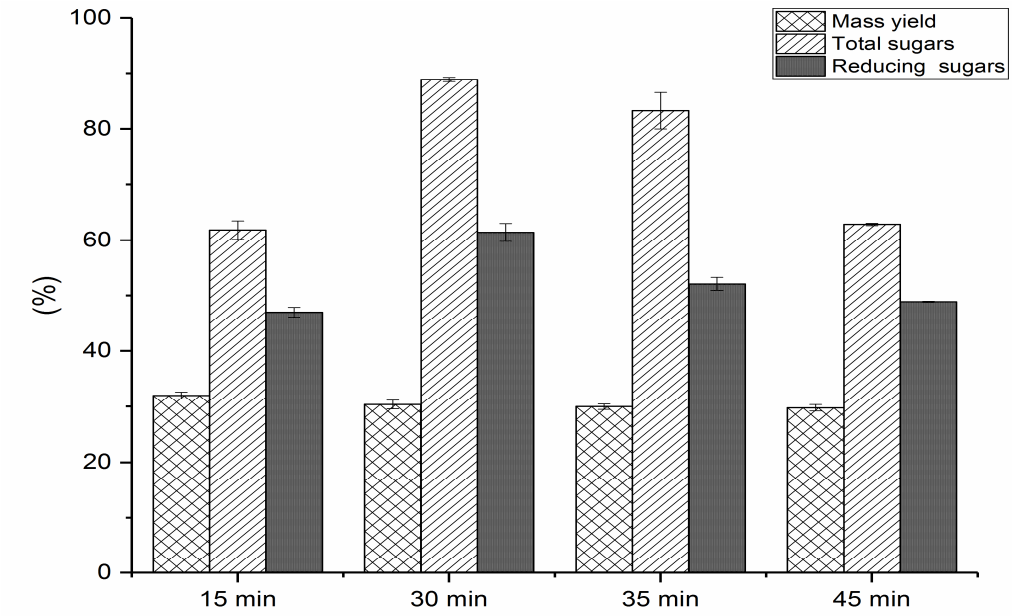


**Figure 3.6:** *Acid hydrolysis of Scenedesmus obliquus (50 g/L) with $H_2SO_4$ 3%v/v at 120°C*

Biomass concentration was set to 50 g/L because viscosity problems occurred with 100 g/L, differently of *Chlorella vulgaris*. A decreasing of the saccharification yield is already demonstrated in the literature when biomass concentration is high and increases significantly the viscosity (Ho et al., 2013). Maximizing biomass concentration is important from an industrial point of view.

## *3.4 Enzymatic hydrolysis*

Enzymatic hydrolysis is an alternative process for carbohydrate extraction and transformation into reducing sugars. Biological proteins with high specificity named enzymes are used. Enzymatic treatment, besides of the high cost of enzymes, provide a

more specific process at middle temperature and pressure (lower heating costs) and decrease the possibility for degradation phenomena to occur.

To apply this last method a pretreatment is required to improve the accessibility of these carbohydrates to enzymatic attack. Several methods for algal cell disruption have been discussed in the literature: ultrasonication, bead beating, microwave, osmotic shock (NaCl) and autoclaving (at 121 °C) and the results are different (Miranda et al., 2012; Jeon et al., 2013; McMillan et al., 2013; Kurokawa et al., 2016; Wang et al., 2016).

### 3.4.1 Enzymatic hydrolysis for *Chlorella vulgaris*

In this case *Chlorella vulgaris* biomass was purchased as dried and milled, thus no pretreatment was required, i.e., enzymes were able to react directly with the biomass because the cells were already broken during the dry treatment.

The experiments were carried out with 100 g/L of microalgal biomass. The enzymatic mix (x), where used in the concentrations of 1x, 0.5x and 0.1x in order to study the effect of enzymes, as show in Figure 3.7. The x means the enzyme mix: Viscozyme: 20 FPU cellulose/$g_{biomass}$, AMG: 100U/$g_{biomass}$, Pectinex: 1000U/$g_{biomass}$.

The enzymatic hydrolysis with enzyme concentration 1x reached the maximum value of 92% of saccharification in 4 hours. With 0.5x a saccharification of 60% after 10 hours was obtained. For 0.1x only 25% was saccharified. This behavior depends on the polysaccharides type present in the biomass. Each enzyme is specialized to saccharify specific polysaccharides. This was evident in the paper published by Kim et al., (2014) where different enzymes (Cellulase, Pectinase, Xylanase, β-glucosidale, Amylase, Chitinase, Lysozyme and Sulfatase) were tested for the enzymatic hydrolysis of *Chlorella vulgaris* (KMMCC-9; UTEX 26) in this case pectinase was the only one to reach a saccharification yield of 78%, the other enzymes were less than 20%. A study investigated the hydrolysis of *Chlorella pyrenoidosa* using Cellulase with a biomass concentration of 20 g/L at 50°C and a saccharification yield of 60% was obtained after 24 hours (Fu et al, 2010). The maximum values found in these works were lower than those of our study.

Interesting was the study performed by Shokrkar et al., (2017) where a microalgal biomass mix with β-glucosidase/cellulose, α-amylase and amyglucosidase was hydrolysate. The three enzymes were added separately in the same hydrolysate one by one and at different times to optimize the pH and temperature for each enzyme. A

synergy of these enzymes was observed and almost 100% of sugars recovery was reached, indicating that an enzymatic mix could be more efficient than the application of simple enzymes, confirming own idea.

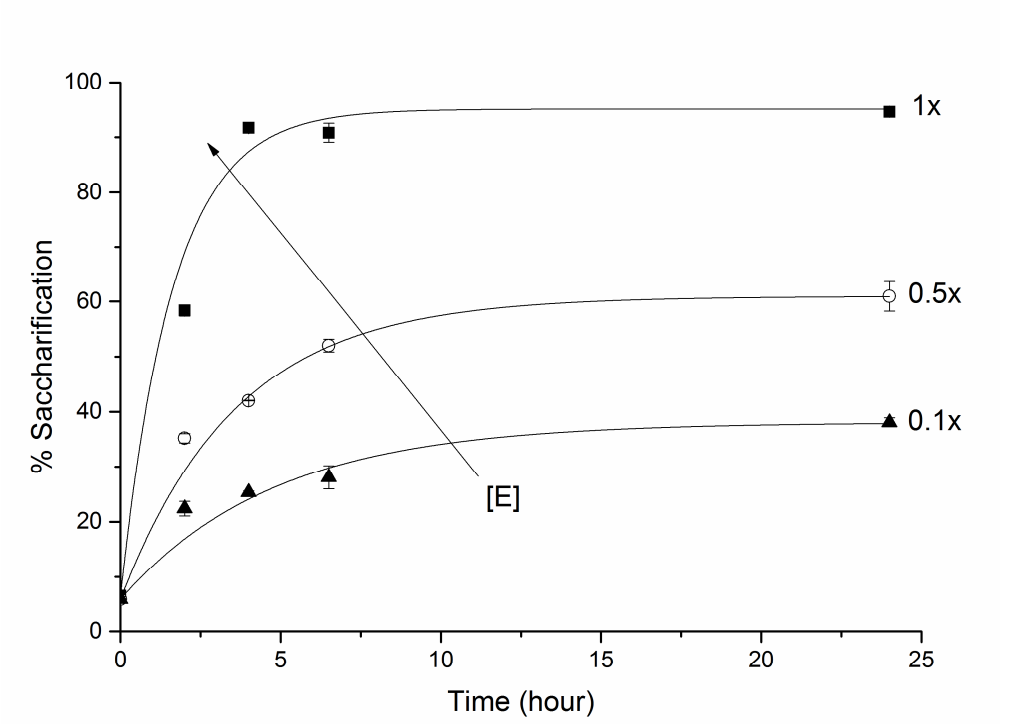


**Figure 3.7:** *Enzimatic hydrolysis of Chlorella vulgaris: effect of enzyme mix concentration on the Saccharification.* (x = Viscozyme: 20 FPU cellulose/$g_{biomass}$, AMG: 100U/$g_{biomass}$, Pectinex: 1000U/$g_{biomass}$) (see §2.5.2).

## 3.4.2 Biomass pretreatment

Ultrasonication was chosen as a pretreatment because it has the advantages of being able to disrupt the cells at relatively low temperatures (lower than microwave and autoclaving), faster extraction, it is suitable for all cell types and does not require beads or chemicals thus keeping production costs low (Jeon et al., 2013), (Byreddy et al., 2015).

A preliminary experiment was run with *Scenedesmus obliquus* to confirm the advantages of ultrasonication with respect to a control condition. The negative control condition was the microalgal biomass without any treatment and the positive control (exploded cells) utilized biomass suspension after autoclaving at 121°C for 20 min (§2.5.2.1). Biomass concentration in all experiments was 10 g/L and optical microscopic visualization was verified before and after the pretreatments with a magnification of 75x

(Figure 3.8). It can be seen in Figure 3.8A the microalgae without pretreatment, in B after ultrasonication, in C the microalgae pretreated with autoclave and in D the biomass after the enzymatic hydrolysis.

In Figure 3.9**Figure 3.**, preliminary results with and without pretreatment are show. It is concluded that sonication improved at least 30% of saccharification yield in comparison with the negative and positive controls (without pretreatment and autoclaving pretreatment) which reached around 70%, while with sonication practically all the carbohydrate content was hydrolyzed in monosaccharides.

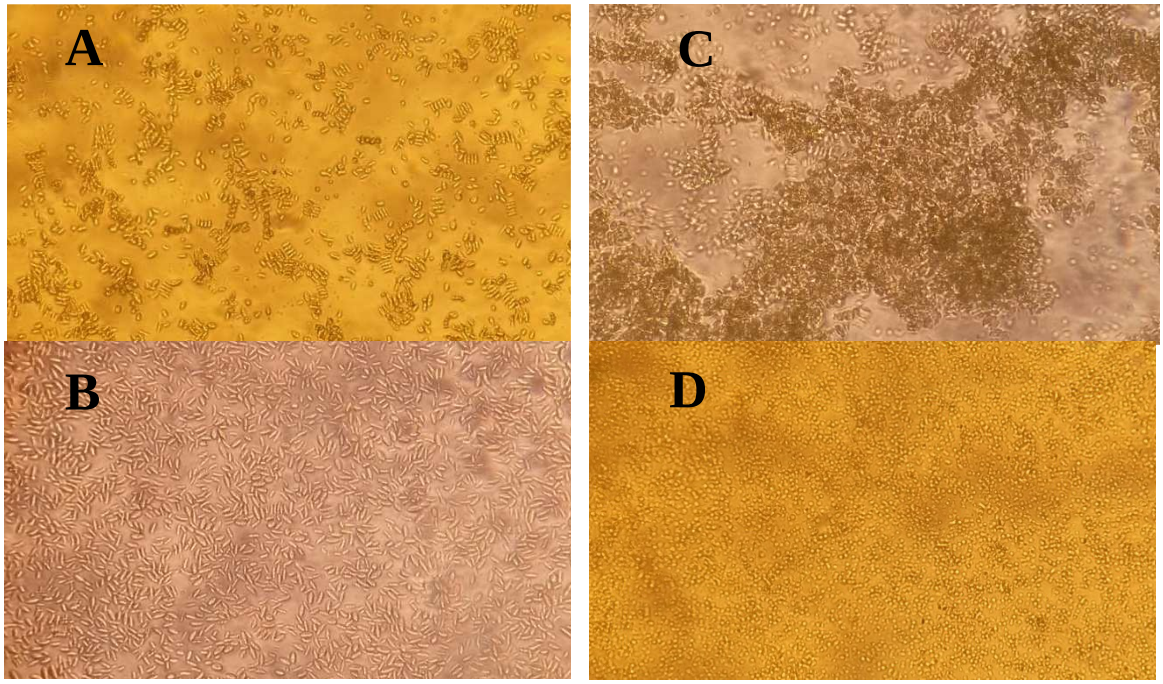


**Figure 3.8:** *Optical visualization after the pretreatments. A) - Control (without treatment), B) Sonication and C) + Control (Autoclave), D) Biomass after sonication and 24h of enzymatic hydrolysis. 10 g/L of biomass concentration and optical magnification of 75x.*

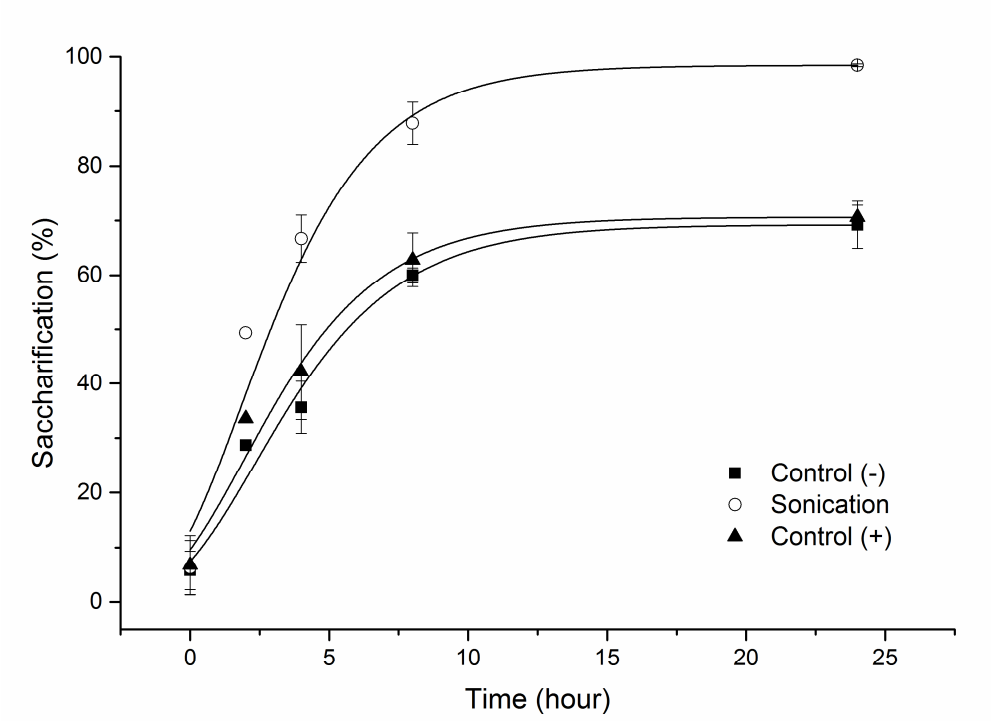


**Figure 3.9:** *Saccharification of the preliminary experiments. Control (-) - without treatment; Sonication – 40% amplitude, 40 kHz for 40 min and Control (+) – autoclaving at 121 °C for 20 min.*

This result is very interesting, because (see Figure 3.9), autoclaving microalgal biomass promotes the completely cell explosion/de-structuration, but does not improve enzyme accessibility, as probably diffusion effects were limited by biomass aggregation (Figure 3.8C). In contrast, some literature mentions that heating methods are more effective to cell disruption and suggest it as the best are to separate biomass fractions and promote enzymatic hydrolysis. We think this is not true (McMillan et al., 2013). On the other hand, after sonication an apparently not significant cell volume reduction is possible (Figure 3.8B), but with a good volume dispersion (homogenization). Sonication promotes fissures and cracks on algal cell surface and consequently enzyme accessibility (Jeon et al., 2013), and a reduction of cell volume may occur or way not (Kurokawa et al., 2016). In Figure 3.8D, an 'apparent' cell reduction is observed after enzymatic hydrolysis, according to what already observed for *C. homosphaera* (Rodrigues et al., 2015).

### 3.4.3 Study of ultrasonication pretreatment

To study the effect of ultrasonication a statistical analysis was done. The variable studied were the *pretreatment time*, the *biomass concentration* and the *sonication intensity* (*amplitude*) (§2.5.2.1).

As a results enzymatic hydrolysis was performed very well in some experiments achieving more than 90% of sugars recovery as monomers. The highest values were those with higher sonication intensity, higher pretreatment time and lower biomass concentration (5 and 6), and achieved near to 95% of saccharification, reaching almost 90% in 4 hours of hydrolysis (Table 3.3).

**Table 3.3:** *Saccharification results of all experiments and hydrolysis time*

| Assay | Time (min) | Sonication parameter Amplitude/Offset (%) | Biomass Concentration (g/L) | Saccharification (%) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Time (h) | | | | |
| | | | | 0 | 2 | 4 | 8 | 24 |
| 1 | 5 | 50/-40 | 10 | 13.00±1.41 | 32.18±1.19 | 36.02±2.01 | 62.26±4.16 | 79.08±5.06 |
| 2 | 25 | 50/-40 | 10 | 10.50±0.71 | 37.54±1.19 | 40.70±0.74 | 82.24±6.24 | 83.71±0.89 |
| 3 | 5 | 50/-40 | 100 | 10.97±0.14 | 66.33±2.74 | 67.85±1.95 | 70.00±4.05 | 79.09±1.35 |
| 4 | 25 | 50/-40 | 100 | 9.00±1.41 | 65.83±0.08 | 77.87±0.38 | 83.06±0.97 | 90.90±0.08 |
| 5 | 5 | 70/25 | 10 | 11.00±1.41 | 81.23±6.69 | 86.66±2.34 | 92.58±4.46 | 92.89±5.51 |
| 6 | 25 | 70/25 | 10 | 10.50±0.71 | 88.50±2.12 | 89.62±9.29 | 93.04±0.74 | 96.38±1.96 |
| 7 | 5 | 70/25 | 100 | 9.89±1.09 | 65.31±7.20 | 68.65±2.02 | 74.21±2.40 | 79.67±9.52 |
| 8 | 25 | 70/25 | 100 | 12.65±0.04 | 67.82±4.46 | 81.26±0.53 | 85.29±0.23 | 87.67±8.54 |
| 9 | 15 | 60/-10 | 55 | 8.83±0.45 | 74.72±4.61 | 71.55±0.40 | 74.02±5.89 | 88.48±7.48 |
| 10 | 15 | 60/-10 | 55 | 9.19±0.05 | 70.32±2.94 | 73.06±1.87 | 75.17±2.14 | 88.38±5.48 |
| 11 | 15 | 60/-10 | 55 | 8.57±0.19 | 77.65±0.74 | 75.12±2.01 | 74.15±6.42 | 86.78±5.35 |

The data were analyzed with a Pareto chart where the influence of the three variables studied on the saccharification (biomass concentration, time of pretreatment and amplitude) were reported. From the statistic plan was determined the magnitude of each variable on the process. If some of them overcame the value of p=0.5 it means that these variables have a major influence on the process compared to the others.

