TÉCNICO LISBOA

Bioethanol Production from Wastes

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Thesis to obtain the Master of Science Degree in

Biological Engineering

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Abstract

The main objective of the present work is to contribute to the development of the biorefinery concept studying the production of bioethanol, a biofuel, from the organic fraction of municipal wastes (OFMSW), through the comparison between two batch systems, one with *Saccharomyces cerevisiae* in suspension and the other with its cells immobilized in a calcium alginate matrix. Since OFMSW is a very complex substrate, both systems were firstly tested using a mineral medium with no organic source of nitrogen and only with glucose as carbon source. Five different initial glucose concentrations were studied: 50, 100, 200, 300 and 400 g/L. Fermentation monitoring was accomplished through analytical methods and by weight loss. Kinetic constants for growth (K_S, μ_{max} and K_i), and biomass and product yields were determined in order to evaluate if there are advantages in using immobilized yeast. The results obtained for the kinetic constants were inconclusive.

After this essay, a typical organic fraction of municipal wastes (OFMSW) was prepared, from which the resulting liquid rich in organic matter was used as substrate for alcoholic fermentation in three concentrations: pure, diluted 1:2 and diluted 1:4. In this essay, the immobilized yeast system was also compared with suspended cells. Diauxic growth was clearly seen in the case of the suspended yeast system. Culture medium with substrate dilution 1:2 seemed to be the best one.

Keywords: Bioethanol; calcium alginate; organic fraction of municipal wastes.

Resumo

O objectivo principal deste trabalho é contribuir para o desenvolvimento do conceito de biorefinaria através do estudo da produção do biocombustível bioetanol a partir da fracção orgânica dos resíduos sólidos urbanos (FORSU), comparando dois sistemas descontínuos, um com *Saccharomyces cerevisiae* em suspensão e outro com a levedura imobilizada numa matriz de alginato de cálcio. Uma vez que a FORSU é um substrato muito complexo, estes sistemas foram primeiramente testados usando um meio mineral e com glucose como fonte única de carbono. Foram estudadas cinco concentrações iniciais de glucose diferentes: 50, 100, 200, 300 e 400 g/L. A fermentação foi monitorizada através de métodos analíticos e por perda de peso. A comparação entre os dois sistemas foi possível através de constantes cinéticas para o crescimento (K_S, μ_{max} e K_i) e de rendimentos em biomassa e em produto. Os resultados obtidos para as constantes cinéticas foram inconclusivos.

Seguidamente, foi preparada uma FORSU sintética, da qual resultou um líquido rico em matéria orgânica que foi usado como substrato para a fermentação alcoólica com células em suspensão e imobilizadas em três concentrações: puro, diluído em 1:2 e diluído 1:4. Verificou-se um crescimento diáuxico no caso das células em suspensão. O meio de cultura com o substrato diluído em 1:2 foi considerado o melhor para o crescimento das leveduras.

Palavras-chave: Bioetanol; *Saccharomyces cerevisiae*; alginato de cálcio; fracção orgânica dos resíduos sólidos urbanos

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Symbols and Abbreviations

$Abs^{540\ nm}$	absorbance at 540 nm
$Abs_{max}^{540\ nm}$	maximum measured value for absorbance at 540 nm
[Biomass]	biomass concentration
$[Biomass]_{Beads}$	biomass concentration inside the beads in g of cells per g of beads
$[Biomass]_i$	initial biomass concentration
$[Biomass]_f$	final biomass concentration
$[Biomass]_{susp.A}$	biomass concentration in suspension A
$[Biomass]_{susp.C}$	biomass concentration in suspension C
COD	chemical oxygen demand
DNS	3,5-dinitrosalicylic acid

DW	dry weight
EDTA	ethylenediaminetetraacetic acid
EE	encapsulation efficiency
EISA	Energy Independence and Security Act
FID	flame ionization detector
GC	gas chromatography
GHG	Green House Gases
$Glucose_c$	consumed glucose
$[Glucose]_i$	initial glucose concentration
GMMs	Genetically Modified Microorganisms
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMDS	bis(trimethylsilyl)amine)
HPLC	high performance liquid chromatography
IEA	International Energy Agency
K_i	inhibition constant
K_S	saturation constant
MTBE	Methyl Tertiary Butyl Ether
NREL	National Renewable Energy Laboratory
OD	optical density
$OD^{beads+cells}$	optical density read in the spectrophotometer
OD^{beads}	optical density of dissolved alginate
OD_{final}	final value of OD used in further calculations
OFMSW	Organic Fraction of Municipal Wastes
OPEC	Organization Petroleum Exportation Countries
$Peak\ area_{max}$	maximum measured value for peak area
RFS	Renewable Fuels Standard
S	substrate concentration
SEM	scanning electron microscopy
SSF	Simultaneous Saccharification Fermentation
t_d	doubling time
TSS	total suspended solids
TSS_{max}	maximum measured value for total suspended solids
UV	ultra violet
V_{beads}	volume of suspension B used for each essay (20 mL)
$V_{cellular\ suspension}$	volume of cellular suspension
$V_{citrate\ sol.}$	volume of 0.1M citrate solution (30 mL)
VSS	volatile suspended solids

VSS_{max}	maximum measured value for volatile suspended solids
$V_{suspA/beads}$	volume of the suspension A used in the 20 mL of suspension B
$V_{susp.C}$	volume of the suspension C
$W_{105°}$	weight of the filter plus sample after drying oven
$W_{after\ muffle}$	weight of the filter plus sample after muffle drying
W_{filter}	weight of the pre-dried filter
W_{sample}	weight of the sample of beads
W_{Beads}^{Total}	weight of beads equivalent to 20 mL of suspension B
X	biomass concentration
YEPG	yeast extract peptone glucose
$Y_{p/s}$	product yield (g of product/g of substrate consumed)
$Y_{x/s}$	biomass yield (in g of biomass produced/g of substrate consumed)
$Y'_{x/s}$	theoretical biomass yield (in g of biomass produced/g of substrate consumed)
$Y''_{x/s}$	semi-theoretic biomass yield
μ	specific growth rate
$\mu_{experimental}$	experimental specific growth rate
μ_{max}	maximum specific growth rate
μ_{model}	Specific growth rate obtained by mathematical model
ρ_{water}	water density

Chapter I

Introduction

Summary

Energy demand has been increasing in the past few years. The majority of the global energy needs are ensured by the combustion of fossil fuels. The extreme consumption of these resources leads to environmental and political concerns. On one hand, the release of greenhouse gases to the atmosphere is a threat to the idea of a sustainable future since it causes global warming, and on the other hand, international political crises regarding Middle East countries have brought, as a consequence, instability to the petroleum market, resulting in oil price fluctuations.

In order to mitigate both problems mentioned, international institutions, such as the United Nations, have taken measures by making agreements in which it commit its parties by setting internationally binding greenhouse gas emission reduction targets. These agreements promote biofuels production since they present an alternative to fossil fuels.

One of the most important concepts and the main motivation for the present study is the biorefinery concept. Biofuels production takes part in this idea since the main objective of a biorefinery is to convert biomass into fuels, power and chemicals, optimizing the use of resources and minimizing wastes, thereby maximizing benefits and profitability.

Bioethanol production will be the object in study of the present work. Bioethanol present good chemical properties, such as higher octane number, evaporation enthalpy and a wider range of flammability, and it can be blended with gasoline, creating an oxygenated mixture that burns more completely. Bioethanol has also some disadvantages compared with gasoline, such as lower energy content, higher latent heat of evaporation and poorer lubricity. Globally, bioethanol production includes three steps: pre-treatment of the substrate, fermentation, distillation and dehydration.

I.1 Fossil fuels: current situation

Nowadays, common people´s lives depend entirely on energy consumption. This energy is mainly provided by the combustion of fossil fuels, which are coal, petroleum and natural gas. According to the World Energy Council these non-renewable energy sources collectively contribute nearly 82% of global energy needs (data from 2013) [1]. The World Watch Institute estimated that the world's oil consumption increased since 2004-2005 and demand increased by 5,3%, typically in China, United States, Canada and United Kingdom [1]. For instance, approximately 97% of the global energy for transportation purposes derived from petroleum [2].

The extreme consumption of these resources, along with continuous growth of the global population, has raised some environment and political concerns. It is now consensus among the scientific community that global warming is a threat to the idea of a sustainable future. This phenomenon is a consequence of the increasing release of greenhouse gases (GHG), which is mostly a consequence of energy generation, as it is shown in Figure I.1**.** The International Energy Agency (IEA) reported that carbon dioxide (CO_2) concentrations in the atmosphere have been increasing significantly over the past century, compared to the pre-industrial era (about 280 ppm). The 2014 concentration of CO_2 was about 40% higher than in the mid-1800s, with an average growth of 2 ppm/year in the last ten years [3].

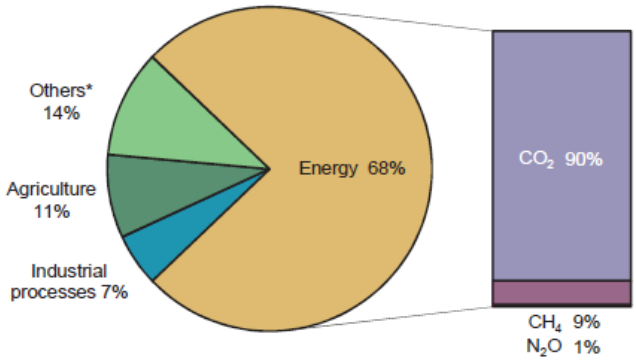

Figure I.1 – Shares of global anthropogenic GHG, 2010. *Others include large-scale biomass burning, post-burn decay, peat decay, indirect N_2O emissions from non-agricultural emissions of NO_x and NH_3, waste and solvent use [3].

Besides this environmental issue, international political crises regarding Middle East countries and the uncertainty about fossil fuel supply (Figure I.2) have brought instability to the petroleum market, resulting in constant oil price fluctuations, as it is shown in Figure I.3.

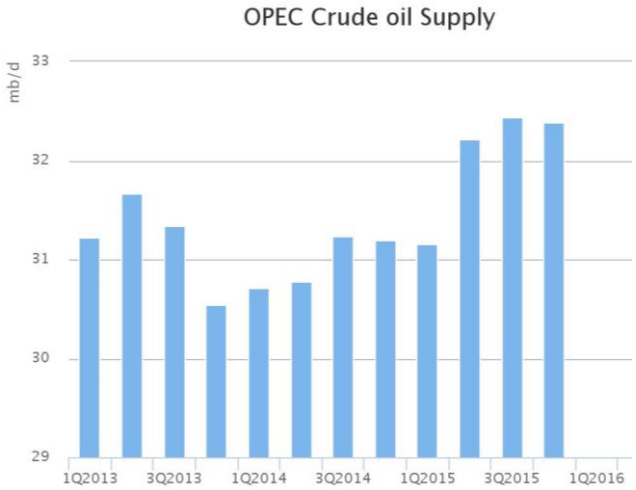

Figure I.2 – Oil supply variations in the last two years regarding the Organization Petroleum Exportation Countries (OPEC). Mb/d thousand barrels of oil per day [5].

Figure I.3 – Crude oil prices (in US dollars/ barrel) variation in the last 5 years (data available on the 31/03/2016) [4].

I.2 Biofuels as an alternative source of energy

In order to mitigate climate change due to global warming, the United Nations Framework Convention for Climate Change has taken measures making agreements such as the Kyoto Protocol (1997) and the Bali Action Plans (2007), in which it commits its parties by setting internationally binding GHG emissions reduction targets [6]. However, to accomplish these goals, there must be an alternative to fossil fuels, equally efficient. Biofuels present a possible solution for both of the problems mentioned, and so studies have been made in order to improve its performance and to guarantee a proper and progressive shift from fossil energy to renewable fuels.

The term biofuel refers to a liquid, solid or gaseous fuel, made from plant matter and residues, and the major biofuels include bioethanol, biodiesel, biogas, biomethanol, biosyngas (CO and H_2), bio-oil, biochar, biohydrogen, Fisher-Tropsh liquids petroleum, and vegetable oil, among of which bioethanol and biodiesel are liquid transportation fuels, usually used as additives [1][7]. The

use of biofuels can contribute to the reduction of GHG emissions since they are mainly produced from green plants that are considered as CO_2-neutral. This means that since green plants are able to fix atmospheric carbon dioxide during photosynthesis by converting it into organic substrates, when the biofuel produced is burned, the CO_2 released will be the same that had been captured by the plant [7]. Moreover, green plants are a renewable source of energy and their very low sulphur content minimizes the acidification of the environment. Besides this, biofuels can be produced from biomass residues, which comes as a solution for the problem of the huge amount of organic wastes that are produced daily around the world. Because of these advantages, biofuels may be an alternative to fossil fuels, providing a prospective of a more sustainable and clean future for our society [7].

However, there are some concerns when it comes to biofuels substrates. Biofuels production can be based upon edible crops, mainly starch, corn and wheat and from sugar crops, such as sugarcane and sugar beet (first generation biofuels). The use of this kind of feedstock for biofuels rise ethical issues since it competes directly with the food market [7]. Furthermore, increased demand for crops to make fuel results in higher global commodity prices that can induce farmers to plow up new ground, including high-carbon ecosystems as tropical forests in South America and Southeast Asia or Peatland in Southeast Asia [8]. Therefore, a second generation of biofuels was developed, which are produced from nonedible lignocellulosic feedstocks. These raw materials result in the production of more fuel per unit of agricultural land, but it is more difficult to convert lignocellulosic biomass into a usable output than other types of biomass, and the developed techniques for biofuel production are highly energy intensive processes [7][9].

Depending on feedstock and farming practices, biofuels production can have significant environmental costs. These include degradation of biodiversity and soil fertility and increased rates of soil erosion, excessive water abstraction and water pollution. In some circumstances, biofuel production can even result in a net increase in GHG emissions [1]. Peer-reviewed research over the last several years, including studies commissioned by the European Commission, indicates that conventional biofuels can directly or indirectly result in substantial GHG emissions through the conversion of forest and grassland to croplands or pasture to accommodate biofuel production. The Directives do not currently account for these emissions in their lifecycle analysis, giving biofuels credit for greater carbon savings than actually achieved [8].

In the US, the Congress passed the Energy Independence and Security Act (EISA) in 2007, in which were established lifecycle GHG emissions thresholds for each category of biofuels (biodiesel and bioethanol), requiring a given percentage of improvement in relation to a baseline of the gasoline and diesel they replace. For instance, biomass based diesel is required to reduce 50% in lifecycle GHG emissions as compared to the baseline petroleum fuel, whereas the cellulosic biofuel derived from renewable feedstock containing cellulose, hemicellulose or lignin must have a lifecycle GHG emissions at least 60% lower than the baseline petroleum fuel [1].

Although there are certain points that need to be clarified with further investigation, the fact is that Governments around the world have proceeded with biofuels promotion, introducing subsidies and policies that motivate its production. International environmental regulations, such as the Kyoto Protocol and Energy Policy Act, promote biofuel utilization, as it is a renewable and clean energy source [10].

The European Parliament and the Council of the European Union decreed in 2009 the Directive on Renewable Energy. It stipulates that the use of biofuels must result in an overall GHG saving of 35%, in order to qualify towards the ambitious 10% biofuels target in the EU27 by 2020. This value rises to 50% from 2017 on for the existing production, and 60% for new installations from 2017 on. This Directive also states that second generation biofuels produced from lignocellulosic materials could enable far greater reductions in GHG, and innovative fuels created from these feedstocks will count double towards the biofuels target of 10% [11].

In the USA, EISA also stipulates a Renewable Fuels Standard (RFS) that required a minimum volume of renewable fuel to be blended into US petroleum fuel in increasing amounts each year until 2022. The EISA renewable fuels standard (known as RFS2), established a target of 36 billion gallons of renewable fuels in US gasoline by 2022 [1].

As a consequence of the policies mentioned, the production and the consumption of biofuels have increased globally in the last few years. Figure I.4 illustrates this trend among European countries, and Figure I.5 shows the same figures worldwide.

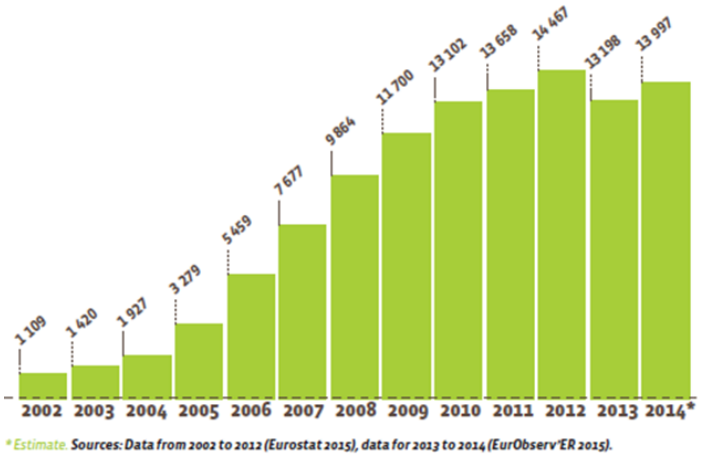

Figure I.4 – European Union (EU-28) biofuel (liquid and biogas) consumption trends for transport trend (in ktoe) [12].