In fact, the Pareto charts represented in Figure 3.10 for each hydrolysis time considered, demonstrate that at the beginning (0 h) no influence of the variables in the pretreatment was observed, i.e., the sugars concentration starts with approximately the same value. This is important, because the temperature in some experiments had a maximum value of 40°C while in other is reached 90°C. Further, all experiments were influenced positively by sonication intensity (amplitude) and negatively due to the linear interaction of sonication intensity and biomass concentration (2L by 3 L), i.e., higher sonication intensity promoted more enzyme accessibility and, consequently, hydrolysis, and lower biomass concentration. However, with respect to the biomass concentration,

from the industrial point of view is not profitable to maintain process with 10 g/L of biomass. Another detail is the energy consumption of the process and how much the hydrolysis yield is influenced by the energy used in the pretreatment process.

Additionally, an interesting information is given by the experiments with 4 and 8 hours of hydrolysis is that the pretreatment time had a positive influence, i.e., if a faster hydrolysis time is required, higher pretreatment time can be used.

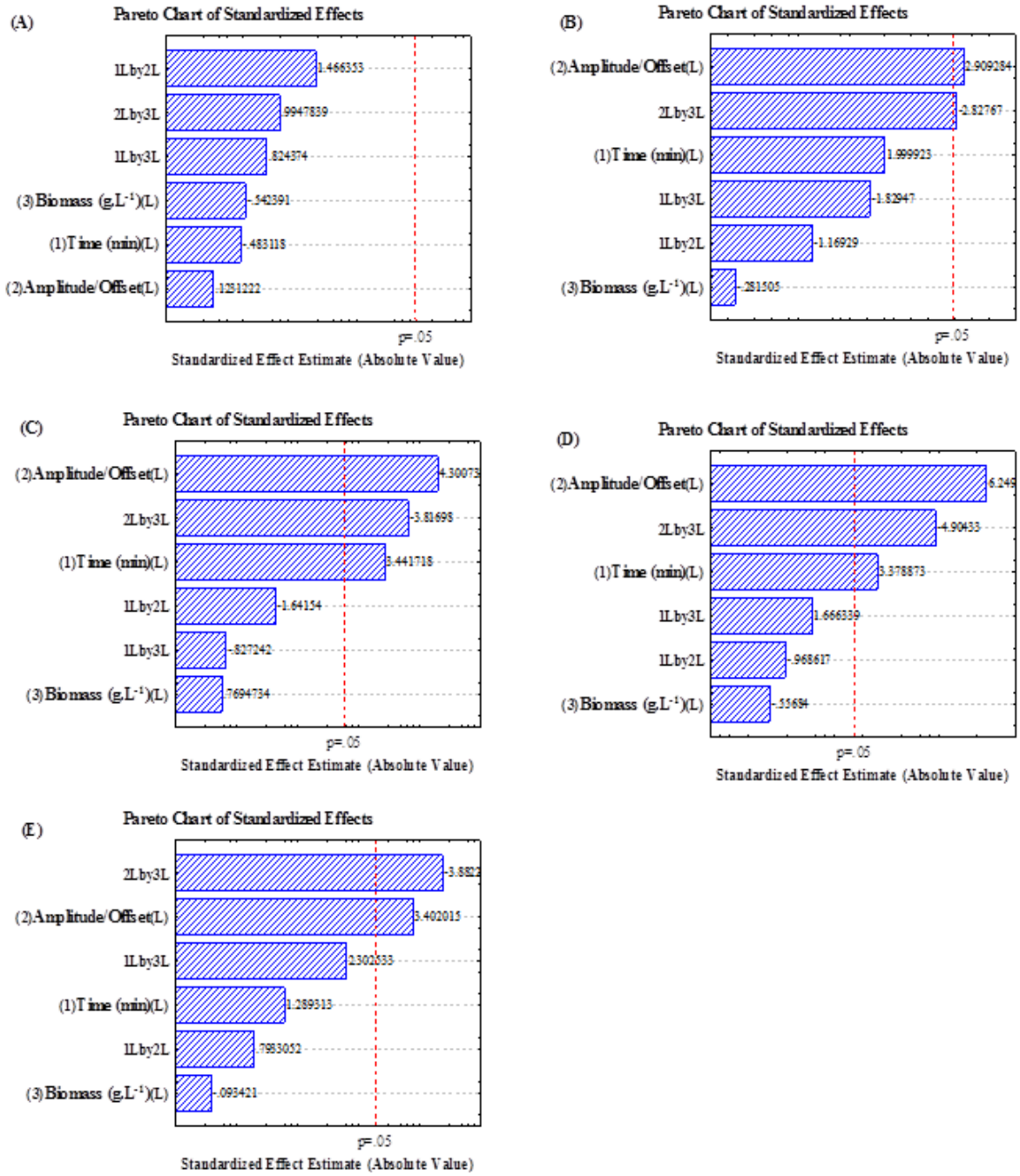


**Figure 3.10:** *Effect of the variables on enzymatic yield for each hydrolysis time. A) 0 hours, B) 2 hours, C) 4 hours, D) 8 hours and E) 24 hours*

At our best knowledge Sonication intensity study (amplitude) applied to algal pretreatment for sugars hydrolysis is not in the literature so then. However, some information is available almost sonication frequency sensitivity to disrupt algal cells: for *Chaetoceros gracilis*, *Chaetoceros calcitrans* and *Nannochloropsis sp.*, using frequencies between 0.02-4.3 MHz, it was demonstrated that values of 2.2-4.3 MHz are efficient in cell reduction (%) (Kurokawa et al., 2016). In addition, *Scendesmus dimorphus* and *Nannochloropsis oculata* using 20 kHz and 3.2 MHz (low and high frequency) to evaluate chlorophyll and lipid fluorescence and consequently extraction and no differences was found in lipid recovery, but the combination of high and low frequencies decrease the pretreatment time (Wang et al., 2014).

Wang and collaborators also verified that the pretreatment time influenced significantly the lipid extraction, proportionally between 1-5 min, reaching value from 50 to 100% of lipid extraction. In the dark fermentation of ethanol, volatile fatty acids (VFA) and hydrogen, the pretreatment time a key-point to promote bioaccessibility/bioavailability of microalgal biomass (*Scenedesmus obliquus* YSW15), reducing the cell surface hydrophobicity and increasing ethanol and VFA production (Jeon et al., 2013). Thus, the additional positive influence of the pretreatment time was expected and confirmed. The regression coefficients are summarized in Equations 1-4, and their surface graph are in Figure 3.11, showing more specifically the visual representation of the variables effect on the saccharification yield.

$$SY\,(\%) = -92.01 + 2.39.\,Amp - 0.0185.\,Amp.\,C_{biom} \quad (2\ h) \tag{3.1}$$

$R^2 = 0.8632$

$$SY\,(\%) = -92.34 + 3.095.\,Time + 2.473.\,Amp - 0.0184.\,Amp.\,C_{biom} \quad (4\ h) \tag{3.2}$$

$R^2 = 0.9243$

$$SY\,(\%) = -22.27 + 0.856.\,Time + 0.164.\,Amp - 0.013.\,Amp.\,C_{biom} \quad (8\ h) \tag{3.3}$$

$R^2 = 0.9515$

$$SY\,(\%) = 31.12 + 0.96.\,Amp - 0.012.\,Amp.\,C_{biom} \quad (24\ h) \tag{3.4}$$

$R^2 = 0.8954$

where: SY – Saccharification yield (%), Time – pretreatment time (min), Amp – Amplitude/Offset (%) and Cbiomass – biomass concentration (g $L^{-1}$).

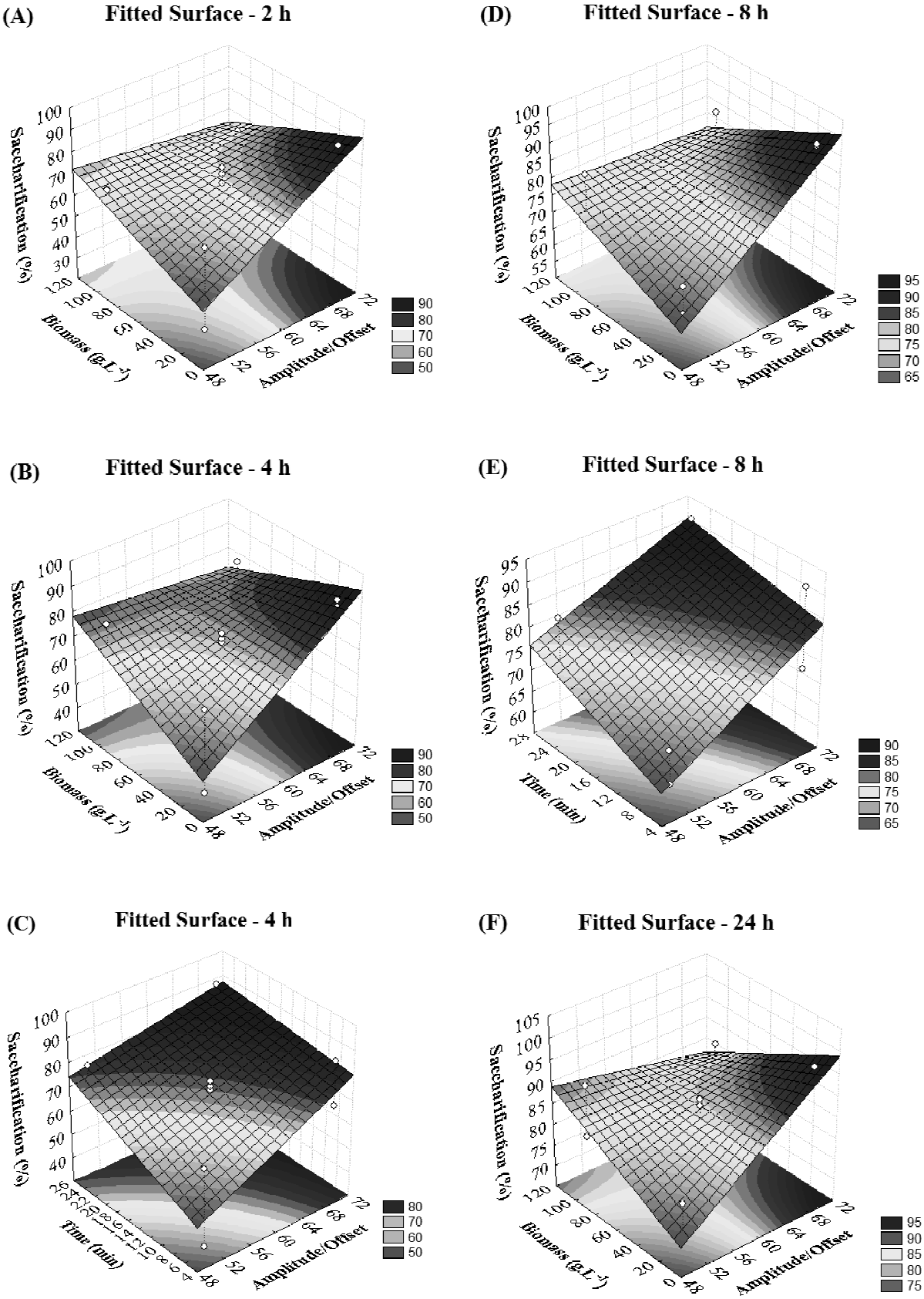


**Figure 3.11:** *Surface graphs of the models obtained by the experimental design*

### 3.4.4 Energy analysis

The energy analysis of the process is important, actually it is a bottleneck, towards an industrial application, thus, optimizing the energy required to provide an efficient saccharification is a must. As seen in Table 3.4Table 3., the ratio energy/biomass changed a lot in the different experiments. Although runs number 5 and 6 reached around 95% of hydrolysis yield, a considerable amount of energy was consumed which makes this choice quite unfeasible. On the other hand, the run number 4 (Table 3.4) achieved 90% of hydrolysis and 83% after 8 hours but using between 30-100 times less energy, i.e. 2.4 kJ/$g_{biomass}$ – MJ/$kg_{biomass}$.

Literature values of energy consumption for microalgal pretreatment using sonication are: 70.6 MJ/kg for *Scenedesmus obliquus* YSW15 (Jeon et al., 2013); 1200 MJ/kg for *Thraustochytrid* strains (Byrreddy et al., 2015) and 44-132 kJ/kg (extrapolated) for *Nannochloropsis oculata*, but with this reduced value demonstrated much lower efficiency was formed in comparison with microwave oven, blender and laser (McMillan et al., 2013). Thus, the value obtained (2.4 MJ/$kg_{biomass}$) represents a promising value, mainly considering the energy content of microalgal biomass, which to generally between 20-22 MJ/kg.

**Table 3.4:** *Energy consumption during sonication pretreatment*

| Assay | Power (W) | Total Energy Consumption (kJ) | Energy/Volume (kJ $mL^{-1}$) | Energy/Biomass (kJ $g_{biomass}^{-1}$) | Final Temperature (°C) | Maximum Yield of Sugars (%)** |
|---|---|---|---|---|---|---|
| 1 | 2-4 | 0.98 | 0.049 | 4.90 | 30 | 79.08 |
| 2 | 2-4 | 6.02 | 0.301 | 30.10 | 42 | 83.71 |
| 3 | 2-4 | 0.92 | 0.046 | 0.46 | 32.1 | 79.09 |
| 4* | 2-4 | 4.79 | 0.240 | 2.40 | 37.9 | 90.90 |
| 5 | 34-55 | 13.00 | 0.650 | 65.00 | 90.2 | 92.90 |
| 6 | 29-58 | 43.20 | 2.160 | 216.00 | 89.5 | 96.38 |
| 7 | 36-50 | 11.30 | 0.565 | 5.65 | 90.2 | 79.67 |
| 8 | 37-59 | 46.90 | 2.345 | 23.45 | 93.2 | 87.67 |
| 9 | 13-21 | 12.60 | 0.630 | 11.45 | 85 | 88.48 |
| 10 | 9-20 | 11.60 | 0.580 | 10.54 | 83.9 | 88.38 |
| 11 | 9-19 | 11.60 | 0.580 | 10.54 | 83.2 | 86.78 |

*25 min, 50% of amplitude and 100 g/L of biomass. **24 h hydrolysis yield.

In Figure 3.12 the energy consumed is plotted versus the % of saccharification for each hydrolysis. It is concluded that the hydrolysis efficiency does not depend on the energy input, but the intensity of amplitude mainly.

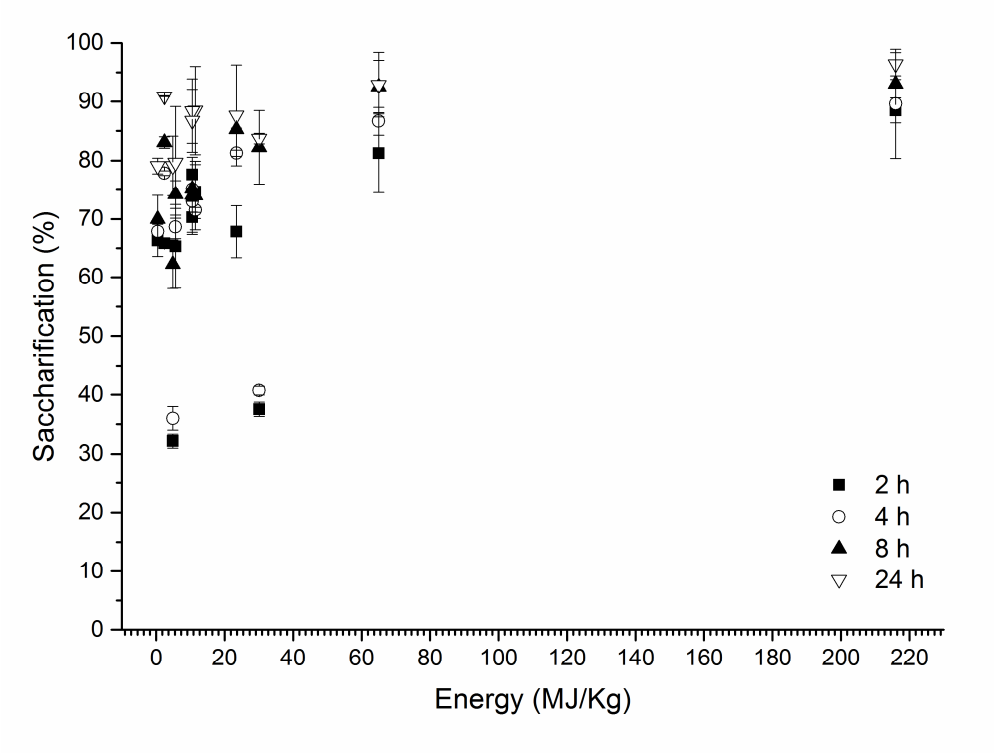


**Figure 3.12:** *Energy consumption versus % saccharification of the experiments*

Ultrasound is a mechanical acoustic wave with the frequency range from roughly 10 kHz to 20 MHz. It imparts high energy to reaction medium by cavitation and secondary effects (both physical and chemical). When ultrasonication is used to break cells, it is important to determine the energy intensity (experimentally represented by a combination of amplitude-power generated and time) and population of active cavitation to promote the specific reactivity with cells and increase the accessibility to the substrate (Kurokawa et al., 2016). The validation of the process was based on enzymatic hydrolysis of microalgal biomass and, in fact, the intensity of sonication showed to be important, but not directly linked to the consumed energy in the pretreatment process. This indicates that physical and chemical changes can be achieved by ultrasound up to a level which is sufficient to perform enzymatic hydrolysis.

### 3.4.5 Enzymatic hydrolysis of Scenedesmus obliquus

*Scenedesmus obliquus* biomass was also contacted with to different concentration of enzymatic mix (as made with *Chlorella vulgaris*), i.e., 1x, 0.5x and 0.1x. The biomass was pretreated with the conditions of experiment number 4 of Table 3.3.

From Figure 3.13, it was noticed that the maximum saccharification level is reached faster by increasing the enzymes concentration. In the concentration 1x the maximum saccharification was 95% which was reached after 8 hours of enzymatic hydrolysis. With the other concentrations (0.5x and 0.1x), the saccharification yield achieved 90 and 75% after 24 hours, respectively. Thus, it is a two-variable system saccharification time and enzyme concentration. It is important to mention that the enzymatic mix was based on literature data but the influence of the specific enzyme and its concentration has still to be optimized.

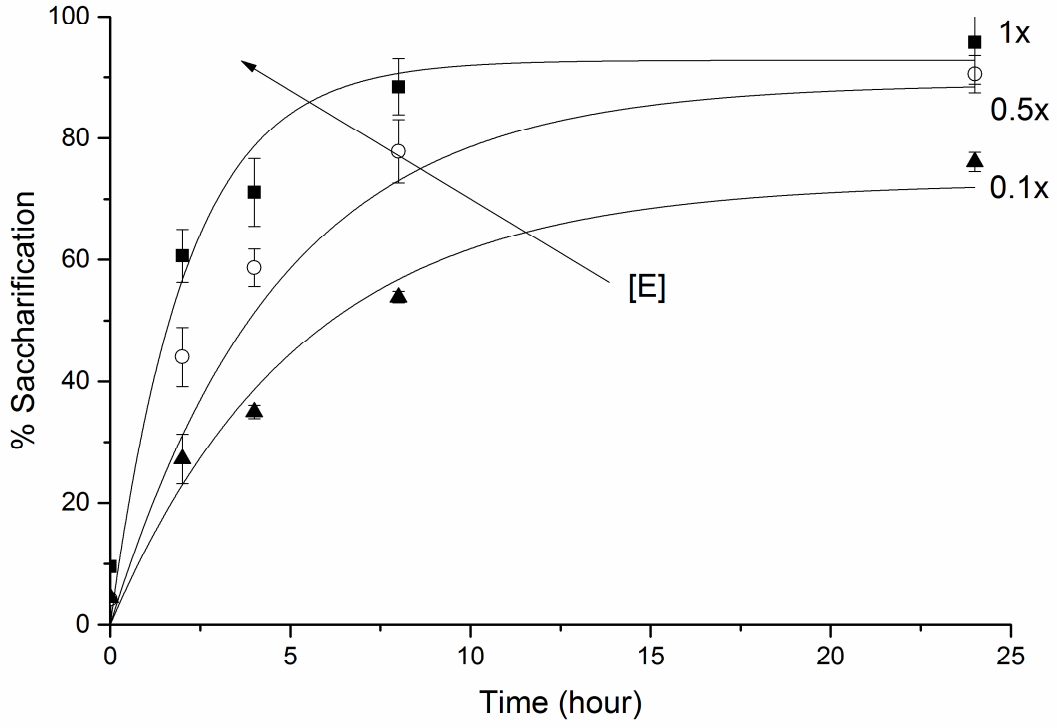


**Figure 3.13:** *Effect of enzymes concentration for Scenedesmus obliquus biomass - 100g/L.* (x = Viscozyme: 20 FPU cellulose/$g_{biomass}$, AMG: 100U/$g_{biomass,}$ Pectinex: 1000U/$g_{biomass}$) (see §2.5.2).

Pancha et al. (2016) studied the effect of enzymes concentration on *Scenedesmus obliquus CCNM 1077* de-oiled (45.23% carbohydrate content). The enzymes used were Amylase, Vyscozyme-L and Cellulase, in the concentration range 5-50 U/$g_{biomass}$. The

best condition after 24 hours was the ones with Vyscozyme-L 50 $U/g_{biomass}$, with a saccharification efficiency of 45% still insufficient for industrial purposes. In our study because more than 90% of sugars recovery was obtained, and it was also demonstrated that by increasing the enzymes concentration, the saccharification yield increase the time needed to reach the maximum saccharification decrease.

### 3.4.6 Acidic vs Enzymatic hydrolysis, final remarks

In literature acidic and enzymatic hydrolysis are the processes most studied in view of bioethanol production process application. According to our results acid hydrolysis showed high efficiency in terms of sugars recovery, mainly for *Chlorella vulgaris* where degradation processes were not detected. On the other hand, this process for *Scenedesmus obliquus* must be carefully used, because the sensitivity of biomass to degradation of sugars was highly evidenced.

The enzymatic hydrolysis process from microalgal biomass ensured lower yield in comparison with acidic treatment and required longer hydrolysis time, (if a biomass concentration value acceptable in view of industrial is considered): for example, it was found 27.4 instead of 93.3% for *Chlorella sp.* (50 $g_{dry\ biomass}$/L and 3 h) (Lee et al., 2015) 64 instead of 96% for *Chlorella vulgaris* FSP-E (40 $g_{dry\ biomass}$/L and 2-3 days) (Ho et al., 2013) and 62.8 instead of 100% for C*hlorococcum* (10-15 $g_{dry\ biomass}$/L and 12 h) (Harun and Danquah, 2011). These results could be emphasize effected by two problems: ineffective pretreatment and/or specificity/concentration of the enzymes. In our study we have demonstrated that enzymatic hydrolyses can compete in terms of sugar recovery with acidic treatment since more than 90% of saccharification was achieved in both treatments. In the Table 3.5, positive and negative points of these two processes are summarized.

**Table 3.5.** *Summarizing of the characteristics of acidic and enzymatic hydrolysis.*

| **Acid hydrolysis** | |
|---|---|
| **Advantages** | **Disadvantages** |
| • Short process time (less than 45 minutes);<br>• High hydrolyzed sugars recovery;<br>• No biomass pretreatment is required. | • High amount of chemicals required;<br>• pH neutralization inhibits the fermentations process;<br>• Efficiency decrease by increasing the microalgal biomass concentration. |

| **Enzymatic hydrolysis** | |
|---|---|
| **Advantages** | **Disadvantages** |
| • Middle conditions of temperature and pressure;<br>• Specificity (other components can be recovered too, almost intact). | • Process time larger than acid hydrolysis (6 to 24 hours);<br>• Organic buffer is necessary;<br>• Enzyme are sensible, additional process control are required to avoid degradation / inhibition;<br>• Pretreatment required. |

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# Chapter 4

# Fermentation of microalgal hydrolysate

In this chapter a systematic study of ethanolic fermentation of acidic and enzymatic microalgal hydrolysate with the goal to understand this process and to improve its productivity. The study was divided in four steps, each one addressing a specific aspect (see Figure 2.9). In a first step, the initial inoculum concentration was optimized to reach a feasible ethanol productivity with two different yeast strains: *Saccharomyces cerevisiae* and *Pichia stipitis*. In the second step the behavior of these yeasts consortium was studied using a medium composed by glucose and xylose, to simulate the hydrolysate which is almost 80% of glucose and 20% of xylose. In the third step the influence of salinity was investigated (to make an effective comparison with saline influence during acidic hydrolysis which requires neutralization to achieve the right fermentation pH). Finally, in the fourth step the ethanolic fermentation of the hydrolyzed microalgal biomass was performed.

## *4.1 Step 1: Inoculum optimization*

The experiments were focused on the research of the best initial inoculum that provide a viable ethanol productivity (defined as the ethanol produced per unit volume, in g $L^{-1}$ $h^{-1}$). Yeasts, *Saccharomyces cerevisiae* and *Pichia stipites* were studied separately. The fermentations were performed in YPD-20 (20 g/L of glucose, 20 g/L Peptone and 10 g/L of Yeast extract) and YPD-50 (50 g/L of glucose, 20 g/L Peptone and 10 g/L of Yeast extract). For each strain four different fermentations were carried out with different initial inoculums: 0.1 g/L, 0.5 g/L, 2.5 g/L and 12.5 g/L.

### 4.1.1 Ethanolic fermentation with *Saccharomyces cerevisiae*

In Figure 4.1 the growth curves for each experiment are reported. The fermentations reached the stationary phase faster when the initial inoculums were higher. For YPD-20 and YPD-50 stationary phase was achieved in less than 6 hours for inoculums with

initial concentration larger than 2.5 g/L. A lag phase of at least 2 hours was required to adapt the microorganisms in the medium conditions.

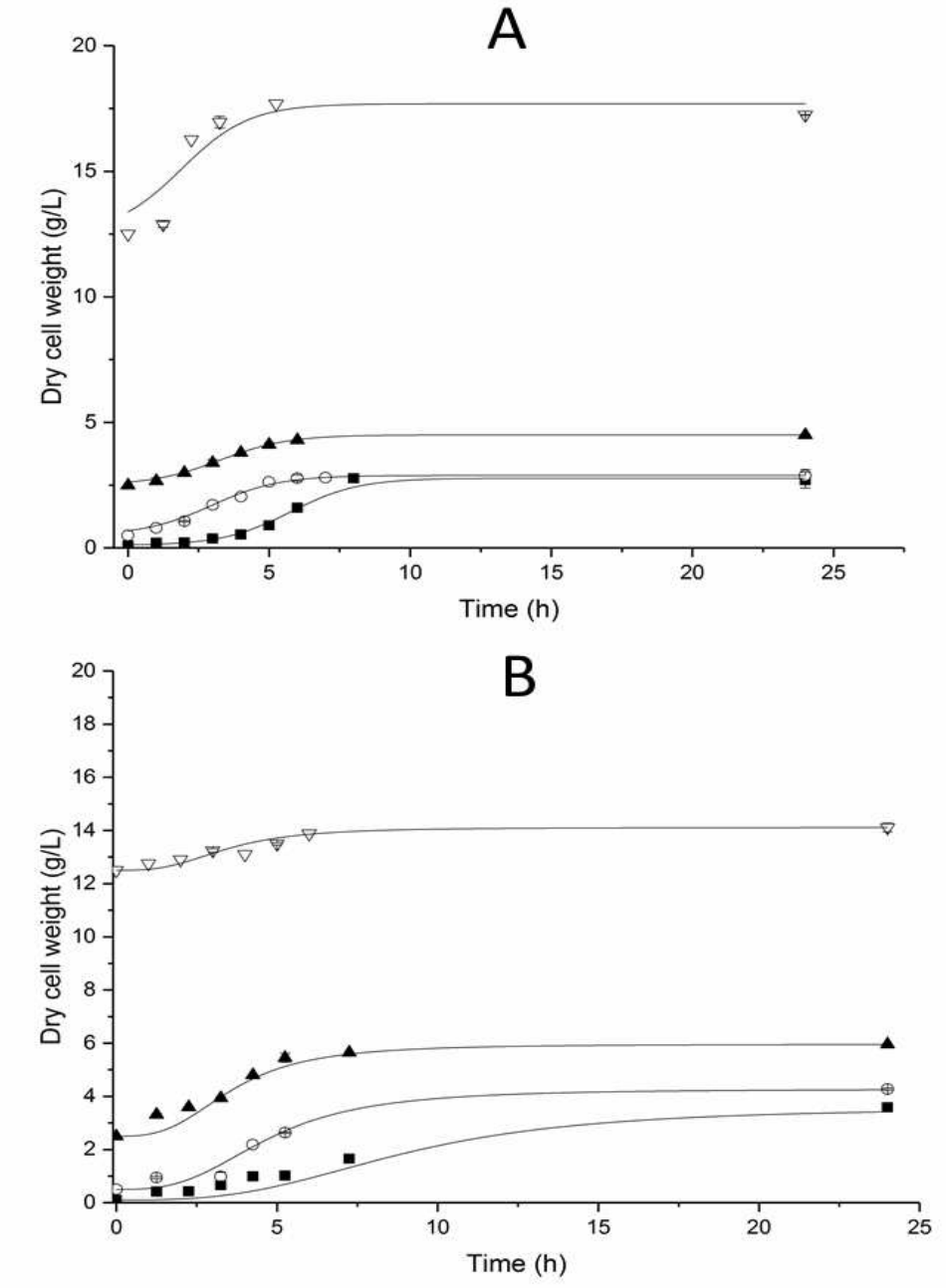


**Figure 4.1:** *Growth curve for the inoculum: (■) 0.1g/L, (○) 0.5g/L, (▲)2.5g/L and (▽) 12.5g/L for YPD-20 (A) and YPD-50 (B)*

In Figure 4.2 growth rates of the experiments are displayed. When increasing the initial inoculums of the yeast's growth rate decreased. The values ranged from 0.57 $h^{-1}$ with 0.1 g/L of inoculum to 0.1 $h^{-1}$ with 12.5 g/L. This behavior is compatible with the

sugars consumption reported in Figure 4.3 where the limitation of sugars determined the limitation of growth. The larger the initial inoculum, the faster the sugars consumption as less nutrient was available for cell growth. In particular the inoculum with 12.5 g/L consumed 90% of total sugars in the medium within 2 hours.

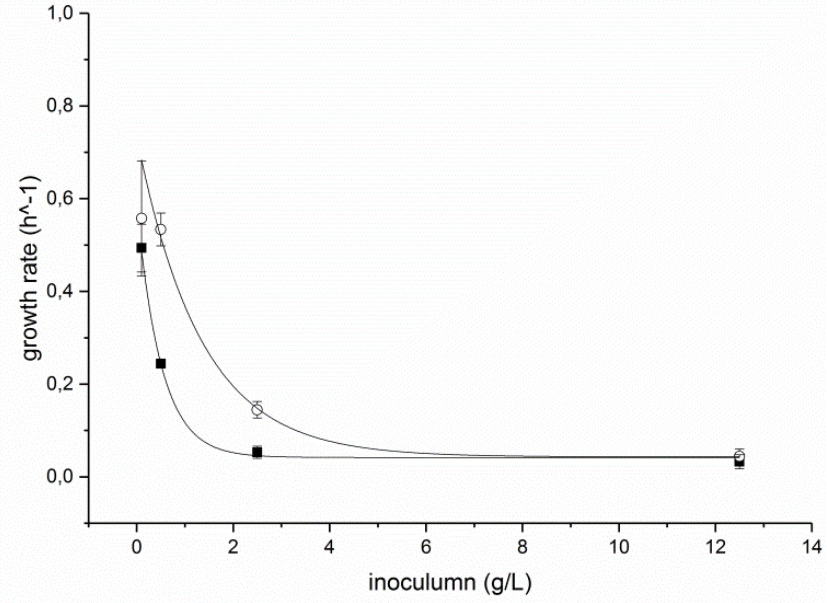


**Figure 4.2:** *Growth rate (■) YPD-20, (○) YPD-50*

Final sugars concentration was practically the same in all experiments, between 2-5 g/L. This emphasizes that the capabilities of the cells to metabolize changed the consumption time, i.e., the higher the inoculum concentration, the faster the sugar consumption (Figure 4.3).

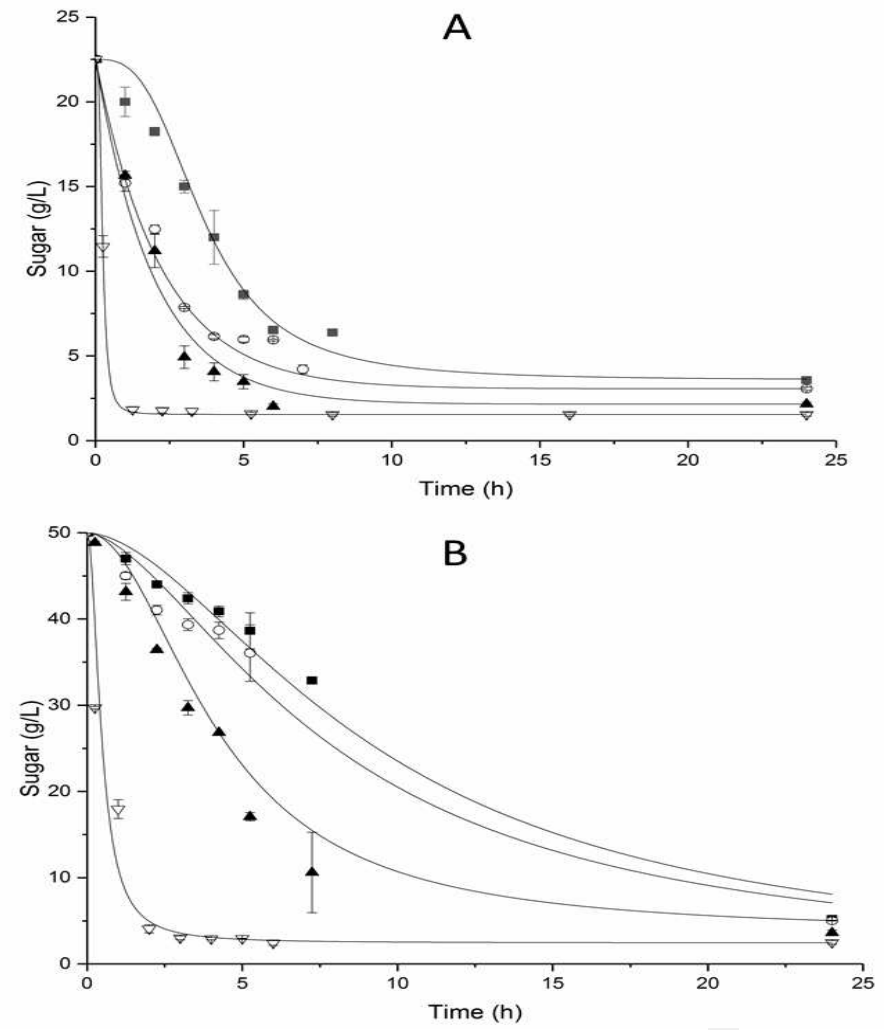


**Figure 4.3:** *Sugars concentration for the inoculums: (■) 0.1g/L, (○) 0.5g/L, (▲)2.5g/L and (▽) 12.5g/L for YPD-20 (A) and YPD-50 (B)*

In Figure 4.4 ethanol concentration profiles along the time are shown. The larger is the inoculum, the faster is the ethanol production as well. The inoculum with 12.5 g/L reached the maximum ethanol concentration in less than 2.5 hours, those with 0.1 g/L and 0.5 g/L needed at least 12 hours. Final ethanol concentration was the same for all experiments. Increasing the initial inoculum, the rate at which the maximum concentration of ethanol is reached increases. Inoculum concentration optimization is one of the most known techniques to improve the efficiency of the fermentation process (Shokrkar et al., 2017).