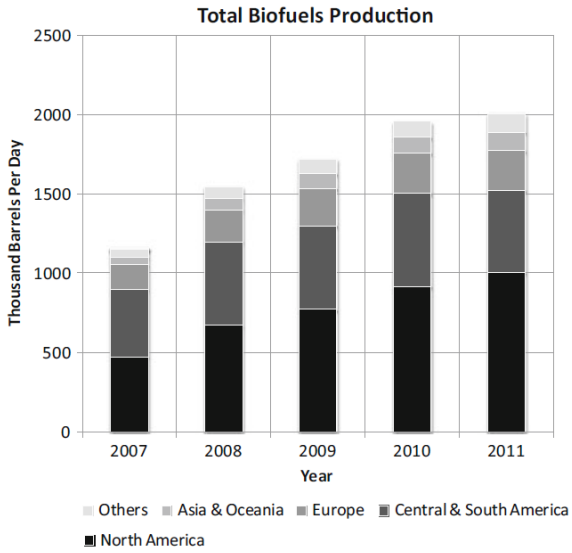

Figure I.5 – Total biofuels production in thousand barrels per day from 2007 until 2011 in the world [7].

I.3 The biorefinery concept

The production of biofuels takes part in a greater concept, in which worldwide economy is based on bioindustry. This model is supported by biorefineries, which according to the American National Renewable Energy Laboratory (NREL) are "facilities that integrate biomass conversion processes and equipment to produce fuels, power and chemicals from biomass" [13]. The aim of a biorefinery is to optimize the use of resources and minimize wastes, thereby maximizing benefits and profitability [9]. This concept is analogous to today's petroleum refineries, in which fossil fuels give origin to multiple fuels and chemicals. Since biomass presents a complex composition, composed of basic carbohydrates, lignin, fats, proteins, dyes, etc, it can give origin to a wide range of products, such as chemicals, materials and fuels [7].

The main advantage of biorefineries is the sustainable processing, since they are based on microbial metabolisms and use of renewable raw materials, such as organic substrates and wastes. Therefore, a biorefinery must be constructed in such a way that its processes take maximum advantage of intermediate and by-products to manufacture additional chemicals and materials. With this configuration, the wastes or by-products of the preceding unit operation could be used as raw materials or source of energy for the following operation, avoiding the generation of wastes [7][9]. In other words, a biorefinery must be self-sustainable.

Moreover, a biorefinery should be built on three different "platforms" to promote different product routes: biochemical platform, thermochemical platform and microorganism platform. The first is based on the biochemical conversion processes and focuses on the fermentation of sugars extracted from biomass feedstocks. The second is currently based on thermochemical conversion processes and focused on the gasification of biomass feedstocks and resulting by-products. The last is based on the production of biofuels from algae using raceway-type pounds and photobioreactors [9][13]. Figure I.6 illustrates the described model for the biorefinery concept.

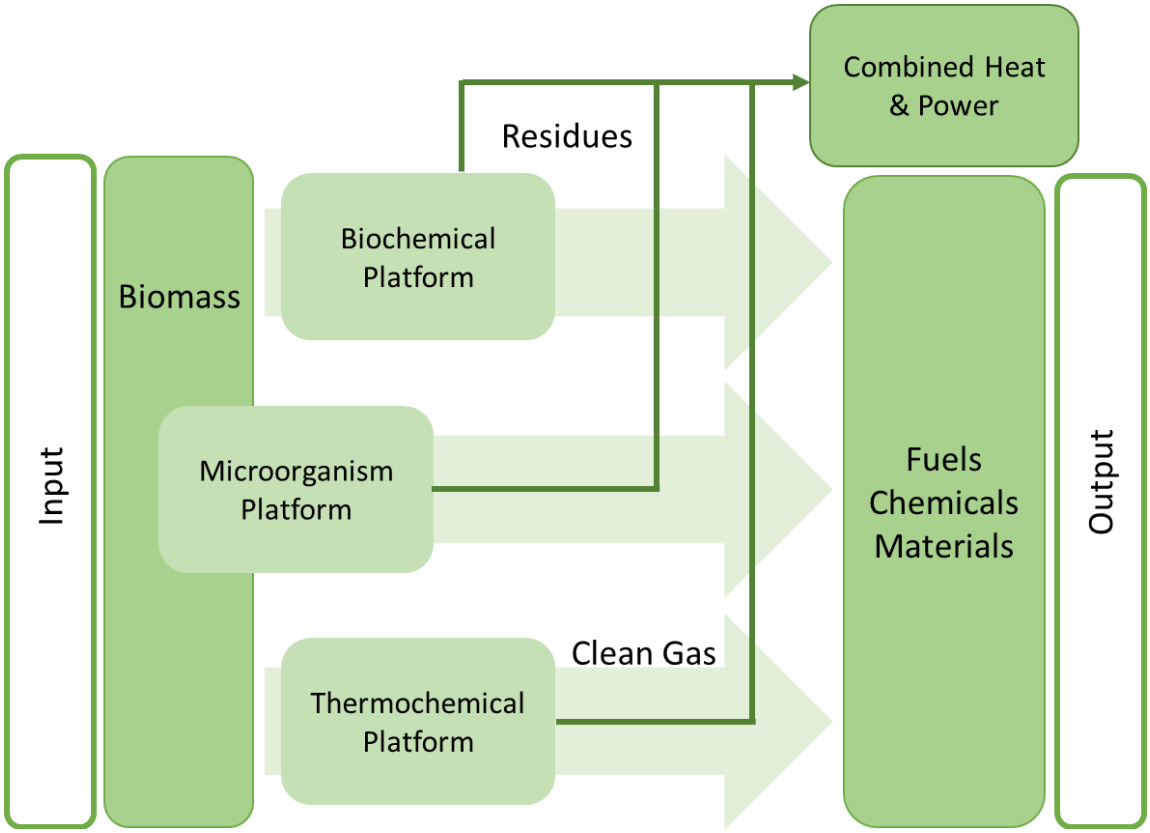

Figure I.6 – The Biorefinery concept. A simple tree describing the three different routes raw materials can take in a biorefinery [9][13].

Another point that must be taken into account is that a successful biorefinery should be balanced in terms of high value/low volume bio-based chemicals and materials with high-volume/low value biofuels. This means a biorefinery might produce one or several low-volume, but high-value, chemical products and a low-value, but high-volume liquid transportation fuel, while simultaneously generating electricity and process heat for its own use and perhaps enough for the sale of electricity. The high-value products enhance profitability, the high-volume fuels help meet national energy needs, and the power production reduces costs and avoids GHG emissions [9].

However, one must consider that an efficient biorefinery is still a concept and its insertion in a market where petroleum based products are well established is very difficult. One of the main commercial challenges is to integrate biorefineries outputs into existing value chains. On one hand, there are biorefineries products that can easily replace petroleum products. In these cases, no or limited change of processing technologies will be required by the customer. An example is green polyethylene made from a renewable resource, namely ethanol from sugarcane. On the other hand, there are bio-based products that are novel or that cannot easily be integrated into existing value chains. Bioethanol, for example, can only be mixed into conventional fuel up to a volume share of around 15% without modification of a standard engine [9].

To sum up, there are still a lot of investigation to carry out so that costs can diminish and to improve biorefineries process efficiency. Biotechnology must be developed in order to create other operations so that petroleum products can be completely replaced by bioproducts.

I.4 Bioethanol

I.4.1 Advantages and disadvantages

Among the renewable fuels, ethanol seems to be the best choice, since it presents good chemical properties: high octane number, evaporation enthalpy, and flame speed and wider range of flammability. Due to these characteristics, fuel ethanol gives higher compression ratio with shorter burning time, eventually providing a better theoretical efficiency than that of gasoline in an integrated circuit engine [15]. Ethanol can also be used to replace lead as an octane enhancer in gasoline [7].

However, there are some drawbacks concerning bioethanol properties. Compared to gasoline, it has lower energy content, higher evaporation enthalpy, poorer lubricity, polar compound soluble in water, which can cause corrosion, and lower vapour pressure. Analysing Table I.1, one can compare the different properties of gasoline, gasohol with various percentages of ethanol, and pure ethanol. It is clear that, for instance, bioethanol has a higher flash point temperature, which means it is safer to store and transport, but it is more difficult to start the car [14]. Moreover, bioethanol has a very high chemical oxygen demand (COD), which leads to harmful effects on the environment when it is accidentally discharged [7].

Table I.1 – Properties of gasoline fuel blended with various percentage of ethanol (average values)

Sample code	% Ethanol	%Gasoline	Flash point (°C)	Autoignition temperature (°C)	Vapour pressure (kPa at 37.8°C)	Energy Density (MJ/L)	Octane number	Specific gravity
E0	00	100	-65	246	36	34.2	91	0.7474
E10	10	90	-40	260	38.9	33.182	93	0.7508
E20	20	80	-20	279	39	32	94	0.7605
E30	30	70	-15	281	38	31.5	95	0.7782
E40	40	60	-13.5	294	35.6	30	97	0.7792
E50	50	50	-5	320	34	29	99	0.7805
E60	60	40	-1	345	31	28	100	0.7812
E70	70	30	0.00	350	28	27	103	0.7823
E80	80	20	5	362	24	26.5	104	0.7834
E90	90	10	8.5	360	18	23.6	106	0.7840
E100	100	00	12.5	365	9	23.5	129	0.7890

Bioethanol can be directly used in the engines or it can be blended with gasoline, resulting in a mixture called gasohol. It can be used as a 5% blend with petrol under the EU quality standard EN 228. The most common blended bioethanol used in USA is E-10 containing a concentration ratio 1:10. In Brazil, on the other hand, pure ethanol is used or a combination of 24% bioethanol and 76% gasoline. With engine modification, bioethanol can be used at higher levels, for example 85% ethanol [7][15]. By blending ethanol with gasoline, an oxygenated fuel mixture is created, that burns more completely, which leads to a reduction of polluting emissions like CO, particulates, hydrocarbons and nitrogen oxides, as well as other GHG besides CO_2. Moreover, due to having lower ambient photochemical reactivity, it reduces the ground level ozone

formation. This fuel energy is also a safer substitute for methyl tertiary-butyl ether (MTBE), a common additive used in gasoline for clean combustion [15].

Bioethanol is, indeed, a renewable source of energy, but CO_2 savings are highly dependent on the type of raw-material and on the type of energy used in the production process [11]. In Figure I.7, it is shown a comparison of the reductions in GHG emissions among the different types of bioethanol.

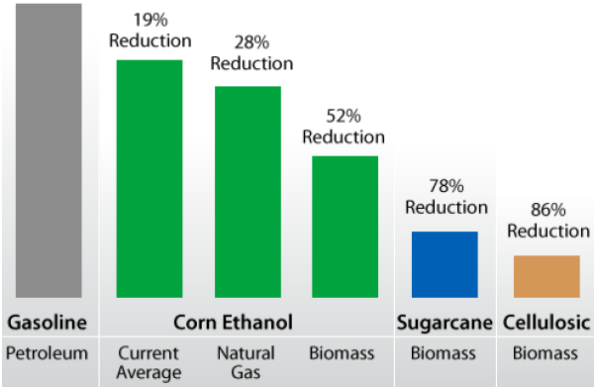

Figure I.7 – Comparison of the reductions in GHG emissions between different types of bioethanol and gasoline, regarding the type of fuel used in processing [16].

I.5 History and statistics

Bioethanol has been used as an alternative transportation fuel since the beginning of the twentieth century. This situation was stimulated during World War II due to the scarcity of petrol supply. Nevertheless, the potential of bioethanol was ignored until the oil crisis of the beginning of the 1970's. Since the beginning of the 1980's, there has been an increased interest in the use of bioethanol and the global market for the biofuel has entered a rapid growth, as it is shown in Figure I.8 and Figure I.9 [7].

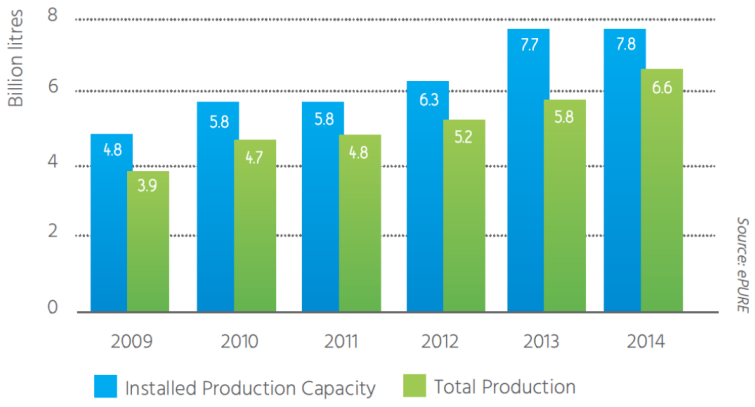

Figure I.8 – European bioethanol market development in the last years. Installed production capacity and total production among European countries. Data from 2015 [17].

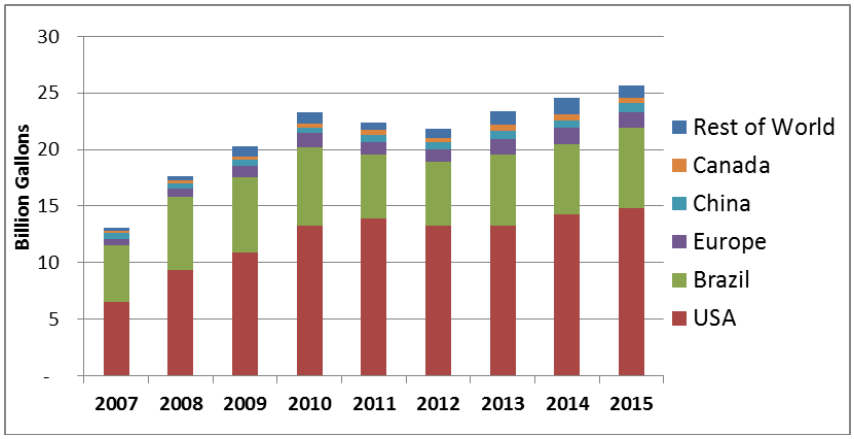

Figure I.9 – Global bioethanol production by country/region and year. Data from 2015 [18].

Ethanol holds promise as an important substitute of gasoline in the transport fuel market, but its costs are higher as compared to fossil fuels [1]. However, its price has been decreasing in the last few years due to processes optimization, as it is shown in Figure I.10.

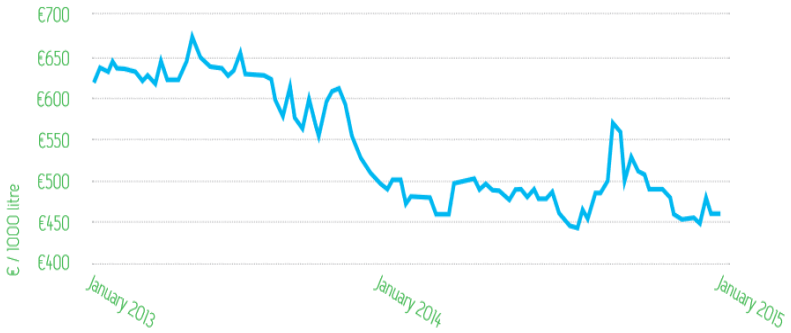

Figure I.10 – Ethanol prices variation in Europe. Data from 2015 [17].

I.6 Bioethanol Production

Globally, the production of bioethanol includes three steps: pre-treatment of the substrate, fermentation, distillation and dehydration [1][7][10].

Figure I.11 presents a general illustration of the process for ethanol production, regarding the three major feedstocks: cellulose, corn and sugarcane.

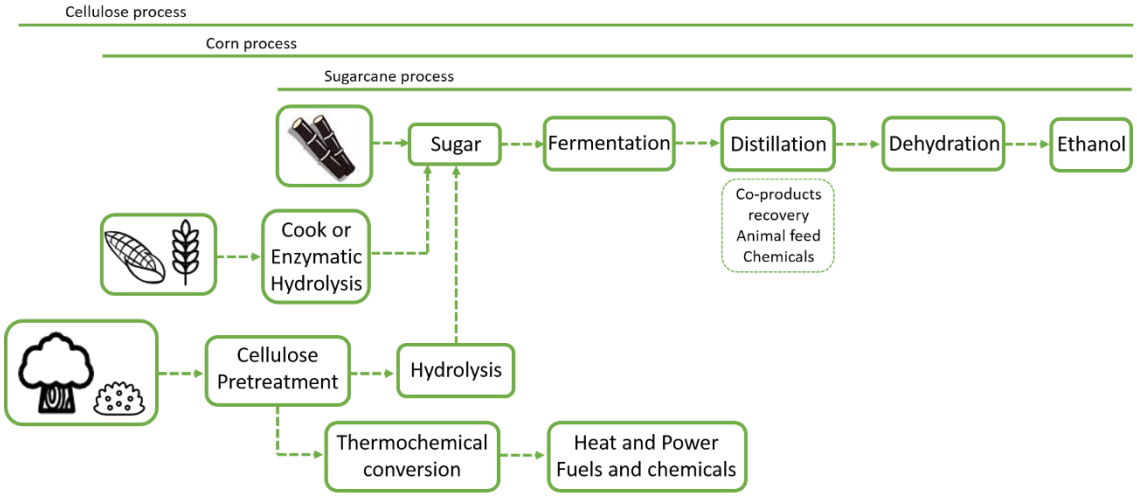

Figure I.11 – Bioethanol production overall process for different raw materials (cellulose, corn and sugarcane) [19].

I.6.1 Feedstocks

Three types of raw materials can be used for bioethanol production: sugars, starches, and cellulose materials. Sugars (from sugarcane, sugar beets, sweet sorghum, molasses, and fruits) can be converted into ethanol directly. Starches (from corn, cassava, potatoes and root crops) must first be hydrolysed to fermentable sugars. Cellulose (from wood, agricultural residues, waste liquor from pulp, and paper mills) must likewise be converted into sugars [20].

About 60% of the global ethanol is produced from sugar crops, while the remaining 40% is produced from starchy grains [15]. However, the use of this kind of substrates raises important ethical issues such as negative impacts on biodiversity, land use and competition with food crops. The increase in demand of this kind of feedstock had an impact on its prices, as it is shown in Figure I.12. The biofuel produced using these materials is called First Generation Bioethanol [21].