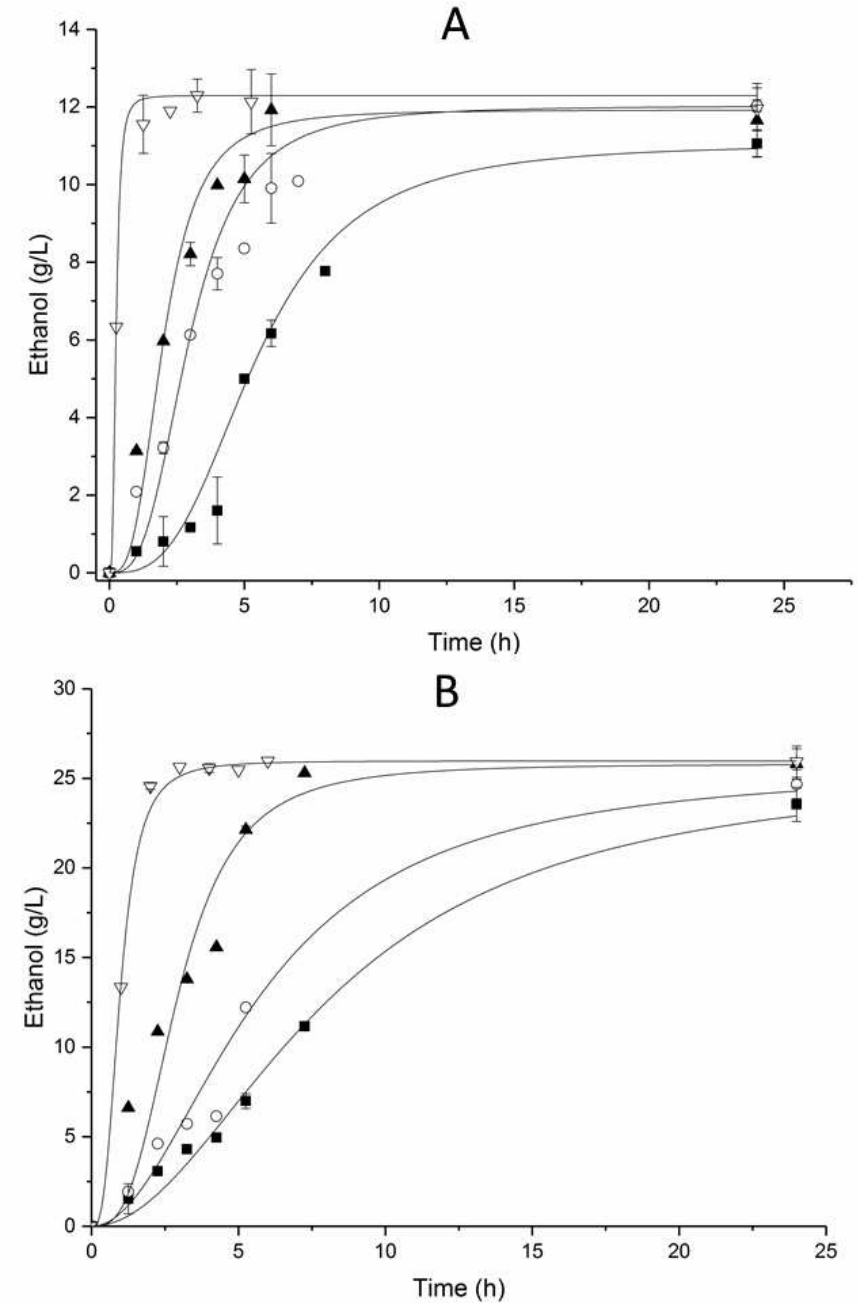


**Figure 4.4:** *Ethanol produced by the inoculums: (■) 0.1g/L, (○) 0.5g/L, (▲)2.5g/L and (▽) 12.5g/L for YPD-20 (A) and YPD-50 (B)*

From an industrial point of view the ethanol produced per unit time is of crucial importance. With reference to Figure 4.5 a broth with 200 g/L (as sugarcane broth) and a fermentation time between 20-24 hours give an ethanol productivity between 4.25 and 5.11 g $L^{-1}$ $h^{-1}$, (thus range was used as a reference value in this thesis). For this achievement an estimated inoculum concentration of 7.5 g/L, which is the average value between 2.5-12.5 g/L was used in the following experiments.

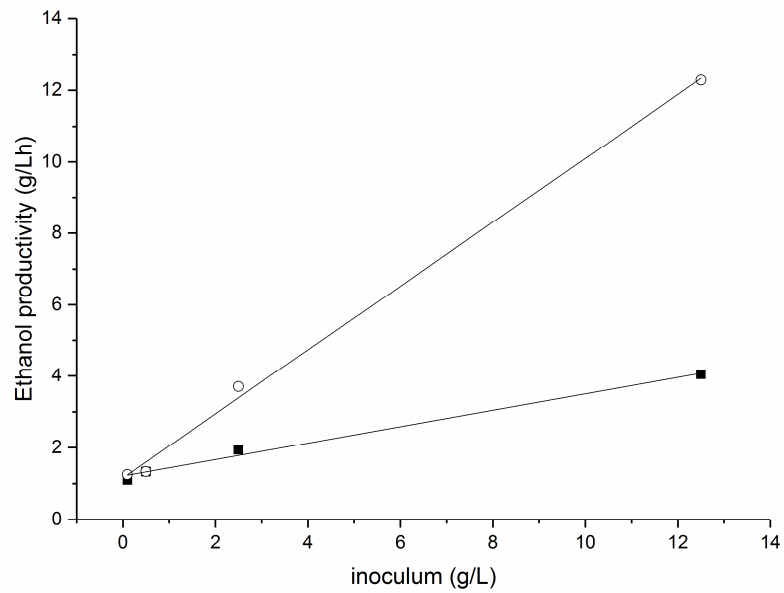


**Figure 4.5:** *Ethanol productivity as the initial inoculums change for (■) YPD-20 and (○) YPD-50*

From Figure 4.5 it is clear that by increasing the inoculum concentration the ethanol productivity can be made layer. This result was also found by Erten et al. (2006): studying on white wines with *Saccharomyces cerevisiae* (Fermiblanc N°SM 102-Gist Brocades) they demonstrated that if the inoculum concentration is increased from $1*10^4$ cells/mL to $1*10^7$ cells/mL the production of alcohols was enhanced. Also, Wanderley et al. (2014) studied the effect of *Saccharomyces cerevisiae* concentration (UFPEDA 1238 and UFPEDA 1324) for ethanol production from sugar cane using inoculums of 0.4 g/L, 4 g/L and 8 g/L. The best ethanol productivity (3.1 g $L^{-1}h^{-1}$) was obtained starting from a strain with 40 g/L of sugars and 8 g/L of inoculum. For the other inoculums the fermentation process was slower and the productivity lower.

### 4.1.2 Ethanolic fermentation with *Pichia stipitis*

Unlike *Saccharomyces cerevisiae*, *Pichia stipites* showed a considerable lag time in all the experiments. The time required by the microorganism to adapt was at least 20 hours. Observing the growth curves reported in Figure 4.6 it can be noted that at high inoculum concentrations the difference between initial and final dry cell weight can be neglected.

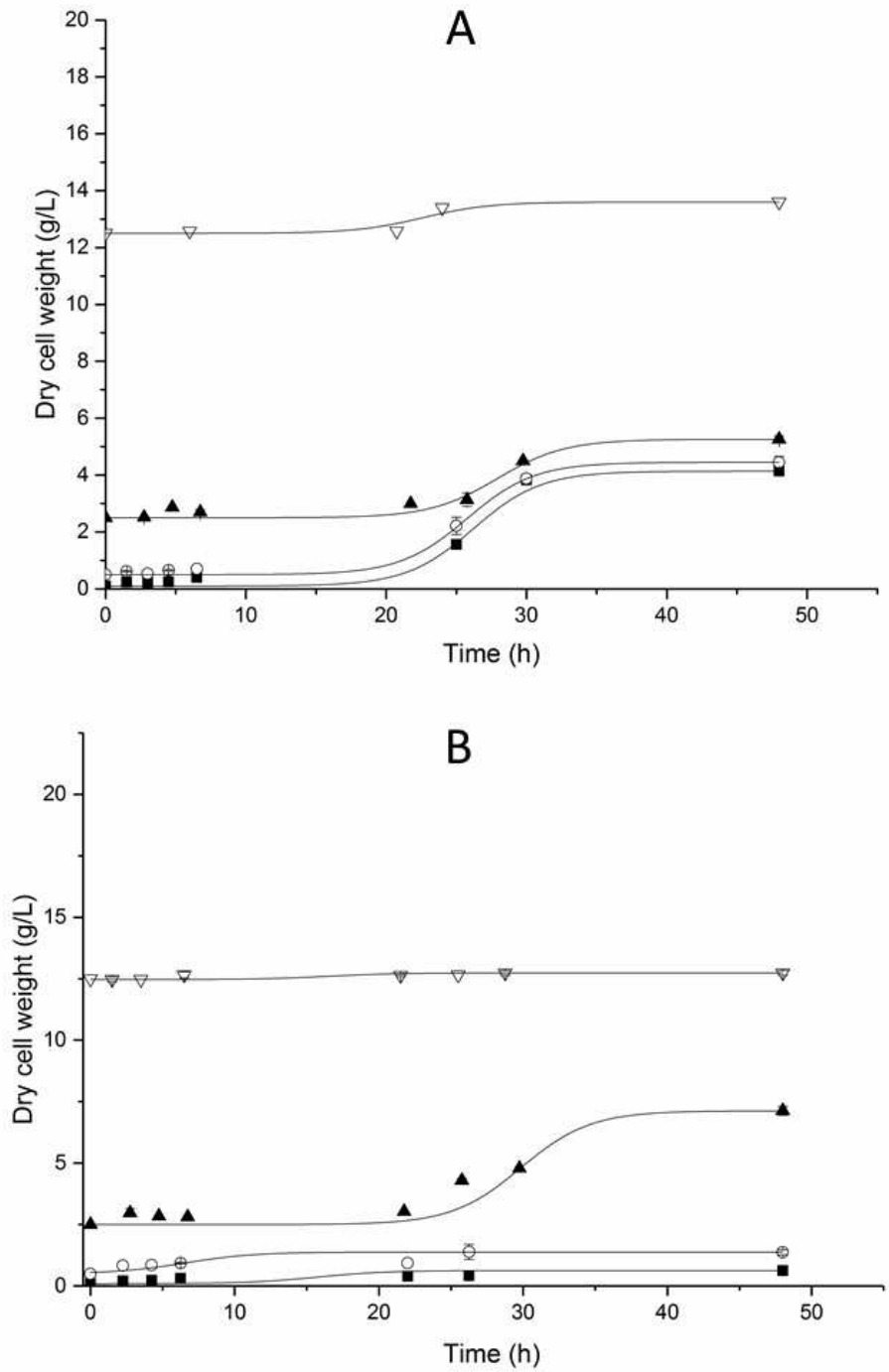


**Figure 4.6:** *Growth curve for the inoculum: (■) 0.1g/L, (○) 0.5g/L, (▲)2.5g/L and (▽) 12.5g/L for YPD-20 (A) and YPD-50 (B)*

The sugars were consumed slowly during the lag time, and path slower after the adaptation depending on the inoculums concentrating (12.5 g/L versus 0.1 and 0.5 g/L respectively). See Figure 4.7 for details. This was more evident for YPD-50 because the glucose concentration was high (50 g/L) and the yeast required a longer period of adaptation, exhibiting also an influence on the sugars concentration. This evidence was

also reported by Silva et al. (2016), who evidenced that *P. stipitis* in a culture medium with 20 g/L xylose, 3 g/L of glucose and 6.7 g/L YNB (yeast nitrogen base) with the inoculum of 0.1 g/L consumed only the 57.5% of sugars after 72 hours.

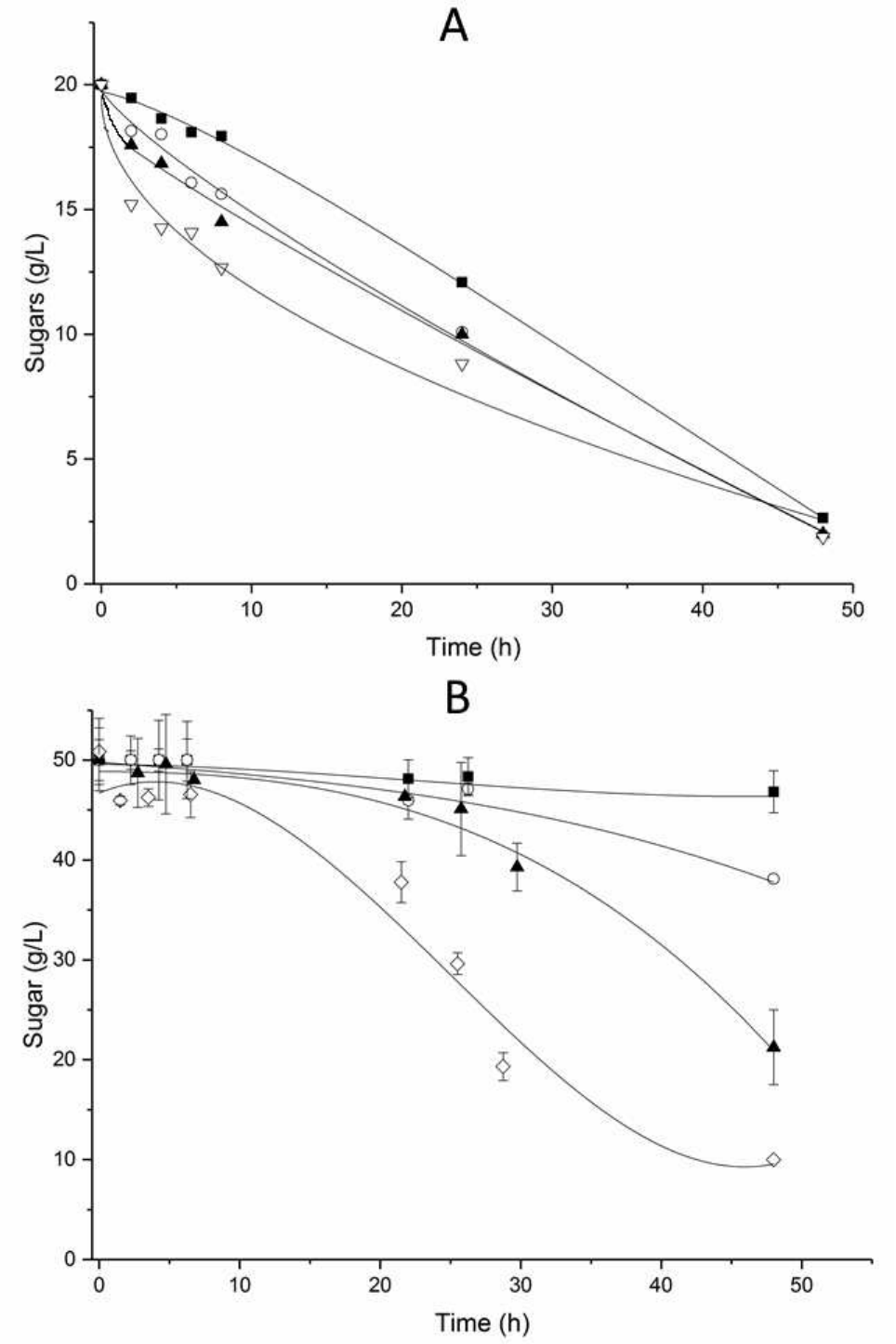


**Figure 4.7** *Sugars concentration along the time starting from different inoculums: (■) 0.1g/L, (○) 0.5g/L, (▲)2.5g/L and (▽) 12.5g/L for YPD-20 (A) and YPD-50 (B)*

As can be seen in Figure 4.8 the ethanol concentration was low during the first hours of fermentation because yeast was in the lag-adaptation time. After 24 hours, the ethanol production rate markedly increased. In Figure 4.9 the ethanol productivity is displayed, indeed a very low value due to the need of the microorganism.

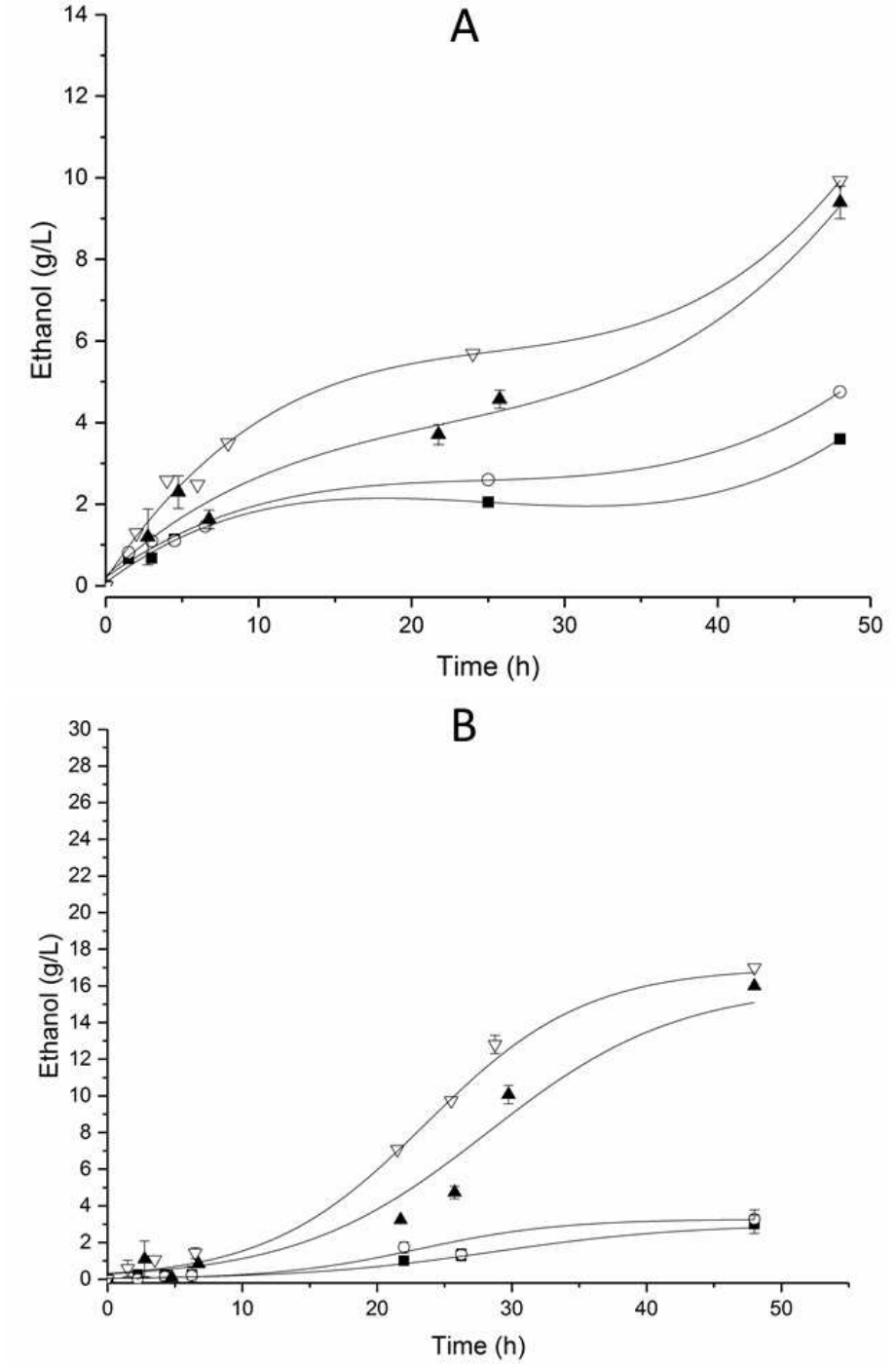


**Figure 4.8:** *Ethanol produced along the time starting from different inoculums: (■) 0.1g/L, (○) 0.5g/L, (▲)2.5g/L and ( ▽) 12.5g/L for YPD-20 (A) and YPD-50 (B)*

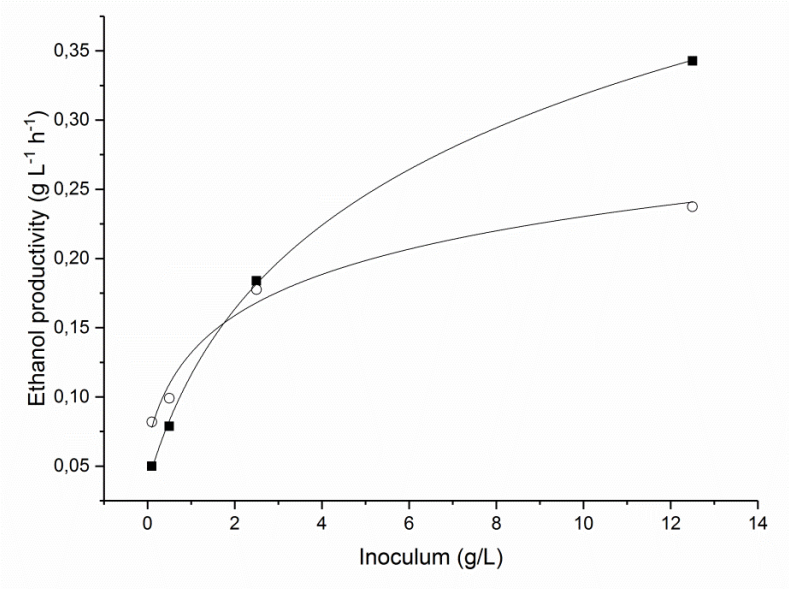


**Figure 4.9:** *Ethanol productivity for different inoculums for Pichia stipitis*

Günan Yücel and Aksu (2015) studied *Pichia stipitis* (NRRL Y-7124) with sugars obtained from beet pulp hydrolysate. They observed low ethanol productivities (0.06 – 0.494 g $L^{-1}$ $h^{-1}$) and long time (50-75 hours) for reaching the maximum ethanol concentration of 37.1 g/L (culture medium with 75.1 g/L of xylose). On the other hand, *Pichia stipitis* (NRRL Y-7124), with inoculum concentration of 7.5 g/L was fermented in a medium with 20 g/L of xylose, 3 g/L of glucose and 6.7 g/L of YNB (yeast nitrogen base) showing an ethanol productivity of 0.03 g $L^{-1}$ $h^{-1}$ after 72 hours, with a maximum ethanol concentration of 4 g/L (Silva et al., 2016).