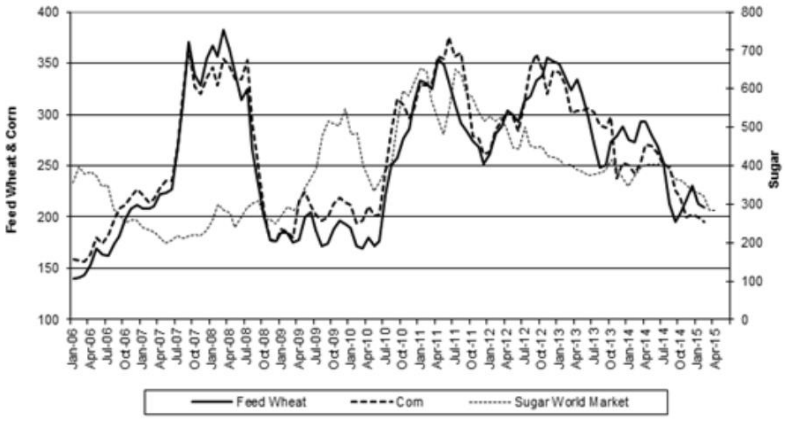

Figure I.12 – Feedstock prices variation in US$/MT among European countries. Based on statistics of IMF and Dutch Agricultural Research Institute. Data from 2014 [22].

These problems lead to the exploitation of others substrates, such as biomass waste, municipal solid wastes, municipal sludge and dairy/cattle manures, which originate second generation ethanol [23]. Lignocellulosic biomass has recently been studied extensively but is still confined to

the laboratory or pilot plant [15]. As a raw material, it is less costly, but the pre-treatment necessary for this feedstock is very expensive. Moreover, a large quantity of wastewaters produced by the fermentation process poses a problem for large-scale production [24].

Concerning the agricultural feedstock, sugarcane is the main carbon source used and it represents 60% of the total ethanol production [10]. Observing the data presented in Table I.2, one can conclude that ethanol produced from sugarcane is also the cheapest.

Table I.2 – Comparison of production cost and bioethanol yield from different energy crops [10]

Type	Yield (t/ha/year)	Conversion rate to sugar or starch (%)	Conversion rate to bioethanol (l/ton)	Bioethanol yield (kg/ha/year)	Cost[a] ($/m^3)
Sugar cane	70	12.5	70	4900	~160
Cassava	40	25	150	6000	700
Sweet sorghum	35	14	80	2800	200–300
Corn	5	69	410	2050	250–420
Wheat	4	66	390	1560	380–480

This happens mainly because it is not necessary to make the transformation from complex carbohydrates to sugars. Another reason is that the majority of this feedstock comes from Brazil, which has the largest amount of open land that is used for or can be converted to farm land, has a very low cost of labour and a tropical climate which allows it to be highly effective at sugarcane production. It is without doubt the most important fermentation process, and as such the most studied [19][26].

Besides this type of substrate, there are several studies in which materials such as the organic fraction of municipal solid wastes (OFMSW) are used as raw materials for ethanol production. In the particular case of OFMSW, it must be noted that currently most of these wastes are disposed of in landfills, losing the opportunity for its valorisation [7]. In Figure I.13 is presented data about the amount of municipal solid wastes produced in the European Union from 1995 to 2003 and the treatment they have received.

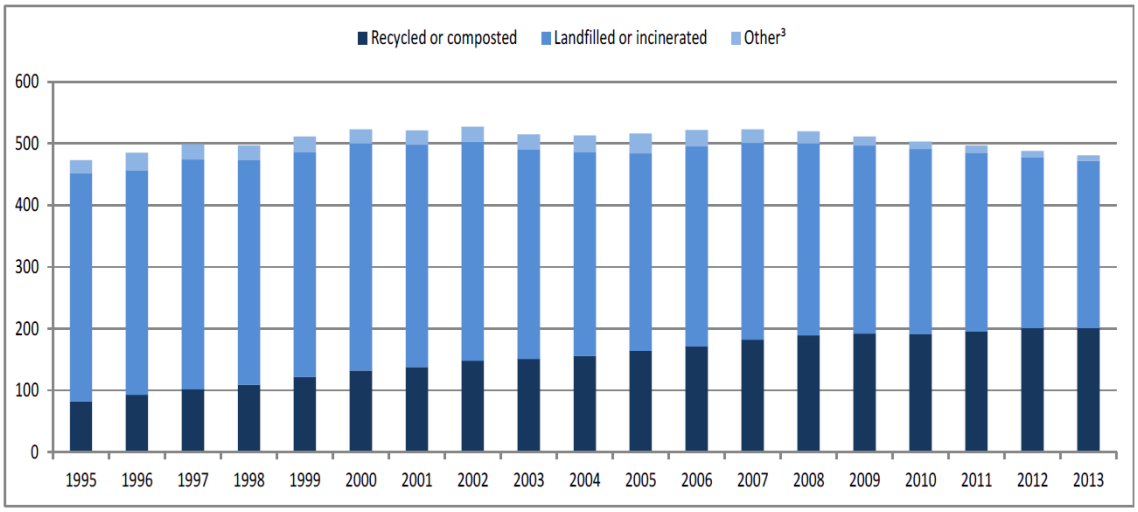

Figure I.13 – Municipal waste generation and treatment in the European Union (in kg per person). European Union refers to EU27 (excluding Croatia) for the years 1995 to 2006 and to EU28 from 2007 onwards. Data from 2015. [27]

Another study (Comelli *et al*. 2016) revealed that wastewaters originated in beverages industries can be used as raw material for bioethanol production, since they are rich in free sugars and generated in large quantities [24].

Therefore, the use of this kind of wastes as a substrate for bioethanol production will contribute to a sustainable solution for the huge amount of devaluated residues produced every year.

There are also ongoing studies about the production of third generation biofuels (biodiesel and bioethanol) using micro-algae. This group of unicellular photoautotrophic organisms have attracted much attention in the last years due to their potential values as renewable energy source. Microalgae produce and store large quantities of triacylglycerols that can be converted in biodiesel through transesterification. The remaining carbohydrate content can also be used for bioethanol production via fermentation. The main advantages are that they are safe, biodegradable, do not compete with arable land in their production, they are highly productive, quick to cultivate and simply require CO_2, sunlight inorganic nutrients and water to grow. Nonetheless, large scale production of microalgae is very costly [9].

Concerning the raw materials mentioned, since each of them present a very complex and variable composition, all of them need a pre-treatment step before they can serve as substrate for a fermentation.

I.6.2 Feedstock Pre-Treatment

The pre-treatment is an important step since it is necessary to convert the complex polysaccharides existing in the substrate to simple fermentable sugars. This happens because ethanol producing microorganisms can use a variety of hexoses, like glucose and fructose, but a limited number of disaccharides (lactose, cellobiose and maltose) and rarely their polymers [19]. Depending on the substrate, the pre-treatment differs.

Regarding sugarcane, the pre-treatment consists of the following steps: milling, washing, cutting into pieces (20-25cm), pressing for the collection of the sugar juice with 10-15% of sucrose, filtration of the juice, chemical treatment and pasteurisation, second filtration of the juice producing vinasse, evaporation of the syrup, cooling, in which a mixture of sugar crystals and molasses are formed, and the separation of the molasses from the sugar crystals by centrifugation. Molasses are fermented so that bioethanol can be obtained, and sugar crystals are dried and ready to be commercialized. During this pre-treatment, one by-product is produced, bagasse, that is a solid waste from the sugarcane and it can be used as a fuel for the bioethanol plant boilers so that it can produce heat and steam on a self-sufficient basis. After fermentation, the broth is centrifuged to recover the yeasts and distilled. During distillation, ethanol is separated from vinasse, which is also produced as a second by-product, considered as a waste product and an environmental hazard due to its viscous nature and high acid content. Some uses include combustion and as a fertiliser [19][28].

Considering feedstocks rich in starch, the most employed method for pre-treatment is acid or enzymatic hydrolysis, in order to break down its long chain into simple glucose molecules. The acid hydrolysis is a rapid process but the major drawback is its high costs, corrosion problems and the products of sugar degradation that inhibit fermentation. During acid hydrolysis, xylose is produced and this component is degraded rapidly to furfural, 5-hydroxymethyl furfural, acetate, hydroxybenzaldehyde, vanillin, among others, that exert an inhibitory effect on yeast growth [25]. Enzymatic hydrolysis has two steps, liquefaction and saccharification. In the first one, a partial hydrolysis of starch occurs, using amylases and other enzymes at high temperature, converting starch into maltose. The conversion to glucose occurs during the saccharificartion step [19].

As to lignocellulosic materials, they are composed of three main components, namely hemicellulose, lignin and cellulose. Pre-treatment is necessary to unwind cellulose from hemicelluloses and lignin in which cellulose is embedded, and make cellulose more susceptible for enzymatic hydrolysis. The net made by the hemicellulose and the lignin can be broken using milling, as a physical pre-treatment, chemically hydrolysed or biologically hydrolysed. Usually, lignocellulosic materials are milled or ground, and then they suffer the chemical or biological treatment. The best alternative is the biological pre-treatment, since it requires less energy inputs, and so, it is less costly. Moreover, biological processes do not consume chemicals and the organisms used are self-sustained. The main disadvantages are the low rates of conversion, which increases the length of the pre-treatment, and that the used microorganisms consume some of the carbohydrates available, which reduces the final yield of the pre-treatment. After this process, the product obtained still need to be hydrolysed in order to convert cellulose into glucose. This can be done chemically or enzymatically. Enzymatic hydrolysis is considered the best option since it requires less energy as compared to acid hydrolysis [1][7].

I.6.3 Fermentation

In every fermentation, the main challenge is to reach the best yield (product formed/substrate consumed) as well as the highest reaction rates and productivity. Furthermore, the synthesis of secondary products must be minimized, so that yields and downstream processes would not be affected [26]. Low equipment cost must be also take into account [19].

In order to accomplish these objectives, some factors must be considered and optimized. They are: pH, temperature, medium composition, fermentation time, initial sugar concentration, agitation rate and aeration. Depending on the microorganisms used, the conditions differ.

A microorganism used in a fermentation should present high growth and fermentation rate, high ethanol yield, high glucose and ethanol tolerance, growth under mild pH and temperature conditions and tolerance to potential inhibitors [19]. Genetically modified microorganisms (GMMs) have been developed in order to obtain higher yield of ethanol by better and wider substrate utilization [1].

Many microorganisms have been studied for bioethanol production. *Saccharomyces diastaticus*, *Kluyveromyces marxianus*, *Pichia kudriavzevii*, *Escherichia coli* strain KO11, *Klebsiella oxytoca* strain P2 and *Zymomonas mobilis* are some of the possibilities. Nevertheless, *Saccharomyces cerevisiae* is the most used and studied. This yeast is very efficient in sugar conversion to alcohol and presents a high tolerance to ethanol. Its optimal conditions are: pH range 4,0-5,0 and temperature 30˚C [15]. Moreover, it has the ability to convert fructose to glucose because of the presence of the invertase enzyme, also known as β-fructosidase, in the yeast periplasm, which regarding many substrates is an advantage [29][30]. However, it can only metabolise hexoses, and so researches aim to find a microorganism as efficient as *S. cerevisiae* but capable of converting other compounds [1]. In Table I.3 are presented values of ethanol concentration, productivity and ethanol yields for microorganisms used in sugar cane processing and each fermentation mode.

Table I.3 – Microorganisms used and fermentation mode for sugar cane [15]

Feedstock	**Fermentation Mode**	**Microorganisms**	**Ethanol concentration (g/L)**	**Productivity (g/L/h)**	**Ethanol yield from sugar (%)**
Sugar cane	Batch	*K. marxianus* DMKU 3-1042	67.9	1.42	60.4
	Batch	*P.kudriavzevii*	71.9	4.00	-
	Batch	S.cerevisiae	89.73	2.48	-
	Batch	*K. marxianus* DMKU 3-1042 and S.cerevisiae M30	77.3-81.4	1.07-1.10	80.23-86.10
	Continuous	Strains of *Saccharomyces* sp.	13.3-19.4	-	80.4-97.30
	Continuous	*S. cerevisiae* IR-2	90	18	99

In the case of *S. cerevisiae*, the metabolic pathway that originates ethanol starts with the degradation of glucose through glycolysis (or the Embden-Meyerhof-Parnas pathway) into two pyruvate molecules [31]. Then, acetaldehyde dehydrogenase converts each pyruvate molecule into acetyl-CoA that originates ethanol by the action of ethanol dehydrogenase [21]. In Figure I.14 and Figure I.15, it is shown the entire metabolic pathway in detail.

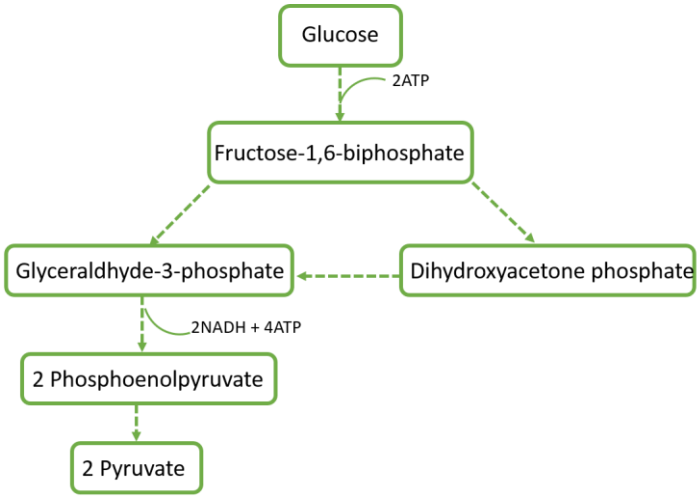

Figure I.14 – Emben-Meyerhof-Parnas pathway [21].

Figure I.15 – Alcoholic Fermentation [21].

The overall equation for ethanol production is:

$$C_6H_{12}O_6 \rightarrow 2CO_2 + 2\,C_2H_6O \qquad \textbf{(I.1)}$$

Alcoholic fermentation can be carried out in a conventional fermenter, where cells are suspended in a medium rich in nutrients such as organic nitrogen, trace metals and vitamins. Nonetheless, it is interesting to explore other ways of producing ethanol in order to diminish production costs [24]. For instance, the use of biofilms for ethanol production has been investigated to improve economics and the performance of fermentation processes [31].

Many researchers have been realising that one way of increasing the production of ethanol is combining enzyme hydrolysis and fermentation in the same step, a process which is called simultaneous saccharification and fermentation (SSF). This method reduces the accumulation of glucose and hence yields increase [1].

As an alternative to the use of cells in suspension, many studies have been investigating the advantages of cells immobilization. Results show that this technique enhances the productivity and ethanol yield and reduces inhibitory effect of high substrate and product concentrations. In addition, immobilization prevents cell washout in continuous fermentation, and hence, cell separation and recycling are not required for maintaining high cell density in the bioreactor. Thus, the bioprocess can be operated more efficiently [15][19]. Besides, the removal of microorganisms from the downstream product stream can be omitted [31].

Nonetheless, the immobilization process changes the environmental, physiological and morphological characteristics of cells, along with their catalytic activity. Internal mass transfer limitations affect the effectiveness of the biocatalysts and the cells deep inside the bioparticle may

become inactive due either to deprivation of some essential nutrients or to accumulation of products to inhibiting concentrations [32].

There are four main immobilization techniques for yeast cells: containment behind barriers, attachment to a supports surface, entrapment within a porous matrix and cell aggregation (flocculation). Containment behind a barrier can be achieved by two main methods: entrapment of the cells in microcapsules or use of a filtration membrane, such as hollow fiber [33]. The first two immobilization techniques are the most extensively studied [34][35]. In Table I.4 are presented types of immobilization, the materials or processes used and applications for each.

Table I.4 – Type of immobilization, materials and processes used and applications for each [26]

Type of immobilization	Materials	Applications
Surface adsortion	Cellulose Cellulose derivates Fragments of vegetables Gluten pellets Wood chips Minerals such as aluminium Ceramics	Winemaking Brewing Ethanol production
Inclusion in a matrix	Polyacrylamide Cellophane Cellulose Alginate Agar Carrageenans	Brewing
Cell retention without external support	Membrane Flocculation aids	Alcohol production Sparkling wine making (second fermentation) Some kinds of beers

Cell immobilization by attachment to different surfaces is a widely used technique because of its simplicity, low costs and high efficiency. However, biofilm detachment from the surface is a commonly observed problem [35].

Several carriers have been reported for cell immobilization including apple pieces, k-carrageenan gel, polyacrylamide, g-alumina, chrysotile and calcium alginate. Immobilization of *S.cerevisiae* can easily be carried out by taking enriched cells from culture media at the log phase of growth followed by entrapping into the carriers [15]. Among the mentioned materials, k-carrageenan and calcium alginate are the most used. Such matrices are more frequently used because of high biomass loading and stability of immobilized microbial cells, but they are not appropriate for industrial-scale production because of the relatively complex procedure [35]. K-carrageenan has been used for this purpose due to its rapid gelification in the presence of potassium ions. Immobilization conditions do not require drastic changes of temperature and pressure, which could affect the activity, and viability of microorganisms [2]. Regarding calcium alginate entrapment, it is a simple, economic and not toxic procedure [34]. Other studies have been made,

in which other kind of immobilization materials have been used. For instance, Borovikova *et al.* (2014) showed that ceramic carriers are a potentially suitable material for immobilization. Some authors also have studied the use of sugarcane pieces, banana leaf sheath and orange peel as supports for immobilization [15]. This subject is extensively explained in the chapter "Yeasts as Biocatalysts" of the book *Yeast in Food and Beverages* (2006) [26].

I.6.4 Distillation

The distillation step aims to recover the ethanol (normal boiling temperature 78,3˚C) to obtain hydrous ethanol at 92-96% v/v due to an azeotrope formation between water and ethanol [19].

An economical evaluation of ethanol fermentation processes revealed that more energy is consumed in the recovery steps conducted by distillation due to low ethanol concentration in fermented broth. Therefore, a way of reducing ethanol production costs would be to increase ethanol content in the broth, and so the energy input during distillation would diminish [15].

I.6.5 Dehydration

The dehydration step is used in order to obtain anhydrous ethanol at ≥ 98,7%. This step is needed so that legal requirements for normalization of bioethanol quality (EN15376) are fulfilled. This process is called bioethanol standardization and it is needed because bioethanol can be produced from different raw materials [19]. In Table I.5 the requirements and the test methods for bioethanol are shown.

Table I.5 – General applicable requirements and test methods for undenatured ethanol [35]

PROPERTY	UNIT	LIMITS		TEST METHOD [a]
		Minimum	Maximum	
Ethanol content + higher saturated alcohols	% *(m/m)*	98,7		EC/2870/2000 - method I, Appendix II, Method B [b]
Higher saturated (C3-C5) mono-alcohols content	% *(m/m)*		2,0	EC/2870/2000 - method III [b]
Methanol content	% *(m/m)*		1,0	EC/2870/2000 - method III [b]
Water content	% *(m/m)*		0,300	EN 15489 [c]
Inorganic chloride content	mg/l		20,0	EN 15484 or prEN 15492 [d]
Copper content	mg/kg		0,100	EN 15488
Total acidity (expressed as acetic acid)	% *(m/m)*		0,007	EN 15491
Appearance		Clear and bright		Visual inspection [c]
Phosphorus content	mg/l		0,50	EN 15487
Non-volatile material content	mg/100ml		10	EC/2870/2000 - method II [e]
Sulfur content	mg/kg		10,0	EN 15485 or EN 15486 [f]

Chapter II

Materials and Methods

Summary

In this chapter is presented a detailed explanation of all the experimental procedures carried out for the study of the influence of glucose concentration on bioethanol production using suspended and immobilized *Saccharomyces cerevisiae*. There is also a subchapter in which are described all the methods used for the determinations of glucose, ethanol and biomass concentrations and chemical oxygen demand. At last, it is described the procedure taken to obtain scanning electron microscopy pictures of immobilized cells.

II.1 Culture media and preculture conditions

An industrial winery strain of *Saccharomyces cerevisiae* BC S103 from Springer Oenologie was used [37]. This strain is commercialized in lyophilized form. In order to obtain a cellular suspension, 2.5 g/L of commercial yeast was added to 50 mL of a sterilized YEPG medium (peptone, yeast extract, glucose, 20, 10 and 20 g/L respectively) and kept in a 100 mL erlenmeyer flask with a cotton stopper for 48 hours at 30˚C. Agitation was carried out by a magnetic stirrer. Samples were taken twice a day, and optical density (OD) was measured, as described in subchapter II.5.

Once the stationary phase was reached, 500 mL of a new medium was prepared (composition in Table II.1) and inoculated with 1mL of the fermentation broth obtained as described above.

Table II.1 – Components of the fermentation medium and its concentration [38]

Component	Concentration (g/L)
$(NH_4)_2SO_4$	6
KH_2PO_4	4
$MgSO_4.7H_2O$	2
Glucose	200
Trace metals solution	2 mL/L

In order to maintain the culture under anaerobic conditions, nitrogen was sparged into the bottle, as shown in Figure II.1, for 5 minutes at the beginning of the fermentation. It was maintained at 30˚C and agitation was carried out by a magnetic stirrer. Samples were taken twice a day. After four days, cells were still active (in the exponential growth phase). At the end of this time, an equal system was prepared again, so that yeast inoculum ready to be used would be always available.

Figure II.12 – System used for inoculum yeast

The trace metals solution used in the new culture medium was prepared according to the method described in [39]. All the components except EDTA were dissolved in 900 mL ultrapure water at pH 6, adjusted using a 20% KOH solution and a pH meter (Crison GLP22). The solution was then gently heated and EDTA was added. In the end, the pH was adjusted to 4 with a 20% KOH solution, and the volume was brought to 1L. This solution was stored at 6˚C. Its composition is presented in Table II.2.

Table II.2 - Components and concentration of salts in the trace metal solution

Component of the trace metal solution	Concentration (g/L)
EDTA	15.0
$ZnSO_4.7H_2O$	4.5
$MnCl_2.2H_2O$	0.84
$CoCl_2.6H_2O$	0.3
$CuSO_4.5H_2O$	0.3
$Na_2MoO_4.2H_2O$	0.4
$CaCl_2.2H_2O$	4.5
$FeSO_4.7H2O$	3.0
H_3BO_3	1.0
KI	0.1

II.2 **First Essay:** Influence of initial glucose concentration on bioethanol production using suspended *Saccharomyces cerevisiae*

II.2.1 Bioreactor system and fermentation conditions

With the purpose of studying the influence of initial glucose concentration on bioethanol production using suspended yeast, a system was used which employed 100 mL erlenmeyers closed with rubber caps perforated with one 1.2 mm x 40 mm needle. This system is shown in Figure II.2.

Figure II.13 – System used as bioreactor.

The culture medium used had the same composition as the one presented in Table II.1, only differing in the initial glucose concentration. Five different initial glucose concentrations were studied (50, 100, 200, 300 and 400 g/L). The culture media were inoculated with 1mL of yeast inoculum, prepared as described in section II.1. The total volume (culture medium + inoculum) was 100mL. Nitrogen was flushed into the vessels at the beginning of the fermentation for 5 minutes, so that anaerobic conditions were guaranteed. The agitation (orbital, 150 rpm) and the temperature (30˚C) in the bioreactors were provided by a thermostatic shaker plate Barnstead Lab-line Max Q 4000 E-class [20]. The fermentation time was 120 hours.

Two different essays were carried out. In the first, CO_2 production was tentatively assessed by weight loss using a precision balance ($\pm$0.01 g), and in the second 5mL samples were taken from the broth and analysed twice a day. There were two experimental replicas for each essay and all samples were analysed in duplicate. Every time a sample was taken, nitrogen was flushed again for 5 minutes into the vessel so that anaerobic conditions were ensured.

For each 5mL sample, optical density (OD), glucose and ethanol concentrations were measured. The analytical methods used for these measurements are described in subchapter II.5 In the weight loss essays, OD was only measured in the beginning and at the end of the 120 hours of

fermentation. OD values were converted into biomass concentration values using the standard curve presented in subchapter II.5.

II.3 **Second Essay:** Influence of initial glucose concentration on bioethanol production using immobilized *Saccharomyces cerevisiae*

II.3.1 Bioreactor system and fermentation conditions

The system used for this essay was the same as the one presented in Figure II.13. The culture medium had the same composition as the one presented in Table II.1, with the exception that five different initial glucose concentrations were studied (50, 100, 200, 300 and 400 g/L).

There were also two series of erlenmeyers flasks, one from which samples were taken and another for which CO_2 production was tentatively measured by weight loss. There were two experimental replicas for each series and samples were taken twice a day and analyses were performed in duplicate. Samples were taken from the culture liquid for glucose and ethanol analyses, and biomass concentration inside the immobilization beads was only measured in the beginning and at the end of the fermentation. Methods used for glucose, ethanol and biomass quantification are described in subchapter II.5. In these essays, for glucose and ethanol detection, samples had to be centrifuged for 10 minutes at 6000 rpm and the supernatants filtered before the readings. Filters of 0.45 µm nominal pore diameter were used for that purpose [31].

The fermentation temperature in this case was 37°C [31]. Agitation rate of 180 rpm (orbital) was enough to maintain all beads in suspension and completely mixed with the culture medium. The fermentation time was also 120 hours.

II.3.2 Immobilization method

A suspension of *S. cerevisiae* (biomass concentration of 0.84 - 1.36 g/L; suspension A) and a 2% solution of sodium alginate in ultrapure water were mixed together at equal volumes (100mL of each solution) and stirred until a homogeneous suspension (suspension B) was visible. Then, with a micropipette, the suspension B was added dropwise to 200 mL of a 0.2M $CaCl_2$ solution prepared in a 0,05M sodium acetate buffer (pH 5.6). Beads were kept in suspension for 30 min at room temperature to harden. To remove calcium ion excess and free cells, beads were washed with 10 mL of a 0.01M sodium acetate buffer of pH 5.6. The beads were used immediately in order to avoid too much contact with oxygen [2]. It was established that each erlenmeyer flask would have a number of beads equivalent to 20 mL of suspension B, in order to guarantee that each experiment had approximately the same amount of biomass [34].

In order to calculate the encapsulation efficiency (EE), beads were made with 20 mL of suspension B, and from these about 100 beads (which is equivalent to more or less 3 g) were weighed in a precision balance (±0.0001 g) and then dissolved in a 30mL sodium citrate solution (0.1M) at room temperature, continuously stirring until disintegration (suspension C) [34]. From

the resulting cell suspension, a sample of 2 mL was taken into an Eppendorf tube and centrifuged for 10 min at 5000 rpm. The supernatant was removed and the *pellet* was re-suspended in 2 mL of acetate buffer in order to dilute the concentration of the alginate in solution. OD was measured. Samples were taken in triplicate. This procedure was used to determine initial and final biomass concentrations in the beads used in the fermentation experiments.

EE was calculated by the equation II.1, in which $[Biomass]_{beads}$ (biomass concentration inside the beads in g of cells per g of beads) is described by equation II.3 [20]. In equation II.1, W_{Beads}^{Total}, $\mathrm{V}_{suspA/beads}$, $[Biomass]_{susp.A}$ represent, respectively, the total weight of the beads equivalent to 20 mL of suspension B, the volume of the suspension A used in the 20 mL of suspension B (which is equivalent to 10 mL) and biomass concentration in suspension A. Since W_{Beads}^{Total} was not measured, its value was calculated from the volume of suspension B used for each essay (20 mL; described by V_{beads}) and water density (ρ_{water}), as it is shown in equation II.2. In equation II.3, $[Biomass]_{susp.C}$, $V_{susp.C}$, W_{sample}, represent, respectively, biomass concentration in suspension C, final volume of the suspension C (described by equation II.4), and the measured value for weight of the sample of beads (around ±3 g). In equation II.4, $V_{citrate\ sol.}$ stands for the volume of 0.1M citrate solution (30 mL).

$$EE(\%) = \frac{W_{Beads}^{Total} \times [Biomass]_{beads}}{\mathrm{V}_{suspA/beads} \times [Biomass]_{susp.A}} \times 100 \quad \textbf{(II.1)}$$

$$W_{Beads}^{Total} = V_{beads} \times \rho_{water} \quad \textbf{(II.2)}$$

$$[Biomass]_{Beads} = \frac{[Biomass]_{susp.C} \times V_{susp.C}}{W_{sample}} \quad \textbf{(II.3)}$$

$$V_{susp.c} = V_{citrate\ sol.} + \frac{W_{sample}}{\rho_{water}} \quad \textbf{(II.4)}$$

There is the possibility of the remaining alginate causing turbidity and bias in the OD results. In order to verify this, beads without cell suspension were made and they suffered the same treatment as the ones with cells. Around 3 g were weighed, dissolved in 30 mL of 0.1M citrate solution, centrifuged and OD was measured. This OD value (0.028) was taken into account for the final values of OD of beads with cells, as described by equation II.5, in which OD_{final} is the final value used in further calculations, $OD^{beads+cells}$ is the optical density read in the spectrophotometer and OD^{beads} is the optical density of dissolved alginate beads.

$$OD_{final} = OD^{beads+cells} - OD^{beads} \quad \textbf{(II.5)}$$

II.4 **Third Essay:** Bioethanol production from the Organic Fraction of Municipal Solid Wastes (OFMSW)

II.4.1 Waste pre-treatment: preparation of the synthetic OFMSW

In order to study the production of bioethanol using the Organic Fraction of Municipal Solid Wastes (OFMSW) as substrate, a mixture of different components was prepared simulating a typical organic fraction of municipal wastes. The composition of this mixture is shown in Table II.3 and it is based on the one presented in reference [40].

Table II.3 – Composition of the synthetic OFMSW

Component	% (w/w)	Mass (g)
Bread	7	35
Banana	7	35
Paper	10	50
Apple	9.8	49
Onion	6	30
Tomato	4	20
Peas	2.6	13
Potato	12.4	62
Carrot	5.6	28
Cauliflower	2	10
Cabbage	10.2	51
Orange	9.8	49
Boiled rice	4.6	23
Boiled pasta	4.6	23
Minced meat	4.4	22
Total	100	500

1.5 L of water was added to these 500 g of raw materials and everything was blended using a Braun® 5000 WH hand blender until a homogeneous mixture was obtained. It was kept in a vessel for 24 hours under anaerobic conditions, since at the beginning of this time nitrogen was flushed into the vessel for 10 minutes. Agitation was carried out by a magnetic stirrer. At the end of the 24 hours, the mixture was filtered using a common strainer (15 cm of diameter) in order to remove bigger particles. Since there still were small particles in suspension, the filtrated was decanted for 12 hours. The resulting supernatant was vacuum filtered using a VWR® 0.7µm glass microfiber filter. Approximately 1L of clarified liquid rich in organic matter was obtained. COD (chemical oxygen demand) was measured and the liquid was stored in a freezer.

In Figure II.3 is shown the procedure adopted for the production of the substrate for the alcoholic fermentation.

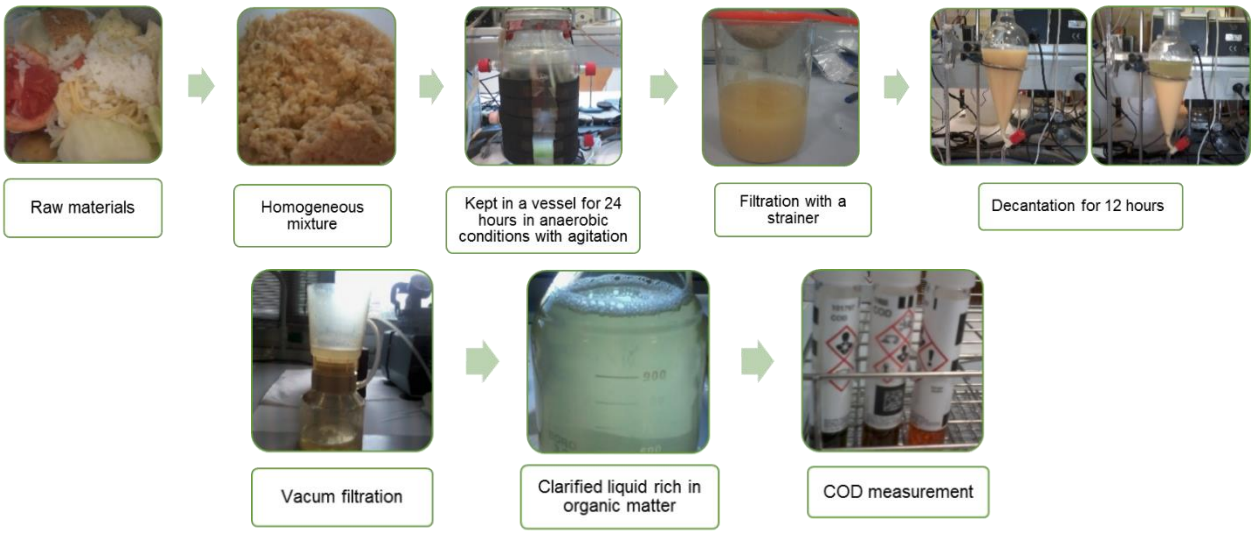

Figure II.14 – Procedure adopted for the production of clarified liquid rich in organic matter.

II.4.2 Fermentation conditions

The liquid rich in organic matter was used as culture medium for alcoholic fermentation. In order to infer if there was any substance that could induce inhibition in biomass growth, this liquid was used in three different concentrations: pure, diluted 1:2 and diluted 1:4. In all cases, salts and the trace metal solution was added to the culture medium in the concentrations presented in Table II.1. The system used as bioreactor was the same as the one presented in Figure II.2. Essays were carried out with suspended and immobilized yeast. The total volume (inoculum + culture medium) was 100 mL. In essays with suspended yeast, inoculum was 1 mL, and it came from the system described in subchapter II.1. In essays with immobilized yeast, each erlenmeyer had an amount of beads equivalent to 20 mL of suspension A. The immobilization method and the procedures to determine EE, initial and final biomass concentration were the same as described in subchapter II.3.

The fermentation time was 96 hours and samples of 5 mL were taken twice a day. Ethanol, glucose and biomass concentrations were measured as described in subchapter II.5. For the determination of biomass concentration, OD was measured using water as zero instead of the liquid rich in organic matter. It was considered that this liquid was clarified enough so that it would not interfere in OD values.

II.5 Analytical methods

II.5.1 Initial biomass concentration

Initial biomass concentration was assessed by OD (optical density) at 600 nm in a Hach DR/4000 spectrophotometer. In order to establish a correlation between OD values and biomass concentration, a standard curve was obtained. For that, a sample of the culture medium was taken and successively diluted in 0,05M sodium acetate buffer (pH 5.6). For each dilution, OD, total suspended solids (TSS) and volatile suspended solids (VSS) were measured and the relation between OD and biomass concentration was established.