By comparing these results with *Saccharomyces cerevisiae, Pichia stipitis* results much less productive. Thus, the only advantage for using this strain is in capacity to increase xylose conversion. The main result of this step is that: larger initial inoculum concentration ensures greater ethanol productivity and fastest the fermentation process.

## *4.2 Step 2: Consortium optimization*

The biomass biochemical characterization (§3.2) highlighted the presence of xylose and other C-5 sugars, with pentose ≈20% of total sugars, in addition to glucose (70-80% of cell carbohydrate dry weight). As *Saccharomyces cerevisiae* is not able to ferment pentose sugars. A lower sugars consumption and a lower process yield are expected (Silva and Bertucco, 2017).

On the other hand, *Pichia stipitis* is naturally able to ferment pentose sugars. However, as seen in the previous step, it reached a remarkably lower ethanol productivity than *Saccharomyces cerevisiae.* The aim of the second step was to optimize a *Saccharomyces-Pichia* consortium in order to maintain a high ethanol productivity and simultaneously maximizing the sugars consumption (both hexose and pentose). The culture mediums were YPD-20 (16 g/L of glucose, 4 g/L of xylose, 20 g/L Peptone and 10 g/L of Yeast extract) and YPD-50 (40 g/L of glucose, 10 g/L of xylose, 20 g/L Peptone and 10 g/L of Yeast extract). (7.5 g/L) It was decided to use the inoculum concentration of the first step, taking into account the previous results, where it was able to consume sugars in less than 8 hours. The second step experiments a consortium between the strains was used: (100% *S. cerevisiae* x 0% *P.stipitis*, 75% *S.cerevisiae* x 25% *P.stipitis*, 50% *S. cerevisiae* x 50% *P. stipitis*, 25% *S.cerevisiae* x 75% *P.stipitis*, 0%*S.cerevisiae* x 100% *P.stipitis*).

In Figure 4.10 the sugars concentration profile is displayed. The fermentations with the consortium made by 75% *S.cerevisiae* was the fastest on both culture medium followed by the ones with 100% and 50% of *S.cerevisiae*. As expected, the worst performance was the one with 100% *P. stipitis*.

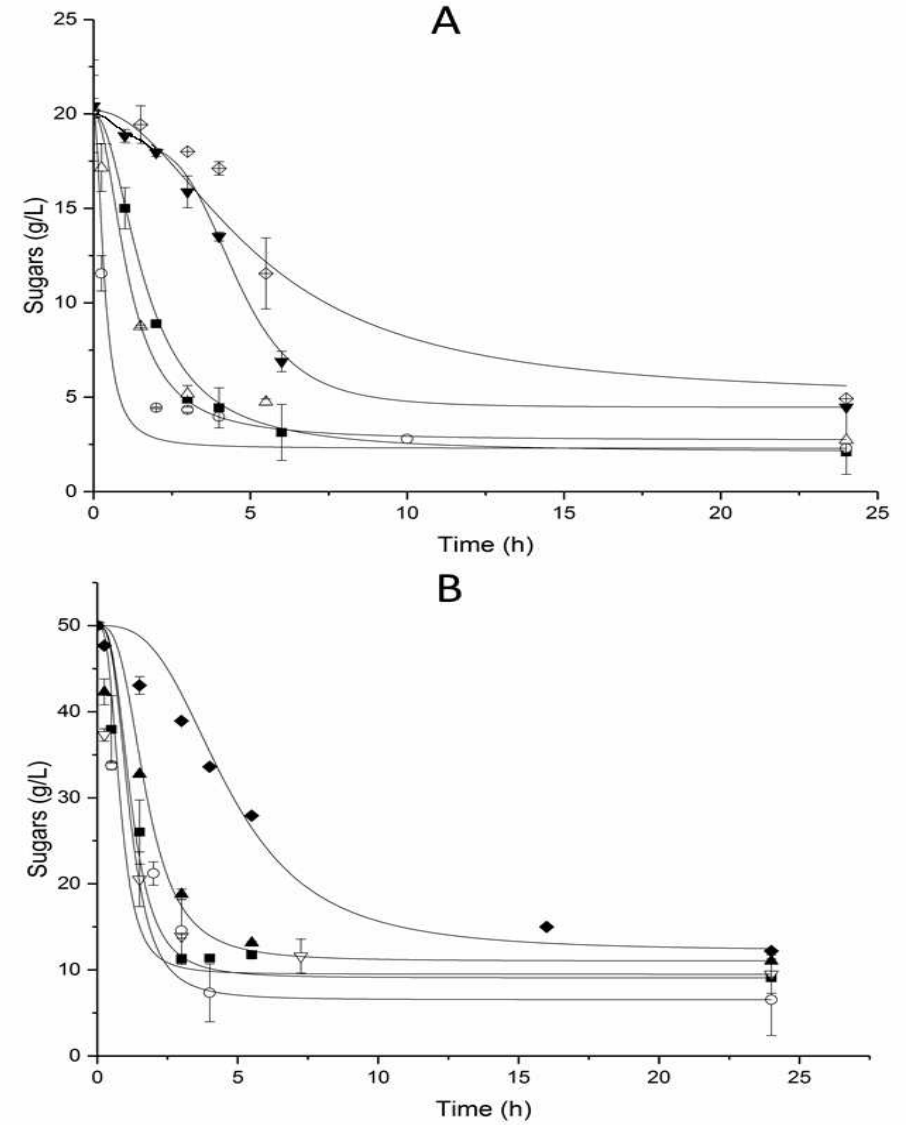


**Figure 4.10:** *Sugars concentration in the time for the consortium: (■) 100% Saccharomyces c. and 0 P. stipitis (○) 75% S. cerevisiae and 25% of P. stipitis, (▲)50% S. cerevisiae and 50% of P. stipitis, (▽) 25% S. cerevisiae and 75% of P. stipitis and (◆) 0% S. cerevisiae and 100% of Pichia stipitis. (A) YPD-20 and (B) YPD-50*

The experiments with 100% and 75% of *Saccharomyces cerevisiae* were the fastest ones to reach the maximum ethanol concentration (10.4 g/L for YPD-20 and 25.5 g/L for YPD-50). In Figure 4.11 are reported the ethanol concentrations profiles are displayed. The outcome of ethanol production profiles was the same of the sugars consumption ones, indicating that experiments with 100 and 75% of *S. cerevisiae* are the most efficient.

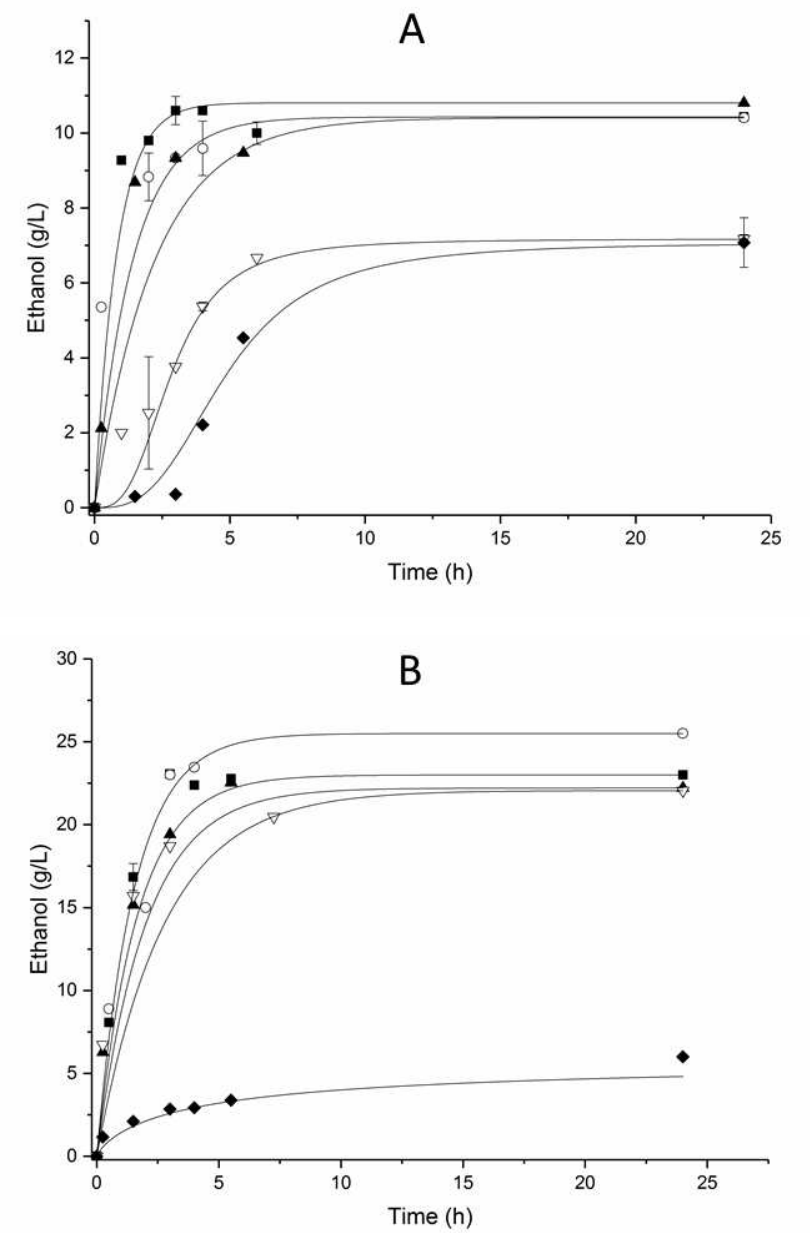


**Figure 4.11:** *Ethanol concentration in time: (■) 100% Saccharomyces c. and 0 P. stipites (○) 75% S. cerevisiae and 25% of P. stipitis, (▲)50% S. cerevisiae and 50% of P. stipitis, (▽) 25% S. cerevisiae and 75% of P. stipitis and (◆) 0% S. cerevisiae and 100% of Pichia stipitis. (A) YPD-20 and (B) YPD-*

In Figure 4.12 the process yield and ethanol productivity for the consortium are shown. These results confirmed that the best consortium was the one made with 75% of *S. cerevisiae*, probably that is the contribution of *P. stipitis* to pentose fermentation. This increased process yield ethanol productivity of 95% and 4.91 g $L^{-1}$ $h^{-1}$ for YPD-20 and 100% and 6.36 g $L^{-1}$ $h^{-1}$ for YPD-50, respectively.

In general, from Figure 4.10 and Figure 4.11it can be concorded that the faster fermentations are those with high content of *Saccharomyces cerevisiae*, in agreement to

what was seen in the previous step (*Saccharomyces cerevisiae* is faster and more productive than *Pichia stipitis*). By increasing the *P. stipitis* fraction in the inoculum (from 25% to 100%) the process yields and the ethanol productivities fell down.

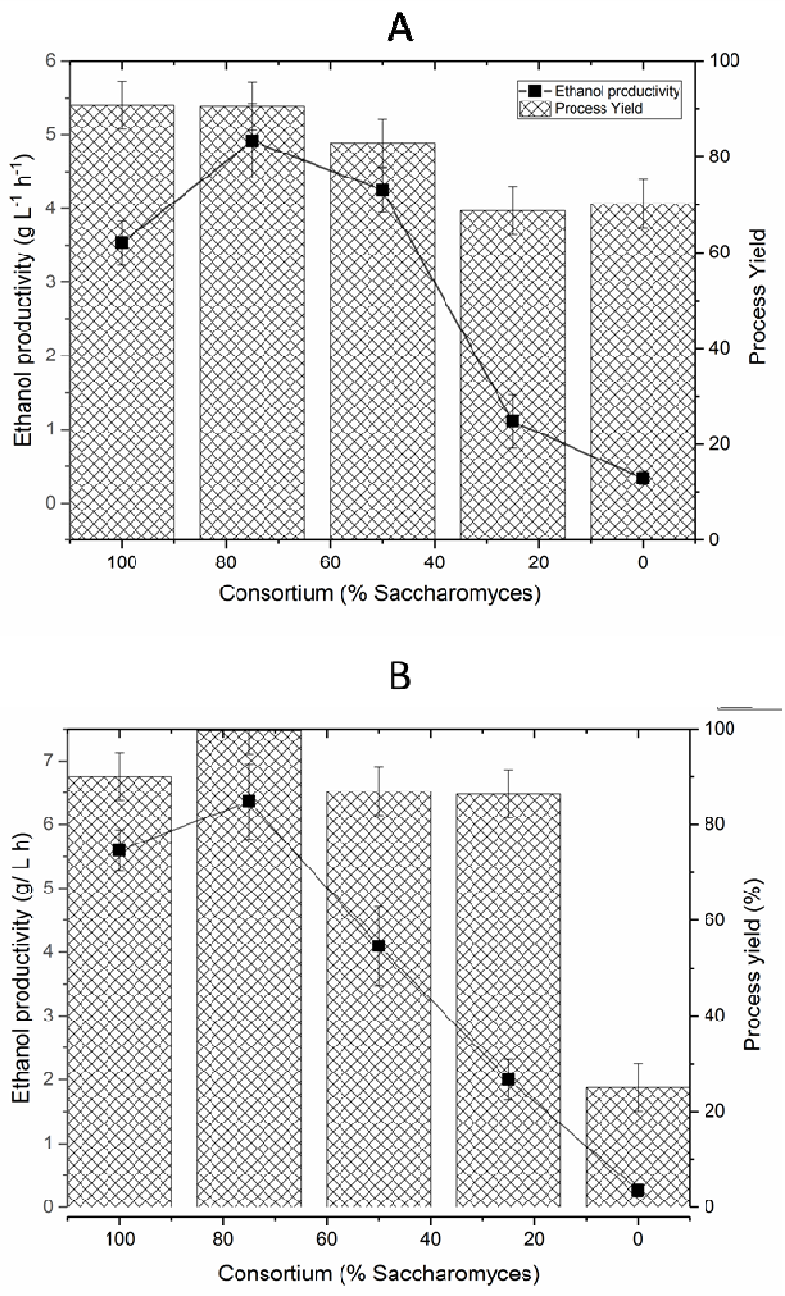


**Figure 4.12:** *Process yield and ethanol productivity for the consortiums in YPD-20 (A) and YPD-50 (B). The percentage of S. cerevisiae is summed with the % of P. stipitis to reach 100%.*

Our results are similar to those published by Ciolfi et al. (2012) who studied the influence of *Pichia guilliermondii* on fermentation for alcohols production, performed with *Saccharomyces cerevisie* and *Saccharomyces uvarum.* Different consortiums were

tested (100 % *S. cerevisiae x S. uvarum*; 100 % *P. guilliermondii*; 10 % *S. cerevisiae x S. uvarum* and 90 % *P. guilliermondii*; 50 % *S. cerevisiae x S. uvarum* and 50 % *P. guilliermondii*; 90 % *S. cerevisiae x S. uvarum* and 10 % *P. guilliermondii*), in a synthetic medium with 200 g/L of sucrose (pH=3.20) and initial inoculum of $2*10^6$ cells/mL. The best consortium was the one made with 90 % *S. cerevisiae x S. uvarum* and 10 % *P. guilliermondii*, it reached the highest alcohols concentration and productivity. The others consortium tested showed a decreasing of alcohols productivity when the *Pichia guilliermondii* fraction increase in the inoculum.

In another study (Kalyani et al., 2013), the advantages of a consortium were shown using an inoculum composed by 50% *S. cerevisiae* ATCC 26603 and 50% *P. stipitis* KCCM 12009 for the fermentation of woody biomass. The hydrolysate with 50 g/L of sugars was neutralized to pH 5 and added with 5 g/L of yeast extract, 10 g/L $(NH_4)_2SO_4$, 4.5 g/L $KH_2PO_4$, and 1 g/L $MgSO_4.7H_2O$ (yeast inoculum was equal to 2% v/v). A sugars consumption of 70% for the fermentation with *S. cerevisiae* was founded, 62.2% with *P. stipitis* and 88% for the consortium. The process yield (based on ethanol) measured 65% for *S. cerevisiae*, 52% with *P. stipitis* and 84% with the consortium.