TSS and VSS were determined by gravimetry and values come in g of dry weight per litre (gDW/L). 50 mL of the cellular suspension were filtered using pre-dried and pre-weighed VWR® 0.7µm glass microfibres filters. This procedure was done in duplicate. All filters were kept for four hours in a 105±3˚C drying oven. At the end of the four hours, filters were cooled in a desiccator and weighed again. TSS was calculated using equation II.6, in which $W_{105°}$, W_{filter} and $V_{cellular\ suspension}$ represent, respectively, weight of the filter plus sample after drying in the oven, weight of the pre-dried filter, and filtered volume of the cellular suspension [41].

$$TSS(gDW/L) = \frac{W_{105°} - W_{filter}}{V_{cellular\ suspension}} \quad \textbf{(II.6)}$$

Then the dried filters were transferred into a pre-heated muffle furnace at 550±0.01˚C for two hours. After being cooled down again, they were weighed and VSS were determined. Equation II.7 describes VSS calculation, in which $W_{after\ muffla}$ represents the weight of the filter plus sample after muffle drying [41].

$$VSS(gDW/L) = \frac{W_{105°} - W_{after\ muffle}}{V_{cellular\ suspension}} \quad \textbf{(II.7)}$$

Although TSS and VSS were both measured, a standard curve between TSS and OD was the one used to express biomass concentration. Both standard curves and their correlation factors are presented by equations II.8, II.9, II.11 and II.12. TSS_{max} and VSS_{max}, described by equations II.10 and II.13, stand for the maximum measured value, above which linearity is not ensured.

$$OD = 1.2717\ TSS + 0.1344 \quad \textbf{(II.8)}$$

$$R^2 = 0.9336 \quad \textbf{(II.9)}$$

$$TSS_{max} = 0.915\ gDW/L \quad \textbf{(II.10)}$$

$$OD = 1.3456\ VSS + 0.1125 \quad \textbf{(II.11)}$$

$$R^2 = 0.9392 \quad \textbf{(II.12)}$$

$$VSS_{max} = 0.880\ gDW/L \quad \textbf{(II.13)}$$

II.5.2 Glucose concentration measurement

After OD measurement, each sample was filtered using 0.45 µm 33 mm nylon Sartorius filters so that glucose concentration could be determined in the filtrate.

To determine the concentration of glucose, a reducing chemical reagent, 3,5-dinitrosalicylic acid 98% solution (DNS), was used. The DNS solution was prepared by dissolving 10g of 3,5-dinitrosalicylic acid in 2M sodium hidroxide solution. A separate solution of 300g sodium potassium tartrate solution was prepared in 300 mL of distilled water. The hot alkaline 3,5-dinitrosalicylate solution was added to sodium potassium tartrate solution. The final volume of DNS solution was made up to 1L with distilled water.

In a test tube, 0.5 mL of DNS and 0.5mL of the sample were added. Test tubes were covered and inserted in a thermostatic bath (100˚C) for 5 min. Then the test tubes were cooled down with running water and 5 mL of water were added to each tube. The solution was homogenized in a vortex and the absorbance at 540nm was measured, against a sample of acetate buffer pH 4.5, that suffered the same treatment as the medium samples [2]. The calibration curve (equation II.14; correlation factor in equation II.15) was prepared with 2 g/L, 1 g/L, 0.5 g/L and 0.25 g/L glucose solutions [2]. $Abs^{540\ nm}$, $[Glucose]$ and $Abs_{max}^{540\ nm}$ (equation II.16) stand for, respectively, the absorbance measured at 540 nm, glucose concentration and the maximum measured value of absorbance at 540 nm, above which linearity is not ensured.

$$Abs^{540\ nm} = 0.5481[Glucose](g/L) - 0.0275 \textbf{ (III.14)}$$

$$R^2 = 0.9981 \textbf{ (III.15)}$$

$$Abs_{max}^{540\ nm} = 1.079 \textbf{ (II.16)}$$

Glucose concentration was also determined by HPLC (Agilent 1100 Series DEGASSER) equipped with ultra-violet (UV) detector. The method was carried out under the following conditions: UV detection was made at 195 nm with column temperature of 30˚C, flow rate of 0.6 mL/min and injections of 20 µL. The mobile phase was acetonitrile and deionised water with a ratio of 75% to 25%. This method was based in the one described in references [20] and [42]. All samples were analysed in duplicate and the sugar concentration was determined according to the standard curve described by equation II.17, where peak area comes in mili absorbance units (mAU) (correlation factor in equation II.18), and [Glucose] is the glucose concentration. $Peak\ area_{max}$ (equation II.19) stands for the maximum peak area value measured, above which linearity is not ensured.

$$Peak\ area\ (mAU) = 91.462[Glucose](g/L) + 400.53 \textbf{ (II.17)}$$

$$R^2 = 0.9969 \textbf{ (II.18)}$$

$Peak\ area_{max}$= 37235.7 mAU **(II.19)**

II.5.3 Ethanol concentration measurement

After OD measurement, each sample was filtered using 0.45µm 33mm nylon Sartorius filter so that ethanol concentration could be determined in the filtrate.

Ethanol was measured by gas chromatography, YL 6100 GC (6000 series GC system controller), equipped with a flame ionization detector (FID) and a SDM 6100 special detector module. Injector and detector temperatures were maintained at 200°C and 220°C, respectively. N_2 was the carrier gas. Standard curve obtained is described by equation II.20 (correlation factor in equation II.21), in which [Ethanol] stands for ethanol concentration. $Peak\ area_{max}$ (equation II.22) stands for the maximum peak area value measured, above which linearity is not ensured.

$$Peak\ area\ (\mu V.s) = 5786.7[Ethanol](g/L) + 74407 \quad \textbf{(II.20)}$$

$$R^2 = 0.9177 \quad \textbf{(II.21)}$$

$$Peak\ area_{max} = 1178960.1 \quad \textbf{(II.22)}$$

II.5.4 COD measurement

COD was measured using a proper kit (Spectroquant® COD cell test). A sample of the clarified liquid obtained was added to the kit solution. This mixture was heated in a thermoreactor Eco 16, cooled down to room temperature and absorbance was measured in a Hach DR/4000 spectrophotometer. Detailed instructions can be obtained from manufacturer.

II.6 Scanning electron microscopy of immobilized cells

SEM (scanning electron microscopy) images were taken from beads before and after the fermentation process. To accomplish that, beads suffered a fixation treatment, followed by dehydration, and finally a chemical drying step [43].

For the step of fixation treatment, a 2% buffered glutaraldehyde solution was prepared previously, adding 5.96 g of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) in 10 mL of a 50% glutaraldehyde solution in water, and the final volume was brought to 250 mL with distilled water.

A small amount of beads (5-10 beads) was immersed in a fixative 2% buffered glutaraldehyde solution overnight. The volume of fixative should be 10 to 20 times the volume of the sample. After this, beads were rinsed with approximately 2 mL of 0,1M HEPES buffer (pH 7.0) 3 times for 5 minutes each with gentle agitation.

The dehydration step was done as follows (the volume used for each step was about 3 mL):

- 50 % ethanol, 2 times for 10 minutes each with agitation;
- 70 % ethanol, 2 times for 10 minutes each with agitation;
- 95 % ethanol, 2 times for 10 minutes each with agitation;
- 100 % ethanol, 3 times for 15 minutes each with agitation.

Chemical drying was done as follows (the volume used for each step was about 3 mL):

- (2 parts 100% ethanol: 1 part HMDS (bis(trimethylsilyl)amine)) for 15 minutes;
- (1 part 100% ethanol: 1 part HMDS) for 15 minutes;
- (1 part 100% ethanol: 2 parts HMDS) for 15 minutes;
- HMDS alone for 15 minutes, 3 times;
- Let the last HMDS evaporate in a fume hood overnight.

Finally, beads were mounted on specimen stubs and examined under SEM FAI Quanta 250.

Chapter III

Results and Discussion

Summary

In this chapter are analysed all the results obtained for the essays described in chapter II. There is also the application of a non-structured model for growth (Jackson and Edwards) to the experimental data in order to determine the kinetic constants of each system. These values were used for a comparison of essays. There was also the determination of biomass and product yield. All these obtained values were compared with the ones presented in literature.

III.1 Culture media and preculture conditions

As it was mentioned in subchapter II.1, the strain used in the present work was *Saccharomyces cerevisiae* BC S103 from Springer Oenologie since it presents a very good alcohol tolerance and low nitrogen requirements [37].

In Figure III.1 is presented the optical density (OD) vs time curve obtained for the yeast growth in the YEPG medium. Experimental data are presented in Table 1 in Annex I.

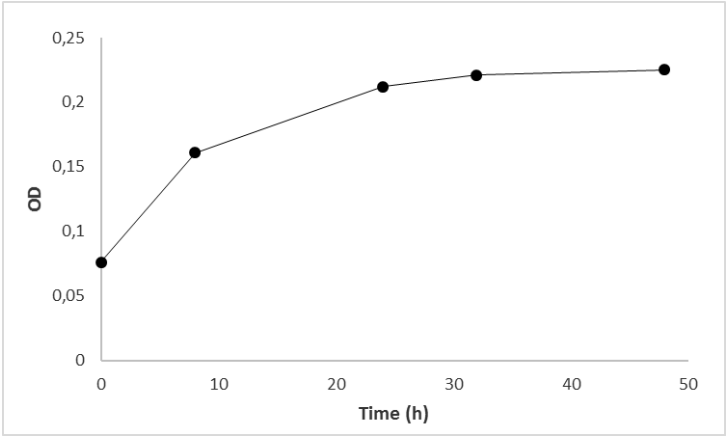

Figure III.15 – Optical density vs time in hours curve obtained for yeast growth in the YEPG medium.

Analysing Figure III.1, one can observe that yeast had a fast growth in the first 8 hours, and started to stabilize from then on, as it is verified by the decreasing slope. Therefore, the first 8 hours were considered to be the exponential phase, and the period of 8 to 48 hours was the stationary phase. Based on this data, specific growth rate (μ) for the conditions used (30ºC, atmospheric pressure, aerobic conditions, perfect agitation) was calculated through the representation of ln(OD) (natural logarithm of OD) vs time, as it is shown in Figure III.2, and its value corresponds to the slope of the obtained trend line (equation III.1). For this representation, only values from exponential phase were used, that is, the first two datapoints, as one can observe in Figure III.2. In Figure III.3 is presented the trend line described by equation III.1.

$$\ln(OD) = 0.0938t - 2.58 \quad \textbf{(III.1)}$$

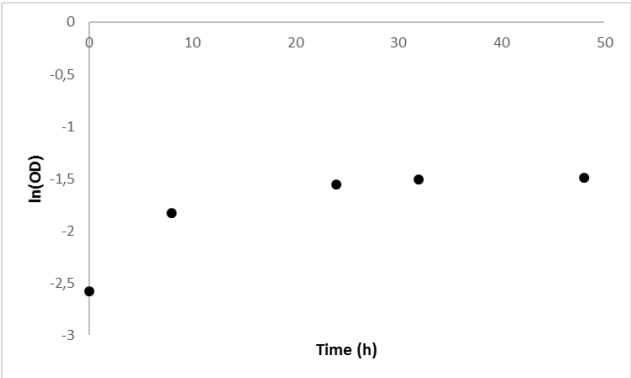

Figure III.2 – ln(OD) vs time for μ calculation in the case of yeast growth in the YEPG medium. Trend line described by equation III.1. Time range: exponential phase.

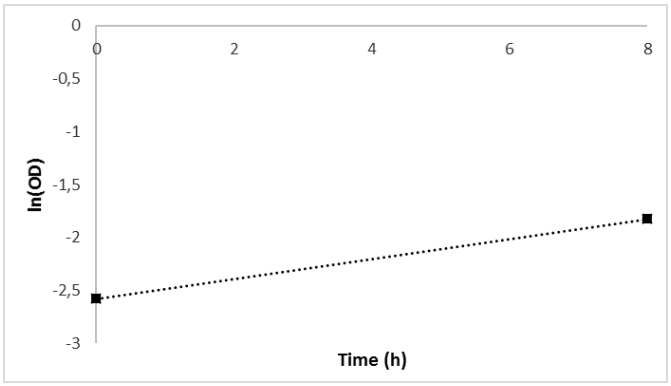

Figure III.3 –Trend line described by equation III.1.

Therefore, the value for specific growth rate is 0.0938 h^{-1}. It was also possible the calculation of the doubling time (t_d; time needed for the population to double its quantity), using equation III.2 [44]. The value obtained was 7.40 h.

$$t_d = \frac{\ln 2}{\mu} \quad \textbf{(III.2)}$$

In Figure III.4 is presented the obtained growth curve for *Saccharomyces cerevisiae* in the new medium (Table II.1). At the end of the 120 hours of fermentation, the medium was renewed and the new growth curve was similar to this one. Experimental data are presented in Table 2 in Annex I.

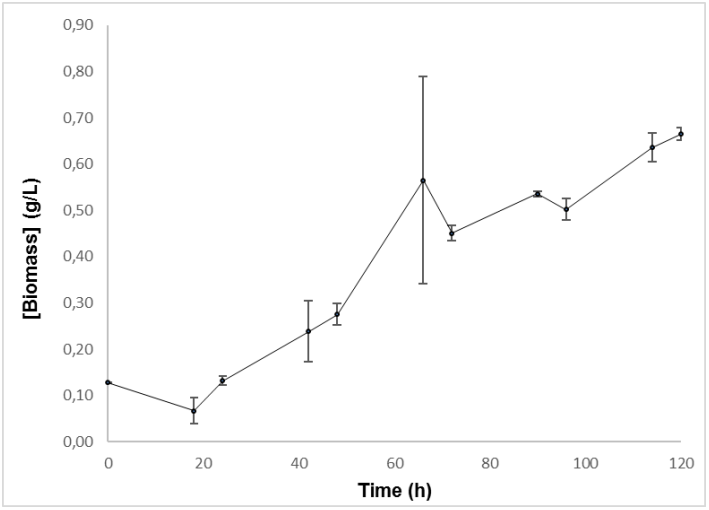

Figure III.4 – Growth curve obtained for *Saccharomyces cerevisiae* in the new medium (Table II.1) in which [Biomass] represents biomass concentration, and error bars represent standard deviation with n=2.

Observing Figure III.3, it is clear that yeast had to adapt itself to the new medium, since a lag phase is prominent at the beginning of the fermentation. This was expected since the inoculum came from a different medium, with different nutrients, and it was collected at late stationary phase. In this growth curve it is also clear that at the end of the fermentation, yeasts were still active, once there is no visible stationary phase.

A specific growth rate value was also calculated for this curve. In this case, it was considered that lag phase took the first 24 hours, and exponential phase began at 24 hours and lasted until the end of the fermentation (120 hours). The linearization is presented in Figure III.5, in which ln([Biomass]) stands for the natural logarithm of biomass concentration. Trend line and correlation factor are described, respectively, by equation III.3 and III.4.

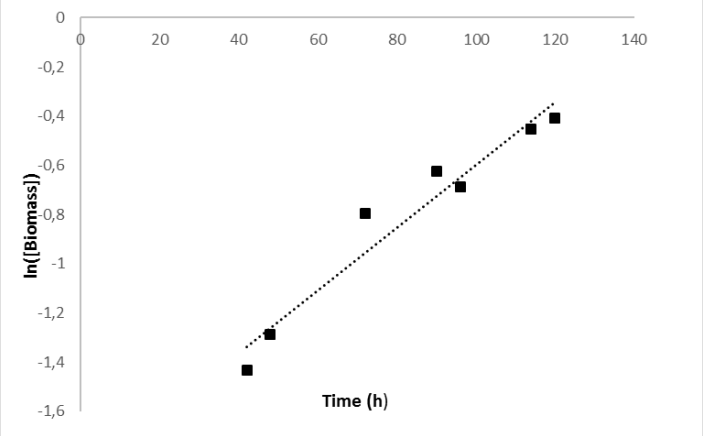

Figure III.5 – ln([Biomass]) vs time for μ calculation in the case of yeast growth in the new medium. Trend line described by equation III.4 and correlation factor by equation III.5. Time range: exponential phase.

$$\ln([Biomass]) = 0.0127t - 1.87 \quad \textbf{(III.3)}$$

$$R^2 = 0.9454 \quad \textbf{(III.4)}$$

Therefore, the values obtained for specific growth rate and for doubling time in the conditions used in this fermentation (30ºC, anaerobic conditions, perfect agitation) are, respectively, 0.0127 h^{-1} and 54.6 h.

Comparing both values obtained, one can conclude that yeast growth was faster in the first medium, since the specific growth rate calculated was higher in the first case than in the second, which means that the number of generations per hour during exponential phase was higher in the first medium. This difference was expected since YEPG medium was designed specifically for yeast growth, which means that has all the substrates yeast needs to grow and in the optimal concentrations. Therefore, yeast growth is expected to be higher in this medium than in the second one. Besides this, the second medium has a very high glucose concentration (200 g/L), which can be inhibitory for yeast growth. Another reason for the result obtained relies on the fact that the first medium is under aerobic conditions and the second in anaerobic conditions. Yeast can either grow in anaerobic and aerobic conditions, but it is slow and inefficient in the absence of oxygen. In aerobic conditions, yeasts mostly use substrate for growth and produces only a negligible quantities of alcohol, whereas in anaerobic conditions the substrate is used essentially for ethanol production [45].

III.2 **First Essay:** Influence of initial glucose concentration on bioethanol production using suspended *Saccharomyces cerevisiae*

III.2.1 Assay monitored by weight loss

The weight loss method was an attempt to establish an easier and more comfortable way of assessing the production of ethanol and biomass. The principle behind this method was the following:

$$Weight\ loss = mass\ of\ produced\ CO_2 \quad \textbf{(III.5)}$$

This principle is valid only if the following considerations are taken into account:

- All CO_2 produced is released to the atmosphere as a gas.
- There is no difference in pressure between the inside and outside of each erlenmeyer, allowing gas release, and therefore the measured weight loss is equivalent to the mass of gas liberated.
- The process does not involve the exchange of other gases with the atmosphere, namely ethanol, volatile acids, water, in significant amounts.