## *4.3 Step 3: Salinity influence on the ethanolic fermentation*

After acidic hydrolysis, the broth needs to be neutralized to a pH = 5.6±0.2, considered as adequate for yeast cultivation. NaOH was used, so that $Na_2SO_4$ was formed, (if acid hydrolysis has been performed with $H_2SO_4$) or NaCl is produced (if acid hydrolysis has been performed with HCl) as follows:

$$H_2SO_4 + 2NaOH \longrightarrow Na_2SO_4 + 2H_2O \quad (4.1)$$

$$HCl + NaOH \longrightarrow NaCl + H_2O \quad (4.2)$$

The aim of this step was to evaluate the influence of salts ($Na_2SO_4$ and NaCl) on the ethanolic fermentation. The salts concentration investigated were: 10 g/L NaCl, 30 g/L NaCl, 10 g/L $Na_2SO_4$, 30 g/L $Na_2SO_4$. From previous results the yeast consortium with 75% *Saccharomyces cerevisiae* and 25% *Pichia stipitis* (7.5 g/L) was used.

In Figure 4.13 and 4.14 the sugars concentration of the YPD-20 and YPD-50 are reported. Compared to the fermentations without salts (control condition) it was evident

that salts inhibited sugars assimilation, being slower when the salt concentration was increased (Figure 4.13 and 4.14 A, B).

The inhibition was less for NaCl showed than for $Na_2SO_4$ (Figure 4.13 and 4.14 A,B)

The same effect was observed on ethanol produced (Figure 4.15 and 4.16). The maximum ethanol concentration (end of fermentation) was obtained in few hours (≈ 5 hours) and was lower than the one without salts.

Fermentations with *Saccharomyces cerevisiae* under salinity stress was studied by several authors and it was demonstrated that salts stress is caused by two different phenomena: osmotic stress and ion toxicity. In osmotic stressed condition *S. cerevisiae* tends to accumulate osmolytes like polyols (glycerol, for example) by wasting energy and consuming sugars present in the fermentation broth (Blomberg, 2000; Logothetis et al., 2007). For this reason, the ethanol produced resulted lower even though most of sugars were consumed. A similar behavior was verified in this work.

From the growth curves reported in Figure 4.17 and 4.18, it was noticed a decreasing of dry cell weight during the first 5 hours in all the experiments (probably part of the cells died due to the osmotic shocking).

During the adaption time, the cells under osmotic pressure accumulate compatible solutes like glycerol, fatty acids and amino acids in cell membranes to minimize the negative effects, because these substances have a recognized osmoprotective action (Logothtis et al., 2007).

In addition, it was demonstrated that the presence of sodium ions in excess are toxic to yeast (Arino et al., 2010; Casey et al., 2013). The anion chloride exhibits more inhibitory effects than sulfate (Casey et al., 2013). This is proven by the growth curves where the final dry cell weight of the fermentations with $Na_2SO_4$ was higher than those with NaCl.