However, there is some important facts that are not being taken into account that introduce error in further calculations. Those are:

- CO_2 solubility in water (as an approximation, culture medium physical constants can be considered the same as water) is 1.25 g gas/kg water at atmospheric pressure at 30ºC (fermentation conditions) [46]. This value is high enough to be an error not to contemplate it in the calculation of actual mass of CO_2 released.
- When CO_2 is dissolved in water, there is a percentage that reacts with water to produce carbonic acid (H_2CO_3). This equilibrium is described by equation III.6. Carbonic acid reacts with water and produces carbonate (CO_3^{2-}) and H_3O^+ that affects the pH of the culture medium, which was not measured. pH would be an important variable, so that total amount of dissolved CO_2 could be determined.

$$CO_{2_{(l)}} + H_2O_{(l)} \leftrightarrow H_2CO_{3_{(l)}} \quad \textbf{(III.6)}$$

$$H_2CO_{3_{(l)}} + H_2O_{(l)} \leftrightarrow HCO_{3(l)}^- + H_3O_{(l)}^+ \quad \textbf{(III.7)}$$

$$HCO_{3\ (l)}^- + H_2O_{(l)} \leftrightarrow CO_{3(l)}^{2-} + H_3O_{(l)}^+ \quad \textbf{(III.8)}$$

Besides all the assumptions described above, there is also the error inherent to the precision balance that was used to measure weight loss. Therefore, this procedure leads to results with a

considerable error. Nevertheless, this method could be a good alternative to the typical procedure (analyses of samples taken from time to time) if other variables were also measured. It is a simple, easy to implement and non-invasive method. For instance, a good procedure could be:

- Built a system similar to the one described for this experiment in which the main objective is to determine a value for biomass yield ($Y_{x/s}$). For that biomass, ethanol and glucose concentration are assessed using analytical methods;
- Reproduce the same system, in which weight loss and actual CO_2 release are both measured, using a precision balance and a mass gas flow meter and analyser, respectively.

Weight loss, initial and final biomass concentration were the variables measured. This data can be used to calculate the stoichiometric amount of consumed glucose and produced ethanol using the macro chemical reaction described by equation III.9. The reduced formula of *Saccharomyces cerevisiae* was taken from reference [47].

$$C_6H_{12}O_6 + aNH_3 \rightarrow bCH_{1.83}O_{0.56}N_{0.17} + cC_2H_6O + dCO_2 + eH_2O \quad \textbf{(III.9)}$$

In order to determine the stoichiometric coefficients (a, b, c, d, e), a value of biomass yield was used (taken from reference [46], in which alcoholic fermentation in anaerobic conditions with *Saccharomyces cerevisiae* was studied). The value used was 0.060±0.005 g of produced biomass/g of consumed glucose and will be mentioned from now on as theoretical biomass yield ($Y'_{x/s}$). The final macro chemical equation is described by equation III.10.

$$C_6H_{12}O_6 + 0.0102NH_3 \rightarrow 0.06CH_{1.83}O_{0.56}N_{0.17} + 1.979C_2H_6O + 1.982CO_2 + 0.0234H_2O \quad \textbf{(III.10)}$$

The amount of ethanol produced and glucose consumed were calculated through stoichiometric relationship with the moles of CO_2 released.

The variation of biomass concentration was calculated using equation III.11, in which $\frac{dX}{dt}$ and $\frac{dS}{dt}$ are, respectively, the variation of biomass concentration and glucose concentration throughout fermentation time.

$$\frac{dX}{dt} = Y_{x/s}\left(-\frac{dS}{dt}\right) \quad \textbf{(III.11)}$$

Since biomass concentration was measured at the beginning and at the end of the fermentation, glucose and biomass variation could be also calculated using a semi theoretical yield value ($Y''_{x/s}$) estimated using the biomass concentration values. All of these values were calculated iteratively, in which the stoichiometric coefficients were recalculated using a given semi theoretical yield, and with these new coefficients, glucose concentration and biomass concentration were calculated again, and this process was repeated until all the values (measured and stoichiometric) were the same. Obtained values for initial and final biomass concentration and for $Y''_{x/s}$ are presented in

Table III.1. Values obtained for new stoichiometric coefficients are presented in Table 1 in Annex II.

Table III.1 – Values obtained for initial and final biomass concentration, and semi theoretical biomass yields for each essay. Average and standard deviation values are given for quadruplicate runs

$[Glucose]_i$ (g/L)	**50**	**100**	**200**	**300**	**400**
$[Biomass]_i$ (g/L)	0.00915±0.00007				
$[Biomass]_f$ (g/L)	0.944±0.057	0.767±0.095	0.867±0.078	0.974±0.081	1.13±0.393
$Y''_{x/s}$ (g/g)	0.0631	0.0631	0.0746	0.0763	0.0887

In Figure III.6 is shown the variation of glucose, ethanol and biomass concentrations, as well as weight loss, for the case of calculations with theoretical yield. In Figure III.7 are presented the values obtained with the semi theoretical yield. Experimental data is presented in Table 2 in Annex II.

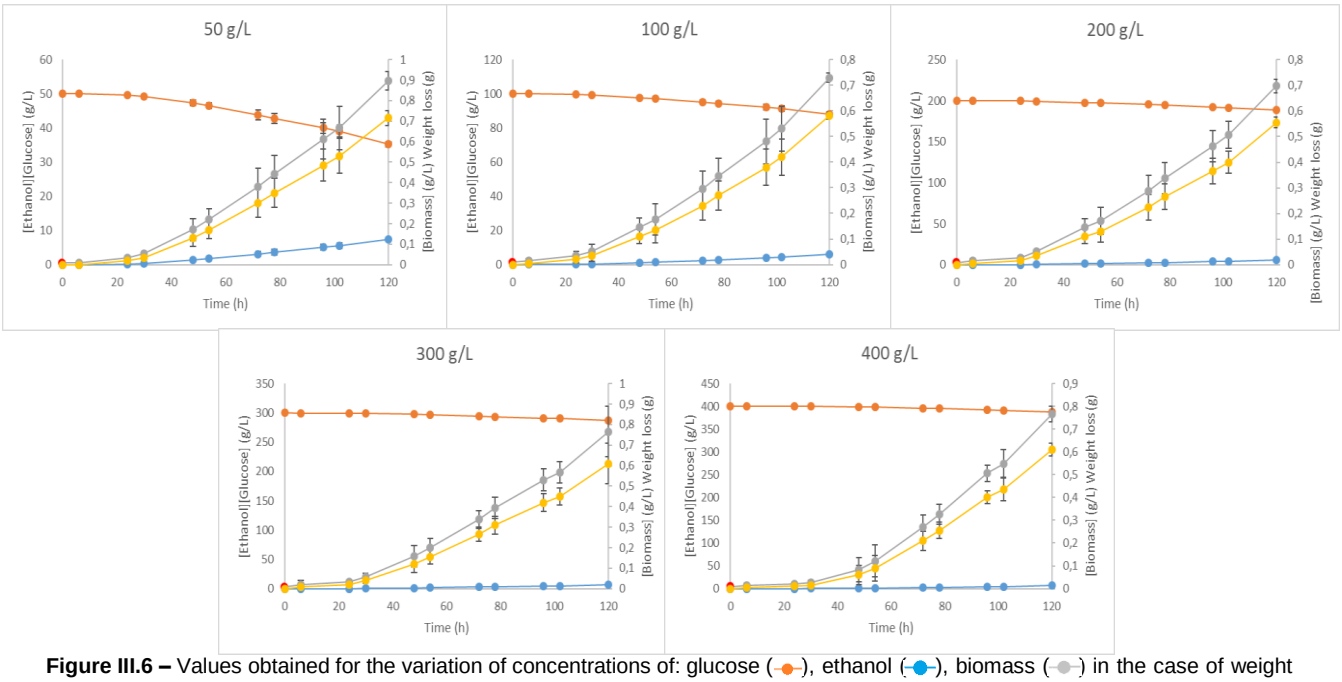

Figure III.6 – Values obtained for the variation of concentrations of: glucose (—●—), ethanol (—●—), biomass (—●—) in the case of weight loss method for suspended yeast essays. Red spots represent experimental values (●). Values obtained calculated using theoretical biomass yield ($Y_{x/s}$). Yellow values (—●—) represent weight loss. Average values and error bars represent standard deviation with n=2.

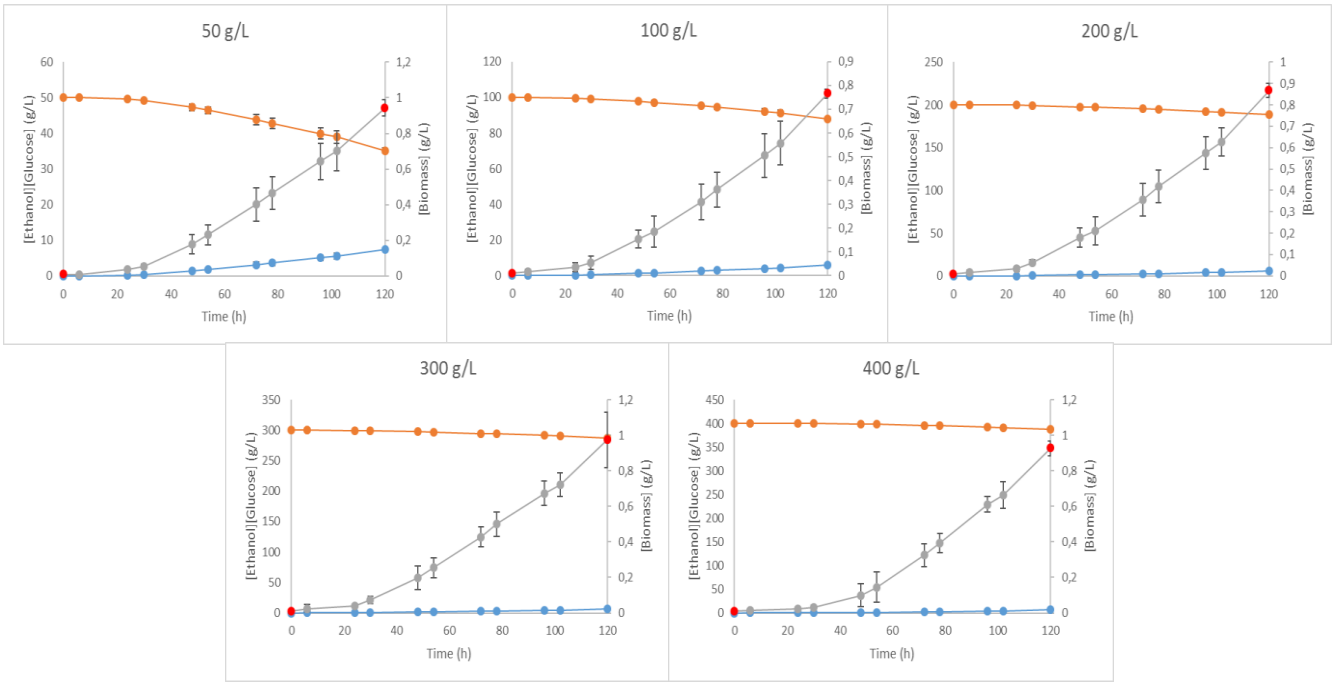

Figure III.7 – Values obtained for the variation of concentrations of: glucose (●), ethanol (●), biomass (●) in the case of weight loss method for suspended yeast essays. Red spots represent experimental values (●). Values obtained calculated using semi theoretical biomass yield ($Y_{x/s}$). Average values and error bars represent standard deviation with n=2.

Since ethanol and glucose concentration were calculated through the stoichiometric relationship, the product yield ($Y_{p/s}$) is the same for all cases, and its value is 0.507 g of ethanol produced/g of consumed glucose. In reference [48] is presented the same value (0.51 g/g) for the stoichiometric product yield. It was expected to find the same value in literature since it is a theoretical one.

From now on, only the values obtained with semi theoretical yield will be presented since they contemplate biomass concentration measurements obtained experimentally.

Regarding the data presented in Figure III.5, it was possible to the determine the μ value for each essay through the linearization of biomass concentration values obtained for the exponential phase. Linearization obtained for data of biomass concentration calculated with the semi theoretical and theoretical yield are presented in Annex III (Figure 1 and equations 1 to 10 for semi-theoretical; Figure 2 and equations 11 to 20 for theoretical case).

In Figure III.8 is presented the relationship between μ and initial glucose concentration ([Glucose]) for the case of the semi-theoretical yield.

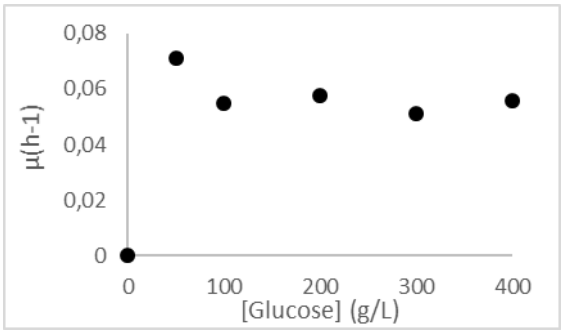

Figure III.8 – Relationship between specific growth rate (μ) and initial glucose concentration for the essay with suspended yeast monitored with weight loss in the case that biomass concentration calculation was accomplished using the semi theoretical yield value.

In Table III.2 are presented values for μ, doubling time (t_d) for semi theoretical calculations for each initial glucose concentration ($[Glucose]_i$), calculated using equation III.3.

Table III.2 – Values of specific growth rate and doubling time for each initial glucose concentration calculated using the semi theoretical yield

$[Glucose]_i$ (g/L)	**50**	**100**	**200**	**300**	**400**
μ (h^{-1})	0.0709	0.0546	0.0574	0.0511	0.0558
t_d (h) (semi theoretical)	9.78	12.7	12.1	13.6	12.4

Regarding data presented in Figure III.6, it was possible to calculate the kinetic parameters, such as μ_{max} (maximum specific growth rate (h^{-1})), K_S (cell growth saturation constant (g/L)) and K_i (inhibition constant (g/L)), using the non-structured model for growth Jackson and Edwards [49] (equation III.12), in which S stands for initial glucose concentration in g/L.

$$\mu = \mu_{max} \frac{S}{K_S + S + \frac{S^2}{K_i}} \quad \textbf{(III.12)}$$

In Figure III.9 is presented the fitting of equation III.11 to experimental data obtained using the semi-theoretical yield. The sum of errors was calculated according to equation III.13. In Table III.3 are shown the values obtained for each kinetic constant. The same was done with the theoretical yield value. Both the fitted model and all the values obtained are presented in Annex III (Figure 3 and Tables 1 and 2).

$$Sum\ errors\ (h^{-1}) = \sum \left(\mu_{experimental} - \mu_{model}\right)^2 \quad \textbf{(III.13)}$$

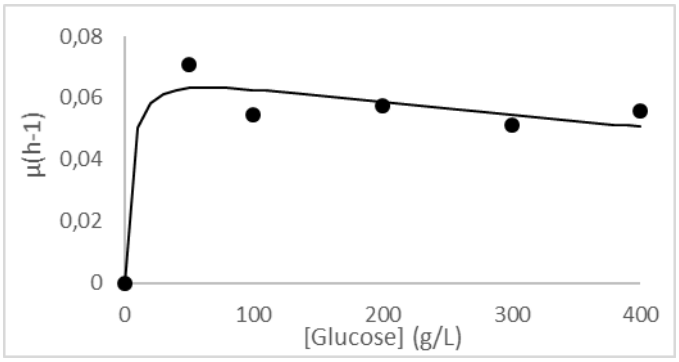

Figure III.9 – Obtained model fitted to the experimental data calculated with the semi theoretical yield. Essay with suspended yeast monitored by weight loss. Sum error: 1.63×10^{-4} h^{-1}. $\mu_{experimental}$ (●) stands for values obtained experimentally, and μ_{model} stands for values calculated through mathematical method described by equation III.11.

Table III.3 – Values for kinetic constants obtained for experimental data obtained using the semi-theoretical yield value

Kinetic constants	Values
K_S (g/L)	4.08
μ_{max} (h^{-1})	0.0715
K_i (g/L)	994

Before analysing the values obtained, it is important to know the conceptual meaning of each kinetic constant for a better understanding of the biological meaning. K_s is the saturation constant (or Monod constant) that reveals the affinity of the microorganism for the substrate. The lower this is, the easier is the uptake of substrate by the microorganism. Values for this constant are in the order of magnitude of mg/L for the case of carbon hydrates [50]. In batch cultures, as those studied in the present work, initial substrate concentrations are a few order of magnitude higher than the respective K_s. μ_{max} stands for the maximum biologically possible value for specific growth rate in a medium with a certain composition and for fixed values of temperature and pH. K_i is the inhibition constant and when its numerical values are two or three orders of magnitude higher than K_S the inhibitory effect is negligible [50].

According to the study presented by Jain (1970) [1] [51], K_s for suspended yeast is 0.187 g/L. On the other hand, Prasad *et al.*[2] [32] reported a value for the same constant of 2.57 g/L (for a suspended cell system), which is of the same order of magnitude of the value obtained in the present work (4.08 g/L). Moreover, as it is stated in reference [50], K_s in batch cultures is a few order of magnitude lower than the initial substrate concentration, which is actually observed in this case.

Regarding the values of μ_{max}, in the study of Jain (1970) the value presented for this constant for a suspended yeast system is 0.500 h^{-1}. According to Auling *et al.* (1984)[3] [52] and Prasad *et al.*[4],

[1] Anaerobic conditions, in batch mode, 30ºC and pH 5.8.
[2] Performed at 30ºC and pH was controlled.
[3] Anaerobic conditions, batch mode, pH 5.0 and 30ºC.
[4] Performed at 30ºC and pH was controlled.

this constant is 0.290 and 0.214 h^{-1}, respectively for a suspended yeast system as well. All these values are one order of magnitude higher than that obtained in this essay. They were obtained for a richer medium (with yeast extract and peptone) and with conditions of pH and temperature well defined. In the present work, pH was not controlled and the medium used did not contain an organic source of nitrogen. Consequently, the comparison between these values is not totally correct because fermentation conditions were not the same. Another work, from Hoek *et al.* (1998) [53], presented a μ_{max} of 0.42 h^{-1} for yeast in a mineral medium with glucose and vitamins. From this gathering of values, one can conclude that the value obtained in this case is much lower than for what was expected. This is a result of the inherent error of the method used (monitoring by weight loss).

Concerning K_i constant, the value obtained is much higher than the one presented in literature (162 g/L; Ortiz-Muñiz *et al.* (2010) [54]).

III.2.2 Assay monitored with analytical methods

III.2.2.1 Assay with colorimetric glucose monitoring

As it was described in Chapter II, glucose concentration was first measured using a reducing chemical reagent, 3,5-dinitrosalicylic acid (DNS). Results obtained with this method are presented in Figure III.10. All experimental data obtained for glucose concentration are presented in Table 1 in Annex V.

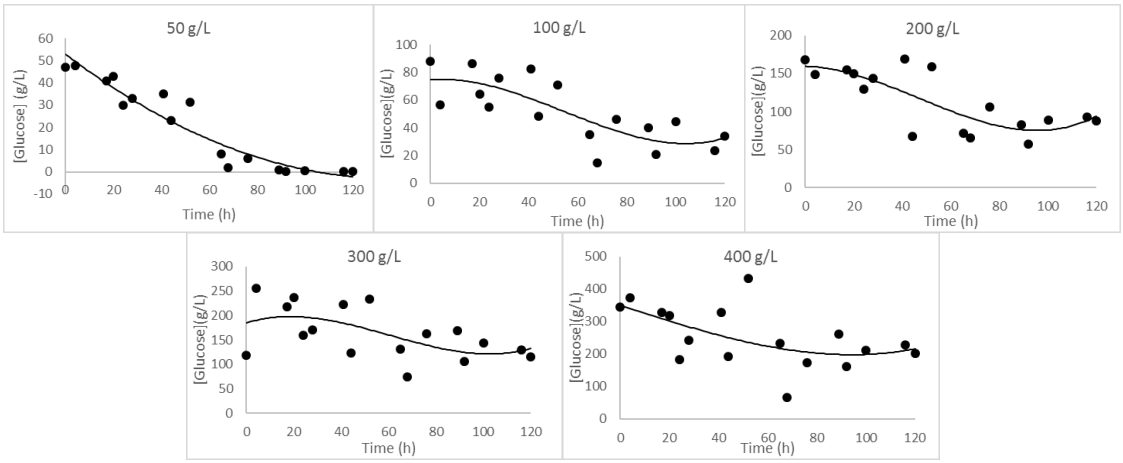

Figure III.10 – Glucose consumption during fermentation with suspended yeast for each initial glucose concentration. Determination by colorimetric method using DNS.

As one can observe, values obtained are very dispersed and so a trend line was calculated in order to better determine the total of consumed glucose. These trend lines were determined considering the ones calculated by *Microsoft Office Excel*® for the best correlation factor. However, values are not highly correlated. Therefore, variance (Var) between experimental data

and trend line values was calculated and it is described by equation III.14 (*x* stands for random variable, $\bar{x}$ stands for the mean between values and *n* is sample size).

$$Var = \frac{\sum(x-\bar{x})^2}{n-1} \textbf{ (III.14)}$$

As a result, the sum of variances was calculated and the polynomial coefficients were recalculated using the *Microsoft Office Excel*® tool *Solver*® for the minimal sum of variances value. Trend lines obtained, as well as each sum of variances and correlation factor, are presented in Annex IV (equations from 1 to 10).

In Table III.4 are presented values obtained for initial and final biomass concentration ($[Biomass]_i$, $[Biomass]_f$), initial and final glucose concentration calculated through equations from 1 to 9 presented in Annex IV ($[Glucose]_i$, $[Glucose]_f$; note that in the case of 50 g/L essay, was considered that all glucose was consumed since the value calculated was below zero), consumed glucose ($Glucose_c$), biomass yield ($Y_{x/s}$) and product yield ($Y_{p/s}$).

Table III.4 – Values obtained for initial and final biomass concentration, initial and final glucose concentration, consumed glucose and biomass and product yield for each essay with suspended yeast and glucose determination with DNS. Average and standard deviation values are given for quadruplicate runs

$[Glucose]_i$ (g/L)	**50**	**100**	**200**	**300**	**400**
$[Biomass]_i$ (g/L)	0.00602±0.00051				
$[Biomass]_f$ (g/L)	1.80±0.06	1.66±0.14	1.60±0.17	1.38±0.08	1.17±0.13
$[Glucose]_i$ (g/L)	53.0	74.5	159	185	350
$[Glucose]_f$ (g/L)	0.00	32.2	94.6	131	216
$Glucose_c$ (g/L)	53.0	42.3	64.4	53.5	134.2
$Y_{x/s}$ (g/g)	0.0338±0.0010	0.0390±0.0033	0.0248±0.0027	0.0257±0.0015	0.00867±0.00099
$Y_{p/s}$ (g/g)	0.0997±0.0082	0.149±0.017	0.0908±0.0130	0.114±0.008	0.0509±0.0008

Observing values obtained for $Y_{x/s}$ and $Y_{p/s}$, there is a maximum value for the case of 100 g/L and a minimum one for the case of 400 g/L. However, one can also observe that the difference between the values obtained for the essays of 50, 100, 200 and 300 g/L is not very significant. Nevertheless, in the case of 400 g/L, there is a clear decrease in the amount of produced biomass and ethanol per consumed glucose. This reveals inhibitory effects in cell growth.

Ortiz-Muñiz *et al.*(2010) [54] reported a maximum value for bioethanol yield of 0.41 g/g for an initial glucose concentration of 150 g/L. The value obtained in this essay is considerably lower than the one presented in literature. In the same study, the maximum value for biomass yield is 0.051±0.001 g/g, which is very similar to the value obtained in the present work.

In Figure III.11 are presented values obtained for ethanol and biomass concentrations. Experimental data is presented in Table 2 (optical density and respective biomass concentration), Table 3, Table 4 and Table 5 (peak area and respective ethanol concentration) in Annex V.

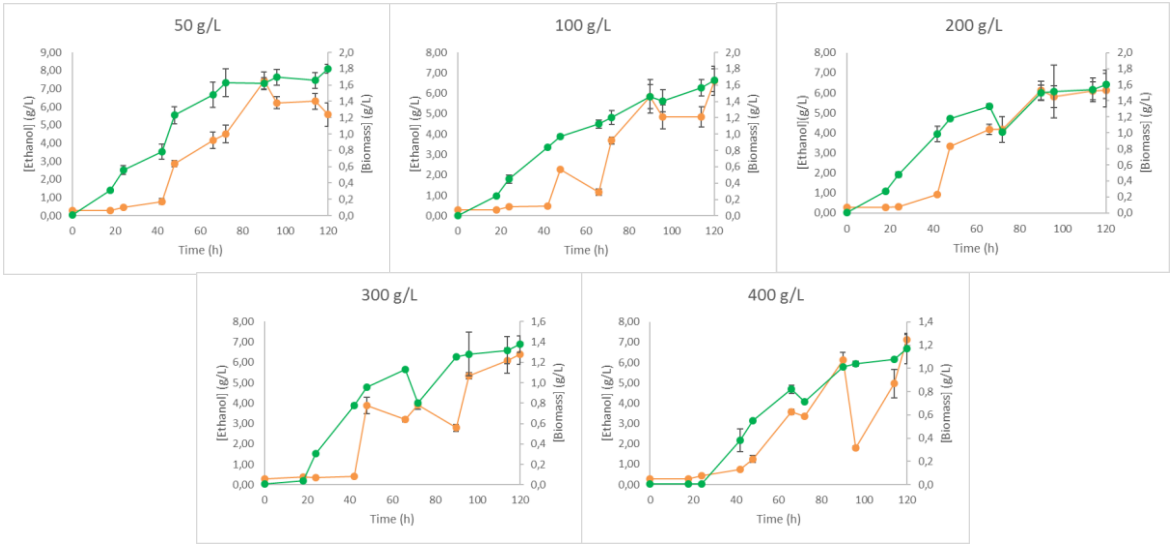

Figure III.11 – Values obtained for the variation of concentrations of ethanol ( ) and biomass ( ) for essays with suspended yeast and in which glucose concentration was measured by DNS. Average values and error bars represent standard deviation with n=4.

Linearization of biomass concentration are presented in Annex III (Figure 4; equations from 21 to 30).

In Figure III.12 is represented the relationship between μ and initial glucose concentration. In Table III.5 are presented values for doubling time (t_d, equation III.3) and for μ for each initial glucose concentration.

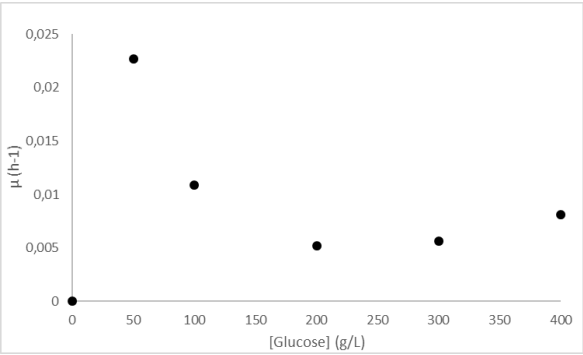

Figure III.12 – Relationship between specific growth rate (μ) and initial glucose concentration for suspended yeast essays (glucose was determined by DNS).

Table III.5 – Specific growth rate and doubling time for each initial glucose concentration for suspended yeast essay in which glucose concentration was measured with DNS

$[Glucose]_i$ (g/L)	50	100	200	300	400
μ (h^{-1})	0.0227	0.0109	0.0052	0.0056	0.0081
t_d (h)	30.5	63.6	133	124	85.6

In Figure III.13 is presented the fitting of equation III.11 to experimental data presented in Figure III.12. In Table III.6 are shown the values obtained for each kinetic constant.

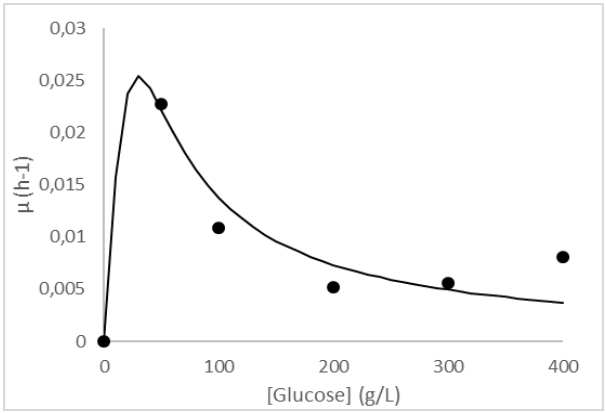

Figure III.13 – Obtained model fitted to the experimental data. Essay with suspended yeast with glucose concentration measured with DNS. $Sum\ error = 3.24 \times 10^{-5} h^{-1}$. $\mu_{experimental}$ (●) stands for values obtained experimentally, and μ_{model} stands for values calculated through mathematical method described by equation III.11.

Table III.6 – Values for kinetic constants obtained for suspended yeast essays in which glucose concentration was measured with DNS

Kinetic constants	Values
K_S (g/L)	1.06×10^3
μ_{max} (h^{-1})	1.88
K_i (g/L)	0.797

III.2.2.2 Assay with glucose monitoring with HPLC

Since DNS method was not the best one for measurement of glucose concentration not only because it had inconclusive results but also it is a hard labour method, glucose concentration was then determined by HPLC, as described in Chapter II. In Table III.7 are presented values obtained for initial and final biomass concentration, initial and final glucose concentration, consumed glucose, biomass yield and product yield.

Table III.7 - Values obtained for initial and final biomass concentration, initial and final glucose concentration, consumed glucose and biomass and product yield for each essay with suspended yeast and glucose determination with HPLC. Average and standard deviation values are given for quadruplicate runs

$[Glucose]_i$ (g/L)	**50**	**100**	**200**	**300**	**400**
$[Biomass]_i$ (g/L)	0.00915±0.00113				
$[Biomass]_f$ (g/L)	1.57±0.08	1.37±0.05	1.42±0.15	1.11±0.01	1.09±0.24
$[Glucose]_i$ (g/L)	53.8±22.5	101±23	216±24	314±50	389±28
$[Glucose]_f$ (g/L)	5.06±0.87	69.2±8.47	91.4±69.6	195±44	203±51
$Glucose_c$ (g/L)	48.8±30.6	31.7±20.6	125±63	118±9	186±32
$Y_{x/s}$ (g/g)	0.0392±0.0225	0.0540±0.0333	0.0142±0.0072	0.00938±0.00060	0.00582±0.00054
$Y_{p/s}$ (g/g)	0.546±0.368	0.657±0.439	0.204±0.156	0.233±0.126	0.0872±0.0080

As one can observe, standard deviation is often of the same magnitude of the value. This happened due to technical problems concerning the equipment used (HPLC and gas chromatograph) and sample conservation.

Observing values of $Y_{x/s}$ and $Y_{p/s}$, 100 g/L essay present the higher value for both constants and 400 g/L present the lower value. In opposite to what was verified in the previous essay (subchapter III.2.2.1), there is a clear increase from 50 g/L essay to 100 g/L, and then values decreased from 100 to 400 g/L, revealing the increasing inhibitory effect. The difference between this essay and the previous one is explained by the fact that glucose concentration was determined by different methods. Values obtained in this essay are closer to the ones found in literature (0.41 g/g for ethanol yield and 0.051 g/g for biomass yield).

The values obtained for this essay are presented in Figure III.14. Experimental data is presented in Table 1 (optical density and respective biomass concentration), Table 2 and Table 3 (peak area

and respective glucose concentration) and Table 4 and Table 5 (peak area and respective ethanol concentration) of Annex VI.

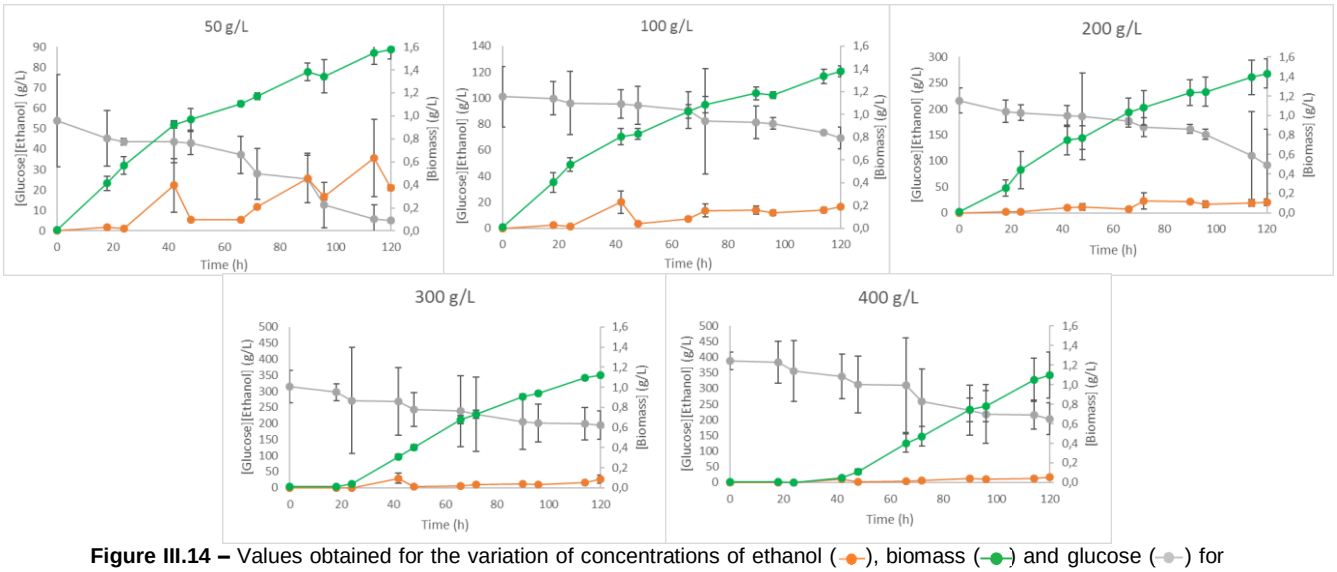

Figure III.14 – Values obtained for the variation of concentrations of ethanol (—●—), biomass (—●—) and glucose (—●—) for essays with suspended yeast. Average values error bars represent standard deviation with n=4.

μ was also determine for this case. Linear correlations are presented in Annex III (Figure 5, equations 31 and 40).

In Figure III.15 is presented the relationship between specific growth rate and initial glucose concentration. In Table III.8 are presented values for doubling time (t_d, equation III.3) and μ for each initial glucose concentration.

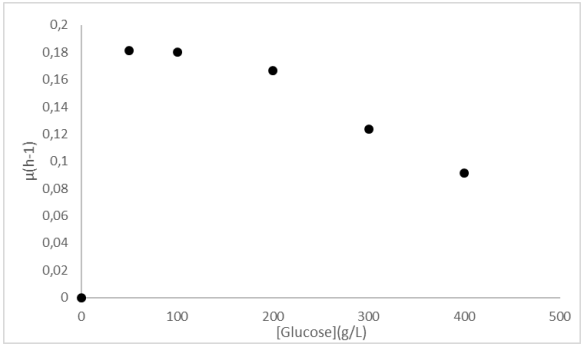

Figure III.15 – Relationship between specific growth rate (μ) and glucose concentration for the essay with suspended yeast and glucose concentration monitoring with HPLC.