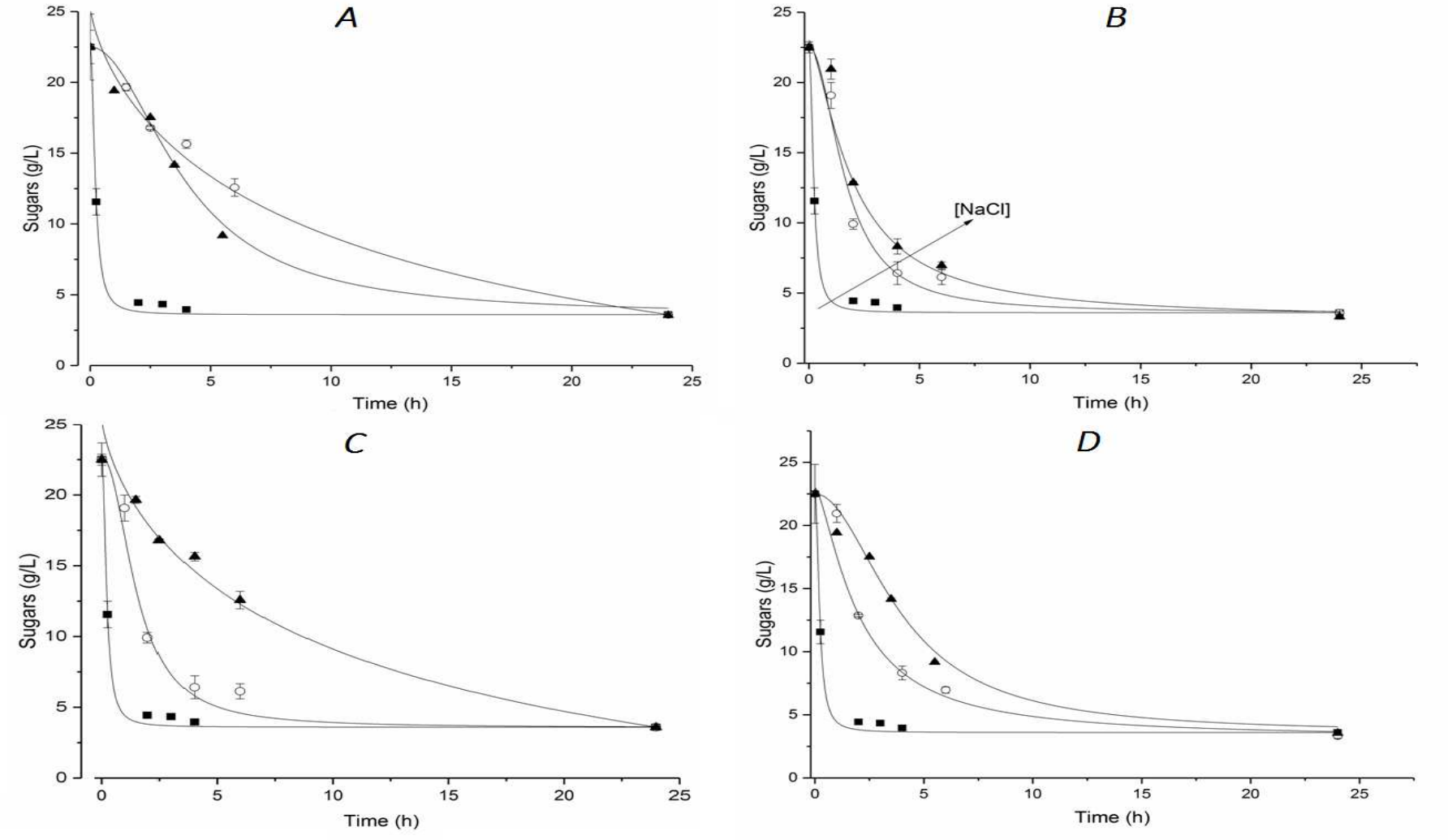


**Figure 4.13:** *Sugars concentration for saline experiments with YPD-20.*

*(A): (○) 10 g/L $Na_2SO_4$, (▲), 30 g/L $Na_2SO_4$, (■) no salts*

*(B): (○) 10 g/L NaCl (▲), 30 g/L NaCL, (■) no salts*

*(C): (○) 10 g/L NaCl, (▲), 10 g/L Na2SO4, (■) no salts*

*(D): (○) 30 g/L NaCl, (▲), 30 g/L Na2SO4, (■) no salts*

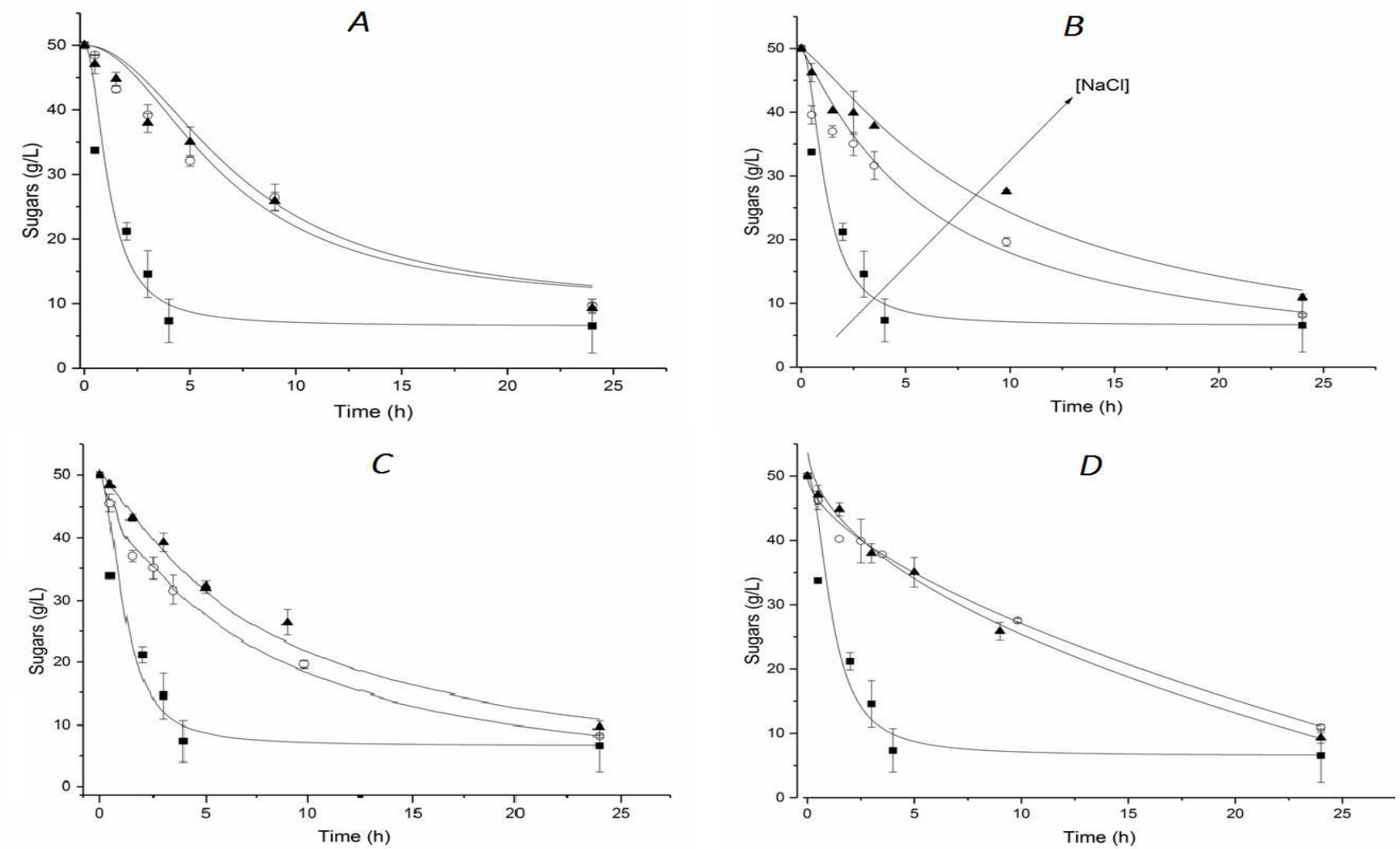


**Figure 4.14:** *Sugars concentration for saline experiments with YPD-50.*

*(A): (○) 10 g/L $Na_2SO_4$, (▲), 30 g/L $Na_2SO_4$, (■) no salts*

*(B): (○) 10 g/L NaCl (▲), 30 g/L NaCL, (■) no salts*

*(C): (○) 10 g/L NaCl, (▲), 10 g/L Na2SO4, (■) no salts*

*(D): (○) 30 g/L NaCl, (▲), 30 g/L Na2SO4, (■) no salts*

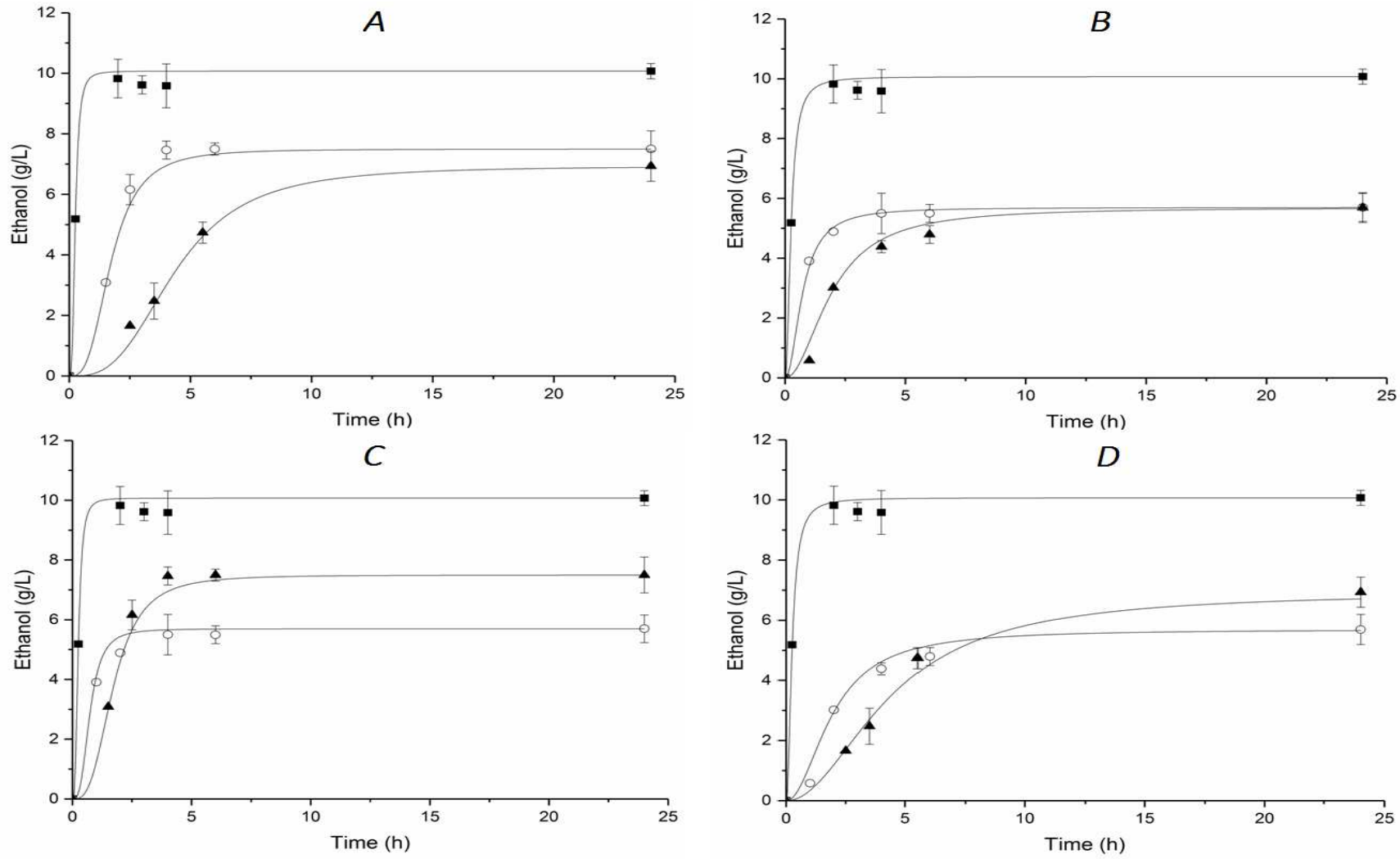


**Figure 4.15:** *Ethanol concentration for saline experiments with YPD-20.*

*(A): (○) 10 g/L $Na_2SO_4$, (▲), 30 g/L $Na_2SO_4$, (■) no salts*

*(B): (○) 10 g/L NaCl (▲), 30 g/L NaCL, (■) no salts*

*(C): (○) 10 g/L NaCl, (▲), 10 g/L Na2SO4, (■) no salts*

*(D): (○) 30 g/L NaCl, (▲), 30 g/L Na2SO4, (■) no salts*

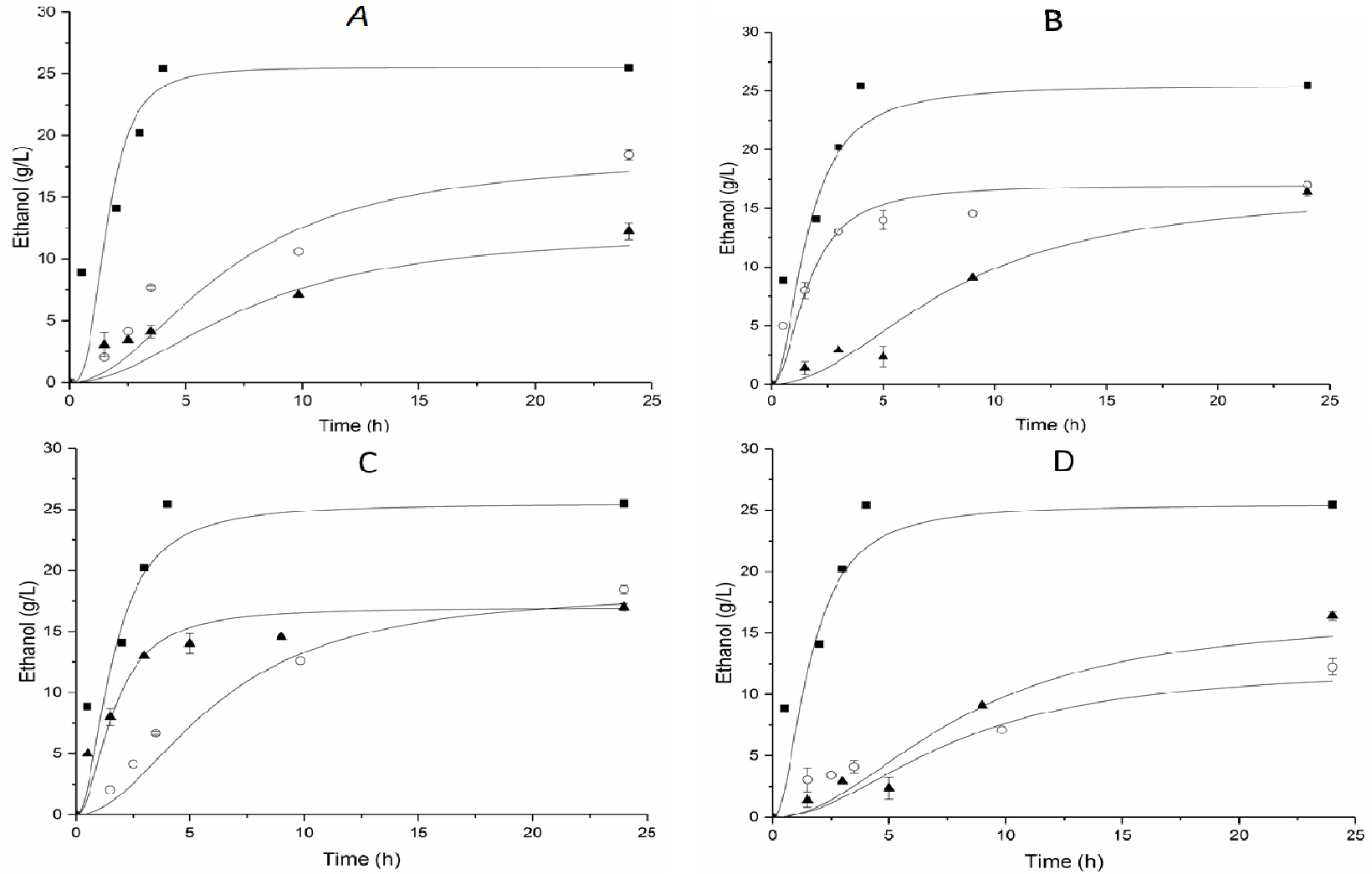


**Figure 4.16:** *Ethanol concentration for saline experiments with YPD-50.*

*(A): (○) 10 g/L $Na_2SO_4$, (▲), 30 g/L $Na_2SO_4$, (■) no salts*

*(B): (○) 10 g/L NaCl (▲), 30 g/L NaCL, (■) no salts*

*(C): (○) 10 g/L NaCl, (▲), 10 g/L Na2SO4, (■) no salts*

*(D): (○) 30 g/L NaCl, (▲), 30 g/L Na2SO4, (■) no salts*

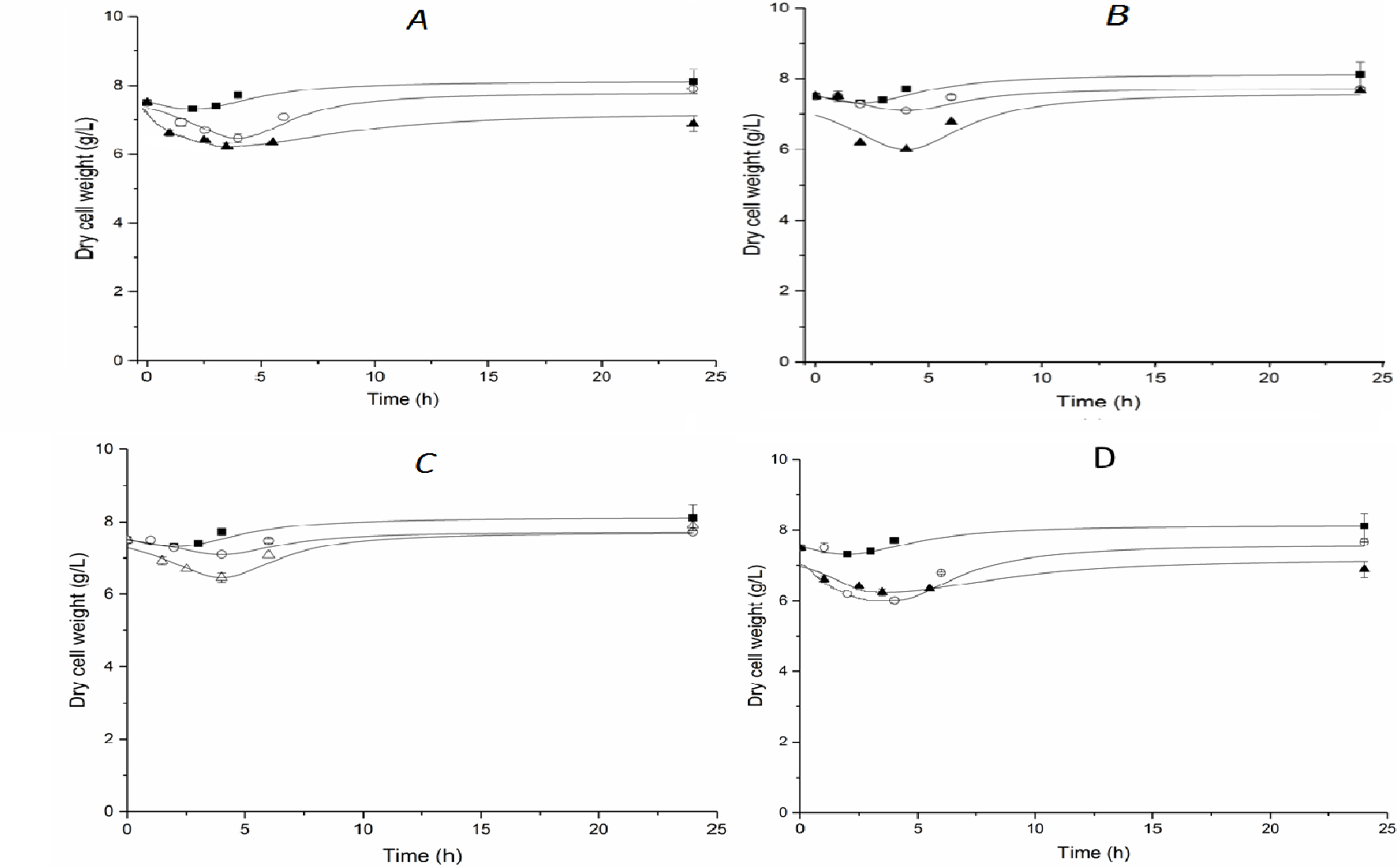


**Figure 4.17:** *Growth curve for saline experiments with YPD-20.*

*(A): (○) 10 g/L $Na_2SO_4$, (▲), 30 g/L $Na_2SO_4$, (■) no salts*

*(B): (○) 10 g/L NaCl (▲), 30 g/L NaCL, (■) no salts*

*(C): (○) 10 g/L NaCl, (▲), 10 g/L Na2SO4, (■) no salts*

*(D): (○) 30 g/L NaCl, (▲), 30 g/L Na2SO4, (■) no salts*

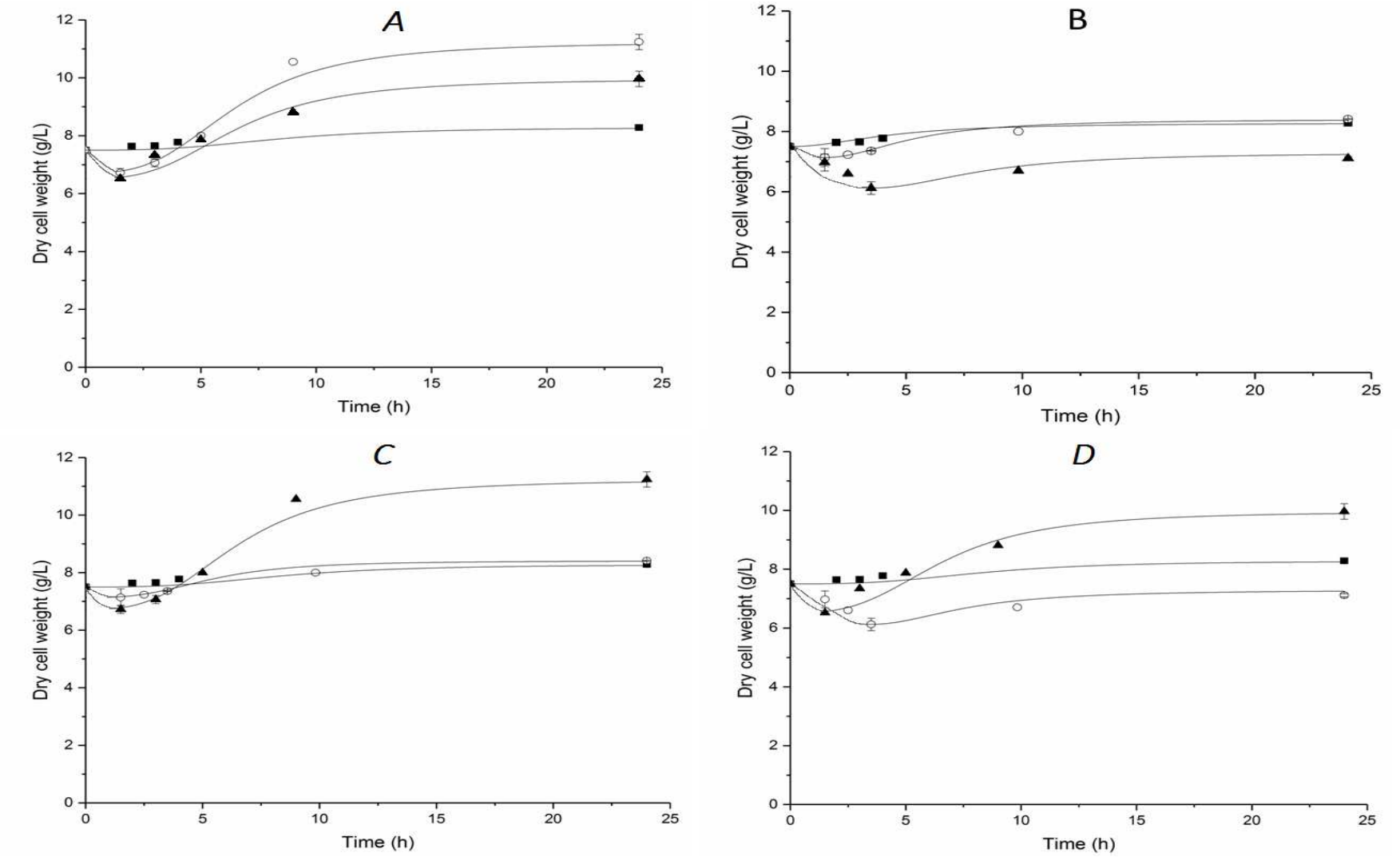


**Figure 4.18:** *Growth curve for saline experiments with YPD-50.*

*(A): (○) 10 g/L Na2SO4, (▲), 30 g/L Na2SO4, (■) no salts*

*(B): (○) 10 g/L NaCl (▲), 30 g/L NaCL, (■) no salts*

*(C): (○) 10 g/L NaCl, (▲), 10 g/L Na2SO4, (■) no salts*

*(D): (○) 30 g/L NaCl, (▲), 30 g/L Na2SO4, (■) no salt*

In Figure 4.19 are shown the process yield and ethanol productivity for the fermentations under salinity stress are reported. Process yield values between 50 and 75% respect to 97% in the control (no salts) despite the sugars had been consumed were achieved for the microalgal hydrolysates. Also, ethanol productivity decreased and this was the consequence of the slower sugars consumption probably caused by the cellular adaptation to the osmotic environment (previously discussed). For YPD-20 the maximum ethanol production was reached after 4 hours for the experiments with 10 g/L of NaCl and $Na_2SO_4$, exhibiting values of 1.37 g $L^{-1}h^{-1}$ and 1.865 g $L^{-1}$ $h^{-1}$, respectively. The fermentations with 30 g/L of salt descreased more the ethanol productivity even more confirming inhibition due the osmotic stress. The productivities measured (after 24 hours, where the maximum ethanol concentration was measured) were 0.237 g $L^{-1}$ $h^{-1}$ for NaCl and 0.288 for g $L^{-1}$ $h^{-1}$ for $Na_2SO_4$. On the other hand, for YPD-50, the ethanol productivities. The values found were 0.77 g $L^{-1}h^{-1}$ and 0.51 g $L^{-1}$ $h^{-1}$ for 10 g/L and 30 g/L $Na_2SO_4$, 0.70 g $L^{-1}h^{-1}$ and 0.65 for 10g/L and 30 g/L of NaCl, respectively.

Those significant reductions of ethanol productivity are not desirable from an economic and industrial point of view.

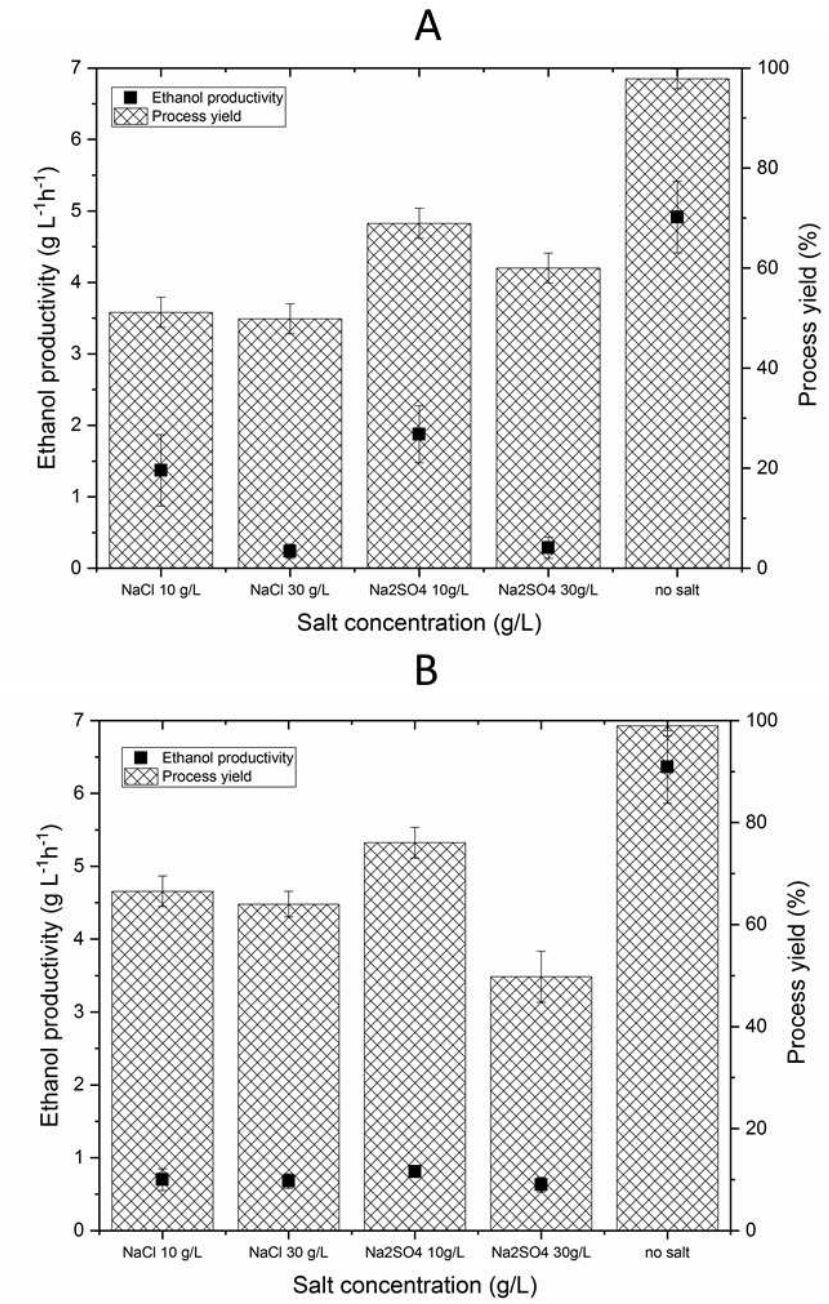


**Figure 4.19:** *Process yield and ethanol productivity for the fermentations under saline stress:*
*(A) YPD-20, for NaCl 10g/L and Na2SO4 10 g/L ethanol productivity were calculated after 4 hours, NaCl 30 g/L and Na2SO4 ethanol productivity were calculated after 24 hours.*
*(B) YPD-50 All the ethanol productivity was calculated after 24 hours.*

## *4.4 Step 4: Microalgal biomass fermentation*

The purpose of this last step was to evaluate the real performance of ethanol production with microalgal hydrolysates. All hydrolysis conditions used in this section were defined in chapter 3. The microalgae used were *Chlorella vulgaris* and *Scenedesmus obliquus*. Both microalgae were submitted to acidic and enzymatic hydrolysis and then fermented.

### 4.4.1 Hydrolysis and fermentation of *Chlorella vulgaris* biomass to ethanol production

Powdered *Chlorella vulgaris* biomass with a carbohydrate content of 23% of the dry biomass (§3.2) was used. The acid hydrolysis was performed at 120°C in autoclave, with $H_2SO_4$ 3%v/v for 30 minutes. The biomass concentration used was 100 g/L (§3.3.1).

The enzymatic hydrolysis was done by means of the enzyme mix: Viscozyme (20 FPU cellulose/$g_{biomass}$), AMG (100U/$g_{biomass}$) and Pectinex (1000U/$g_{biomass}$). The reaction was performed at 50±1 °C for 24 hours with no biomass pretreatment because the biomass was powdered (cells were already broken). The results of saccharification after hydrolysis are resumed in Table 4.1 and both showed efficient sugars recovery.

**Table 4.1:** *Reducing sugars extracts from Chlorella vulgaris after hydrolysis processes*

| | Reducing sugars (g/L) | %Saccharification |
|---|---|---|
| Acid hydrolysis | 19.16 ± 0.18 | 83.3 ± 0.51 |
| Enzymatic hydrolysis | 21.45 ± 0.43 | 93.27 ± 1.89 |

The hydrolysates were fermented with inoculum concentration of 7.5 g/L (consortium 75% *Saccharomyces cerevisiae* and 25% *Pichia stipitis*).

Before starting the fermentation, acidic hydrolysate pH was adjusted to 5.6±0.2 by adding NaOH 10% v/v. In Figure 4.20 it can be seen that the fermentation obtained from the enzymatic hydrolysate is faster than the one from acid hydrolysis. The sugars conversion to ethanol were 82% for acid and 93% for enzymatic hydrolysate.

As seen in the previous step, the salinity affects a lot the fermentation process. Also, the enzymatic hydrolysate has a lower but significant salts concentration (sodium citrate).

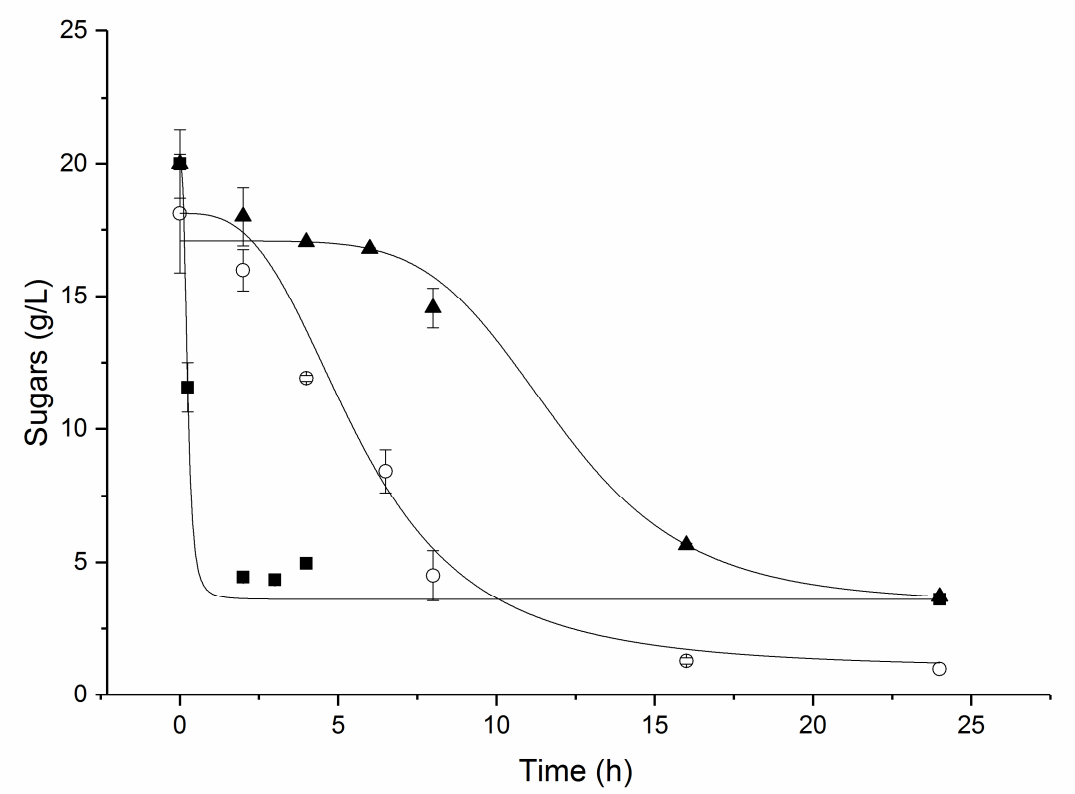


**Figure 4.20:** *Sugars concentration for the fermentation of Chlorella vulgaris hydrolysates: (■) control (YPD-20), (○) enzymatic hydrolysate, (▲) acid hydrolysate.*

In Figure 4. the growth curves for the two fermentations and the control with YPD-20 21 are reported. The microalgal biomass hydrolysate used contains a significant concentration of salts. The behavior of the growth curves represented in Figure 4.21 were similar to the growth curve seen in Figure 4.15 in the salinity fermentation with an initial decreas of cell concentration (lag phase).

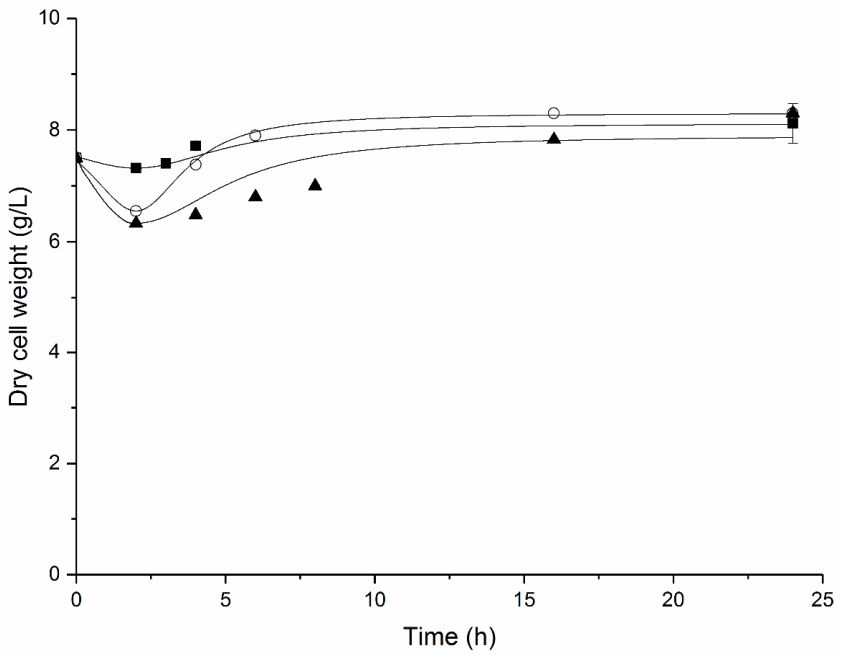


**Figure 4.21:** *Growth curves for Chlorella vulgaris hydrolysate: (■) control (YPD-20), (○) enzymatic hydrolysate, (▲) acidic hydrolysate.*

Kim et al., (2014) investigated the fermentation of *Chlorella vulgaris* after enzymatic hydrolysis. The fermentation process was done in continuous (fed with 0.03 mL/min, residence time of 5.55 hours) with *Saccharomyces cerevisiae* at 30°C. At steady state condition 89% of sugars conversion was reached and an ethanol yield and productivity of 78% and 0.11 g $L^{-1}$ $h^{-1}$, respectively. Shokrkar et al., (2017) performed the fermentations of acid and enzymatic hydrolysates of a biomass of an algae mix. For the enzymatic hydrolysate with 13.5 g/L of reducing sugars a process yield of 89.5% was achieved after 24 hours of fermentation with a maximum ethanol concentration of 6.2 g/L. From the acid hydrolysate (13 g/L of sugars content) 4.96 g/L of ethanol were produced with a process yield of 75% after 24 hours. Practically all the sugars were consumed.

Ho et al., (2013) fermented *C. vulgaris* hydrolysate with *Zymomonas mobilis* (ATTC 29191) at 30°C. The initial sugars concentration was 23.6 g/L. After 12 hours, almost all sugars were consumed and a process yield of 91% was reached. In addition, a control fermentation was done with a 20 g/L of glucose medium and a faster sugars consumption and ethanol production were verified, thus it was achieved that microalgal hydrolysates are fermented slower than simple sugars obtained from traditional crops (sucrose and glucose, for example).

### 4.4.2 Hydrolysis and fermentation of *Scenedesmus obliquus* biomass to ethanol production

The carbohydrate content of the dry biomass used was 45% (of the dry mass). The acid hydrolysis was performed at 120°C in autoclave, with $H_2SO_4$ 3%v/v for 30 minutes. The biomass concentration was 50 g/L (§3.3.2).

The enzymatic hydrolysis was done by using the enzyme mix: Viscozyme (20 FPU cellulose/$g_{biomass}$), AMG (100U/$g_{biomass}$) and Pectinex (1000U/$g_{biomass}$). The process was operated at 50±1 °C for 24 hours. The microalgal biomass was pretreated with ultrasonication for 25 minutes with an amplitude of 50% and offset of -25% (§3.4.3). The biomass used for enzymatic hydrolysis was 100 g/L. The results of the saccharification after hydrolysis process are summarized in Table 4.2.

**Table 4.2:** *Reducing sugars extracts from Scenedesmus obliquus after hydrolysis processes*

| | Reducing sugars (g/L) | %Saccharification |
|---|---|---|
| Acid hydrolysis | 11.47 ± 1.56 | 50.98 ± 2.3 |
| Enzymatic hydrolysis | 41.19 ± 0.91 | 91.55 ± 0.62 |

After hydrolysis, microalgal broth were fermented with the same inoculum concentration used for *C. vulgaris* hydrolysate. Sugars consumption profiles are reported in Figure 4.22. The sugar conversion after 24 hours was 92% for acid and 97% for enzymatic hydrolysates. As seen in chapter 3 (§3.3.2), *Scenedesmus obliquus* is very sensible to thermal degradation, so that the saccharification yield obtained was low compared *with Chlorella*'s acid hydrolysis.

The fermentation with the acidic hydrolysate was compared with the control YPD-20 (50 g/L of biomass with almost 50% of carbohydrate content can recovery at maximum 25 g/L of sugars). The fermentation with enzymatic hydrolysate was compared with the control YPD-50 (100 g/L of biomass and 50% of carbohydrate content).

As demonstrated by *Chlorella vulgaris*, *Scenedesmus obliquus* fermentation exhibited inhibition probably due the medium salinity. This is evident by observing Figure 4.23A where the growth curve has the same shape of the saline fermentation seen in Figure 4.15 and 4.18. Enzymatic fermentation was slower than the control condition as well (Figure 4.22B).

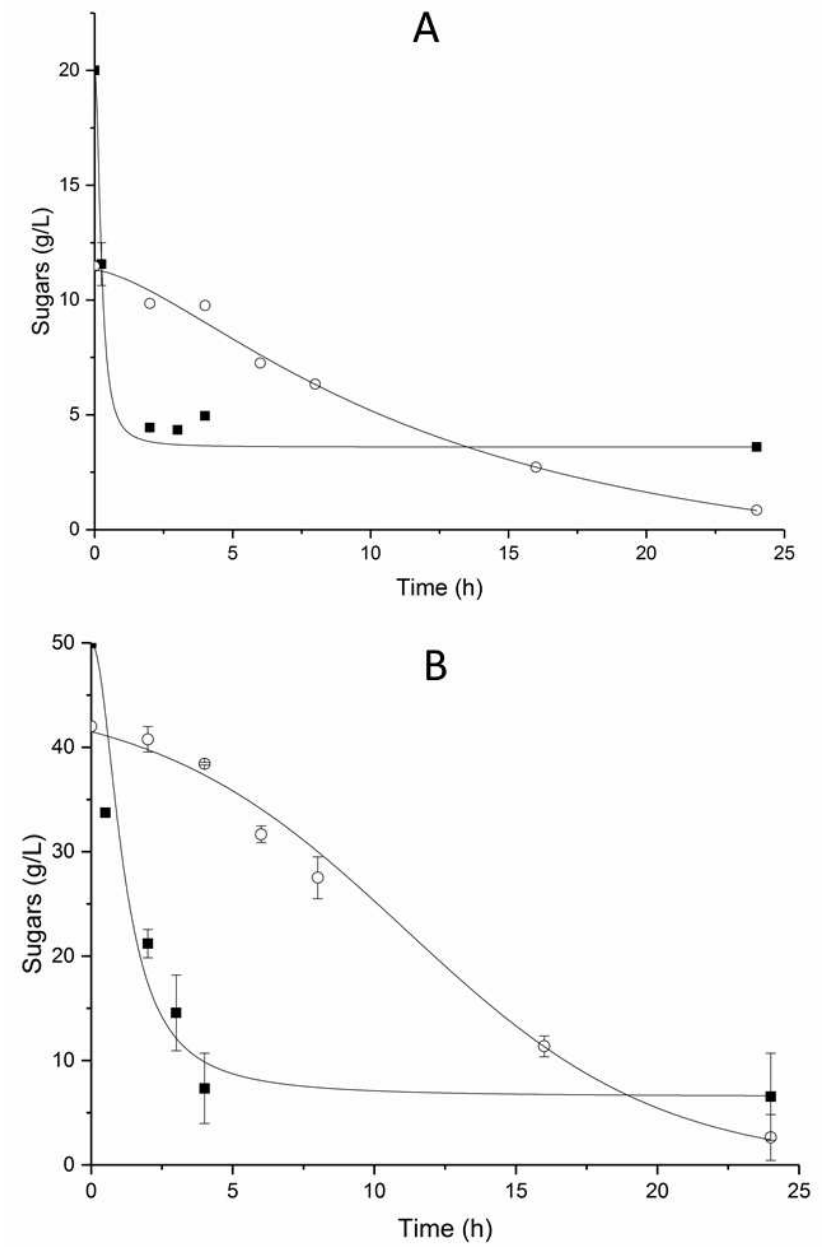


**Figure 4.22:** *Sugars concentration for Scenedesmus obliquus hydrolysate fermentation.*
*(A) Acid hydrolysate: (■) control (YPD-20), (○) acid hydrolysate.*
*(B) Enzymatic hydrolysate: (■) control (YPD-50), (○) enzymatic hydrolysate.*

In Figure 4.23 the growth curves of the fermentations are displayed. The acid hydrolysate caused a decrease of the dry cell weight due to the cellular adaptation to the salinity and the osmotic stress. This did not occur for the enzymatic hydrolysate.

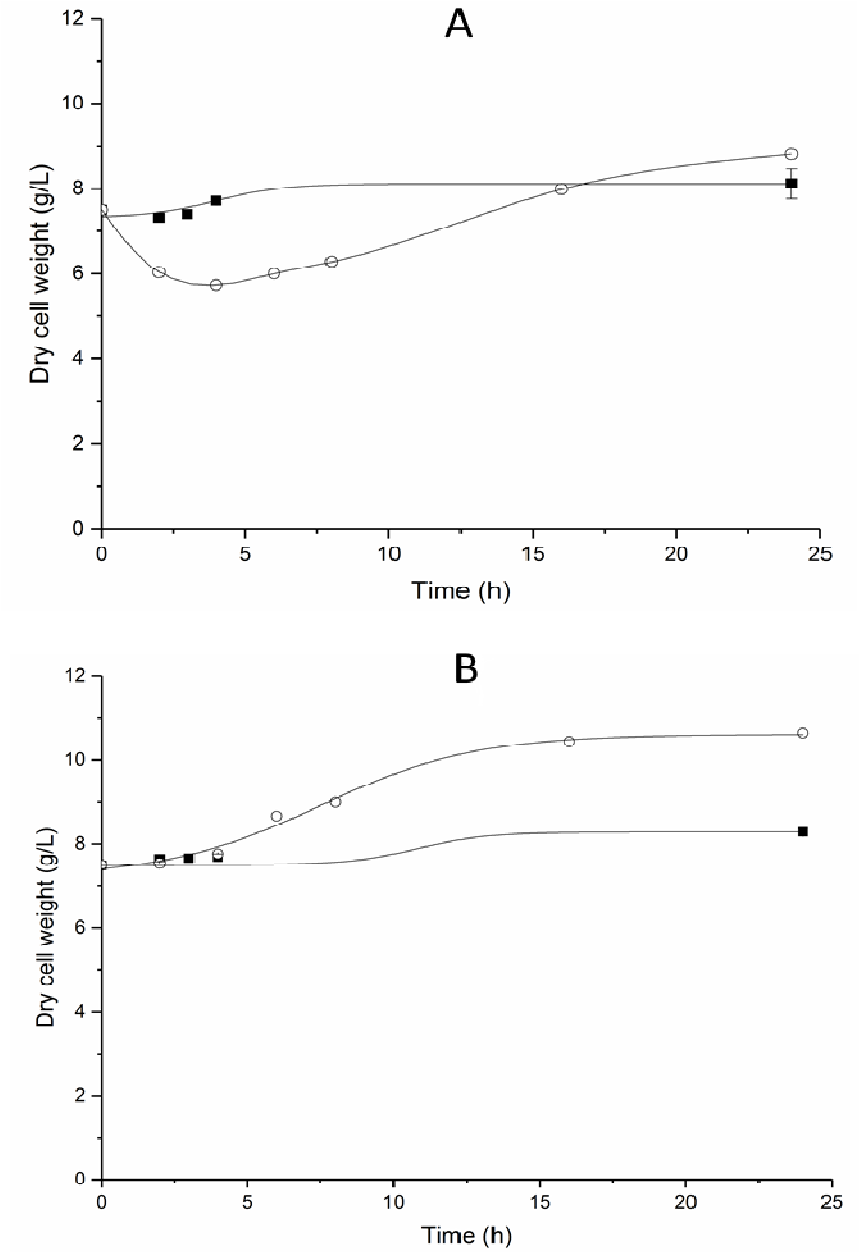


**Figure 4.23:** *Growth curves for Scenedesmus obliquus hydrolysate fermentations.*
*(A) Acid hydrolysate: (■) control (YPD-20), (○) acid hydrolysate.*
*(B) Enzymatic hydrolysate: (■) control (YPD-50), (○) enzymatic hydrolysate.*

According to the literature (Ashokkumar et al., 2015), *Scenedesmus bijugatus* after acid hydrolysis obtained 100 g/L of reducing sugars and they were fermented with *Saccharomyces cerevisiae* (0.01 g/L as initial inoculum). The sugars conversion after 24 hours was 30% (due to the small inoculum used) and after 120 hours was 70%. The process yields were 39% and 72% after 24 and 120 hours, respectively.

*Scenedesmus obliquus* acid hydrolysate (16.5 g/L of sugars) fermented with 0.7 g/L of *Zymomonas mobilis* (ATCC 29191) (30°C) obtained an ethanol concentration of 8.55 g/L after 4 hours with an ethanol productivity of 2.13 g $L^{-1}$ $h^{-1}$ (practically all the sugars were consumed) (Ho et al., 2013). The results were good because the acid hydrolysis was done at a lower biomass concentration and a lower acid concentration ($H_2SO_4$ 2% v/v, 40 g/L, 20 minutes), with respect to the conditions used in this study ($H_2SO_4$ 3%v/v, 50 g/L, 30 minutes). In addition, the pH neutralization after acid hydrolysis was done with $CaCO_3$ (in this study with NaOH). The main advantage is that the presence of cation $Ca^{2+}$ in solution can be less toxic than $Na^+$, leading it a lower inhibition.

Finally, we note that there were no ethanol data available for this last step because the method used for the determination was not reliable due the matrix characteristics (microalgal hydrolysate). Samples will be analyzed in an external laboratory with HPLC.

## *4.5 Fermentation, final remarks*

The main results obtained in this chapter can be summarized as follow:

- The ethanol productivity could be increased by increasing the yeast inoculum.
- The higher the initial inoculum, the fastest is the fermentation process.
- *Saccharomyces* + *Pichia* consortium can be able to ferment the additional fraction of pentose sugars and can increase the fermentation yield.
- The presence of salts in solution, even little quantities (10 g/L for example) leads to inhibition effects on cell growth, sugars consumption and ethanol production
- The hydrolysis process influences the hydrolysate characteristics and interferes directly the fermentation process (salinity, inhibitors, degraded sugars …).
- However, almost all sugars present in the hydrolysates were consumed ensuring that ethanol or a secondary component were produced.

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# Conclusions

In this study, the problem of production bioethanol from microalgae was addressed.

It was shown that microalgae can be saccharified efficiently with both hydrolytic methods (acidic and enzymatic) reaching values higher than 90% of sugars recovery. In the acidic hydrolysis, *Scenedesmus* showed higher susceptibility than *Chlorella* to degradation processes, more difficult, to solubilize, and for this reason lower biomass concentration was used (50 g/L for *Scenedesmus* instead of 100 g/L applied for *Chlorella*). The best condition was found as 120 °C, 3% of sulfuric acid and 30 min of reaction time. During enzymatic hydrolysis, ultrasonication was very efficient as pretreatment, and in the best condition the energy duty of 2.4 MJ/kg of biomass was spendend. The enzymatic mix with amylase, cellulase and pectinase with concentration of 100, 20 and 1000 U/g were sufficient to perform the process. In the fermentation, sugars concentration between 20-50 g/L (20% of pentose) were used and an inoculum concentration of 7.5 g/L and a consortium composed by 75% of *Saccharomyces cerevisiae* and 25% of *Pichia stipitis* was determined as the best. Saline concentration (10-30 g/L of NaCl and $Na_2SO_4$) showed a significant contribution to decrease the productivity and ethanol yield, i.e., leading to and redirecting the metabolism to use the energy obtained by sugars consumption in other cellular processes instead of ethanol synthesis. Microalgae hydrolysates were fermented and the sugars were almost completely consumed suggesting their conversion to ethanol. Final analysis of ethanol concentration will confirm the efficiency of the fermentation process. As a final result, it is possible to conclude that hydrolysis and fermentation processes can be efficiently performed using microalgal biomass. Fermentation needs further studies to understand better what is the inhibitory factor and how is possible to reduce its effect.

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