Table III.8 – Specific growth rate and doubling time for each initial glucose concentration for suspended yeast essay in which glucose concentration was measured with HPLC

$[Glucose]_i$ (g/L)	**50**	**100**	**200**	**300**	**400**
μ (h^{-1})	0.181	0.180	0.167	0.124	0.0913
t_d (h)	3.82	3.84	4.16	5.59	7.59

Regarding the information presented in Figure III.15, it was possible to fit equation III.11 to experimental data and find out Ks, μ_{max} and K_i. In Figure III.16 is presented the model obtained and in Table III.9 are shown the values for the kinetic constants.

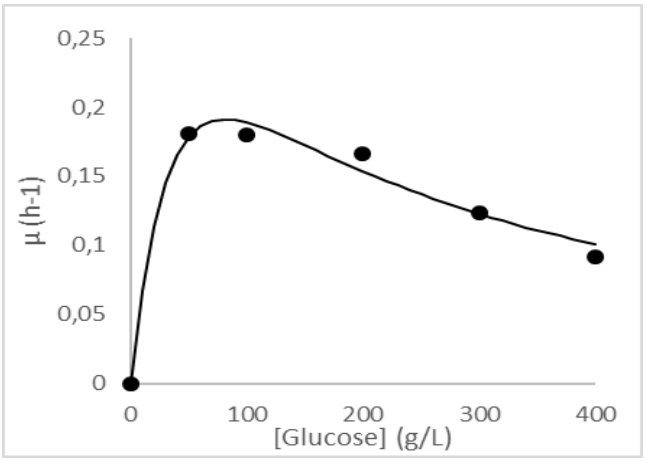

Figure III.16 – Obtained model adjusted to the experimental data. Essay with suspended yeast with glucose concentration measured by HPLC. Sum error: 7.45×10^{-4} h^{-1}. $\mu_{experimental}$ (●) stands for values obtained experimentally, and μ_{model} stands for values calculated through mathematical method described by equation III.11.

Table III.9 – Values for kinetic constants obtained for suspended yeast essays in which glucose concentration was measured with HPLC

Kinetic constants	Value
K_S (g/L)	56.5
μ_{max} (h^{-1})	0.455
K_i (g/L)	119

III.2.3 Comparison of the methods

In order to better discuss all the kinetic constants obtained, all values for the first essay are presented in Table III.10.

Table III.40 – Kinetic constants obtained for all the essays executed with suspended yeast

	Suspended yeast		
Kinetic constants	**Semi theoretical**	**DNS**	**HPLC**
K_S (g/L)	4.08	1.06×10^3	56.5
μ_{max} (h^{-1})	0.0715	1.88	0.455
K_i (g/L)	994	0.797	119

In the case of the suspended yeast essay, there was an analysis of the same system with different methods. Therefore, it was expected to obtained similar kinetic values. Observing data presented in Table III.10, one can conclude that all values are very different from each other. This means

that the essays were not reproducible. This discrepancy is a result of experimental errors and technical issues verified during the execution of each essay.

In every essay values of K_s are an order of magnitude of g/L, which goes against what was mentioned to be stated in reference [50]. Both values obtained for the DNS and HPLC essays are much higher than expected, since it was expected to be a few order of magnitude lower than the initial substrate concentration.

Regarding μ_{max}, only the HPLC essay is of the same order of magnitude of the ones presented in literature (0.214, 0.290, 0.500 and 0.42 h^{-1} as mentioned in subchapter III.2.1). μ_{max} obtained for the case of DNS essay is too high for this constant, since a typical value for this constant, in the case of fermentation with yeasts and in the mentioned conditions, should be one order of magnitude lower than the obtained.

In the study of Ortiz-Muñiz *et al.* (2010) [54], K_i is reported to be 162 g/L for alcoholic fermentation with suspended yeast. HPLC essay was the one closer to the literature value. Essay monitored by weight loss presented a value too high for the constant, and DNS essay present a very low value for the constant.

To sum up, kinetic constants obtained in DNS essay were the ones that presented a bigger discrepancy of the values reported by other studies. This was expected since values obtained for biomass concentration (Figure III.9) are highly inconstant and present a considerable error. The reason for this relies on experimental errors and problems regarding equipment calibration. Although essay monitored by weight loss is the one that present a higher inherent error, as it was mentioned in subchapter III.2.1, it was the essay with the best approximation to values reported in literature.

III.3 **Second Essay:** Influence of initial glucose concentration on bioethanol production using immobilized *Saccharomyces cerevisiae*

Due to technical problems, samples taken from the essay with immobilized yeasts could not be analysed.

III.3.1 Encapsulation efficiency

Encapsulation efficiency (EE) was calculated for both methods (weight loss method and analytical method) using equation II.1, presented in subchapter II.3.2. Values obtained are shown in Table III.11.

Table III.11 – Values obtained for encapsulation efficiency for both weight loss and analytical method

Method of measurement	Encapsulation Efficiency (%)
Weight loss method	47.7±0.7
Analytical method	62.4±7.1

Analysing EE obtained, one can conclude that although experimental conditions were the same in both cases, there were uncontrolled factors that led to this different values. Besides this, EE obtained are lower than the ones presented by the reference [55], in which EE for the same sodium alginate concentration was 91.10±0.85%. The disparity between this values can be caused by the approximations that needed to be done for the calculation of EE (mentioned in subchapter II.3.2). These are the major source of error. Besides this fact, in the case of the reference, the method used for determination of biomass concentration (colony forming units counting via a pour-plate method) was different from the one used in the present study, which can be a source of disparity.

In Table III.12 are presented values obtained for biomass concentration inside beads at the beginning and at the end of the fermentation ($[Biomass]_{ibeads}$; $[Biomass]_{fbeads}$; equation II.3 in subchapter II.3.2.) for both weight loss and analytical methods and for each initial glucose concentration ($[Glucose]_i$), calculated using equation II.3 presented in subchapter II.3.2. All experimental data are presented in Table 1 and Table 2 (biomass concentration inside beads) in Annex VII.

Table III.12- Values of initial and final biomass concentration inside beads for both weight loss and analytical methods, and for each initial glucose concentration

Method of measurement	$[Biomass]_{ibeads}$ (g/L)	$[Biomass]_{fbeads}$ (g/L)				
		$[Glucose]_i$ (g/L)				
		50	100	200	300	400
Weight loss method	$2.00 \times 10^{-4} \pm 6 \times 10^{-6}$	$5.09 \times 10^{-3} \pm 5.8 \times 10^{-4}$	$5.47 \times 10^{-3} \pm 6.4 \times 10^{-4}$	$4.00 \times 10^{-3} \pm 1.1 \times 10^{-4}$	$4.32 \times 10^{-3} \pm 3.9 \times 10^{-4}$	$3.11 \times 10^{-3} \pm 4.0 \times 10^{-4}$
Analytical method	$4.25 \times 10^{-4} \pm 4.8 \times 10^{-5}$	$1.66 \times 10^{-3} \pm 8.1 \times 10^{-4}$	$1.70 \times 10^{-3} \pm 8.1 \times 10^{-4}$	$2.77 \times 10^{-3} \pm 7.1 \times 10^{-4}$	$1.17 \times 10^{-3} \pm 5.8 \times 10^{-4}$	$1.06 \times 10^{-3} \pm 4.6 \times 10^{-4}$

III.3.2 Assay monitored by weight loss

All the assumptions and considerations explained for subchapter III.2.1 are equally valid for this case. Stoichiometric equation used in this case was the same as in the case of suspended yeast essay (equation III.9). In Table III.13 are presented values obtained semi theoretical biomass yield determined as described in subchapter III.3.2. In Figure III.17 is shown the variation of glucose, ethanol and biomass concentrations (theoretical) and weight loss. In Figure III.18 are presented values obtained for semi theoretical case. These values were calculated iteratively, as explained in subchapter III.2.1. Experimental data is presented in Table 3 (weight loss values) in Annex VII. In the case of 100 g/L initial glucose concentration, one of the replicas had a weight loss that lead to a consumption of glucose superior to the initial one, so values were negligible. This happened because the stopper was not well fitted and so there was evaporation of medium. As to the product yield ($Y_{p/s}$) it is again 0.511 g/g in every case, which corresponds to the theoretical one, as it was mentioned in subchapter III.2.1.

Table III.13 – Values obtained for semi theoretical biomass yield for each initial glucose concentration for weight loss method in the case of the essay with immobilized yeast

$[Glucose]_i$ (g/L)	50	100	200	300	400
$Y''_{x/s}$ (g/g)	3.45×10^{-4}	4.57×10^{-4}	3.49×10^{-4}	3.43×10^{-4}	2.46×10^{-4}

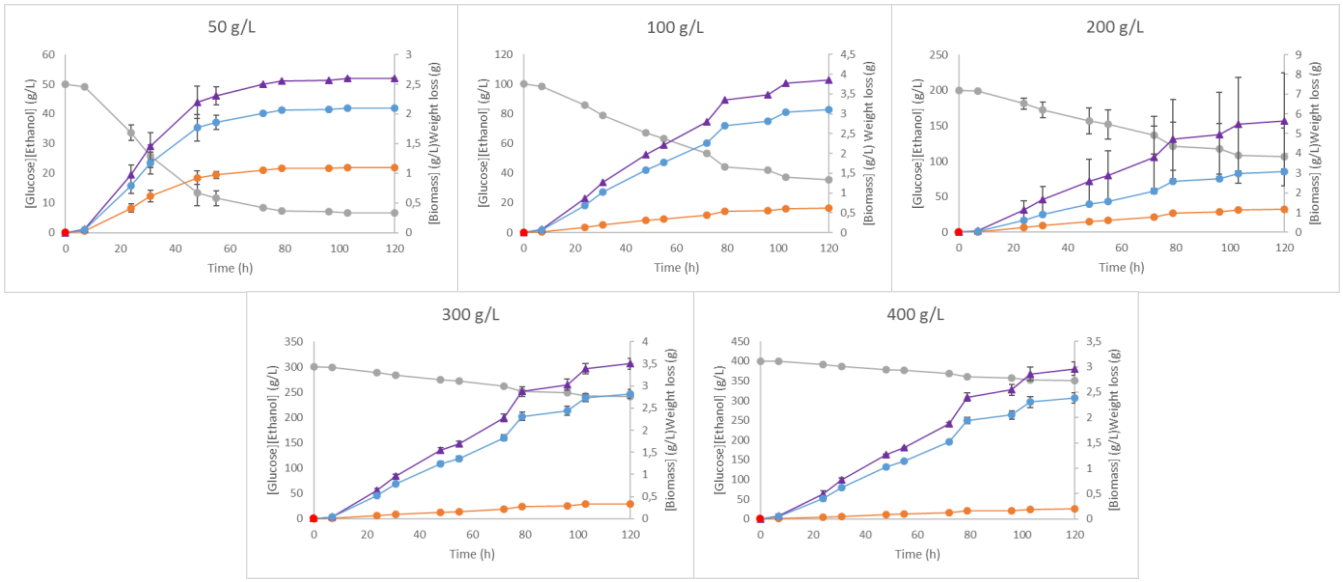

Figure III.17 – Values obtained for the variation of concentrations of: glucose (⚪), ethanol (🟠), biomass (🔺) , for the essay with immobilized yeast and monitored by weight loss. Red spots represent experimental values (🔴). Biomass concentration calculated using theoretical biomass yield ($Y_{x/s}$) and blue spots (🔵) represent weight loss. Average values error bars represent standard deviation with n=2.

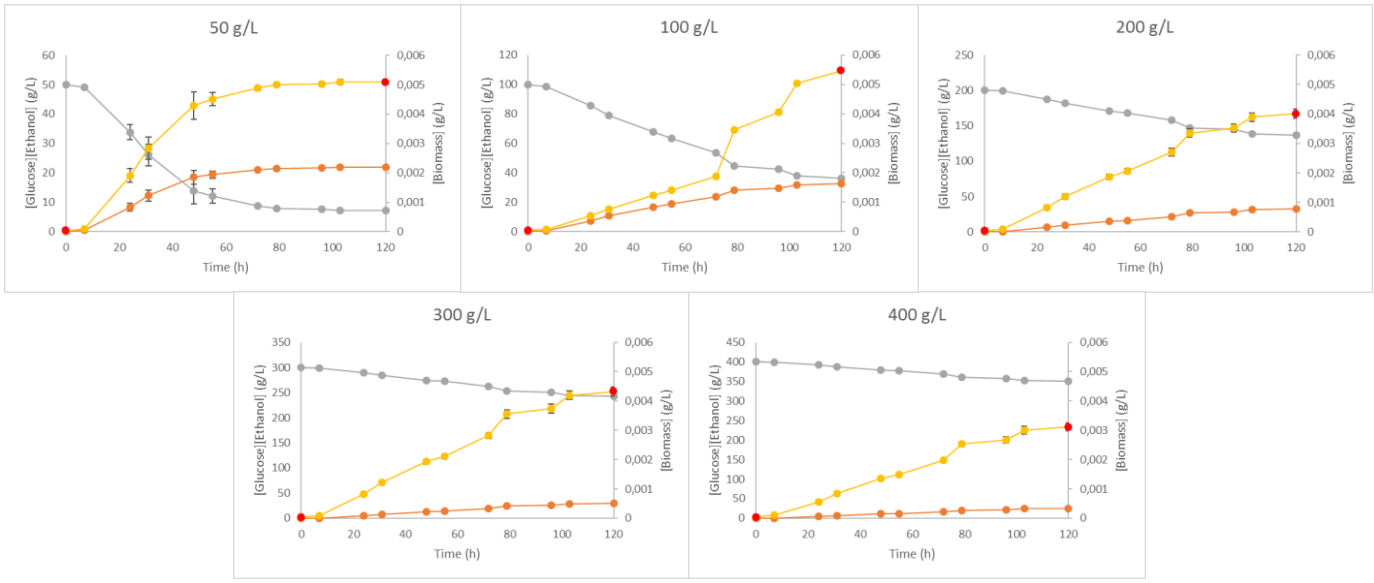

Figure III.18 – Values obtained for the variation of concentrations of: glucose (⚪), ethanol (🟠), biomass (🟡) , for the essay with immobilized yeast and monitored by weight loss. Red spots represent experimental values (🔴). Biomass concentration calculated using semi theoretical biomass yield ($Y_{x/s}$) and blue spots (🔵) represent weight loss. Average values error bars represent standard deviation with n=2.

As it is clear observing Figure III.17 and Figure III.18, values obtained for biomass concentration using theoretical yield and semi theoretical yield are completely different: biomass concentration calculated using theoretical yield is 10^3 times higher than biomass concentration calculated using semi-theoretical. There are two reasons for this disparity, that are the following:

- Approximations in the calculation of biomass concentration inside beads (as mentioned in subchapter II.3.2.) that lead to a considerable error in final values.
- Theoretical yield is 10^2 times higher than semi-theoretical one.

The growth of cells and their metabolic activity are altered when they are immobilized in a polymer matrix. There are mass transfer limitations that not only prevent inner cells (cells that are in centre of the beads) to receive the substrate they need, but also interfere with the release of toxic fermentation products [34]. Therefore, cell growth in this case will not be the same as in the free cell system, as it was observed with the lower values of biomass concentration determined experimentally. This fact is not taken into account when theoretical biomass yield is used for biomass concentration calculation, and thus, this will be much higher than the ones obtained experimentally. However, in reference [32], biomass yield coefficient inside the gel matrix was determined, and the value is 0.0774 g/g, which is in the same order of magnitude of the theoretical yield used for calculations. Therefore, values obtained experimentally are below of the ones expected.

Regarding experimental values obtained for initial, final biomass concentrations and weight loss, one can observe that although this method has a considerable error, as it has already been mentioned, there was a significant loss of weight comparing to the values observed in the suspended essay (10 times higher). Since there was almost no growth, this loss of weight is essentially due to ethanol production, and it was considerable higher in this case than with suspended yeast. Nonetheless, this conclusion should be verified by data collected by sample analysis.

μ and kinetic constants were determined for both semi theoretical and theoretical case. Linear correlation between ln([Biomass])) and time for both cases are presented in Annex III (Figure 6 and Figure 7; trend lines equations from 41 to 50 and 51 to 60, respectively). Figure III.19 show the relationship between specific growth rate and glucose concentration only for the case of semi theoretical yield. In Table III.14 are presented values obtained for μ and doubling time for each case.

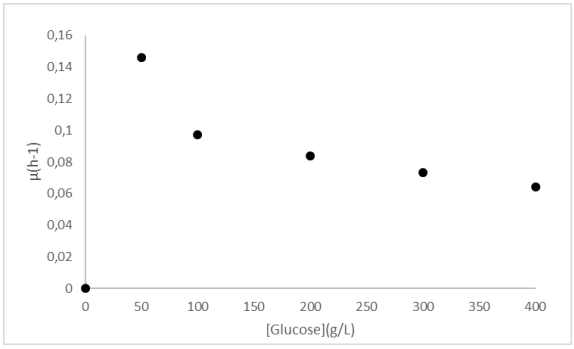

Figure III.19– Relationship between specific growth rate (μ) and glucose concentration for the essay with immobilized yeast and monitored by weight loss for semi theoretical case.

Table III.14 – Specific growth rate and doubling time for each initial glucose concentration for immobilized yeast essay monitored by weight loss

$[Glucose]_i$ (g/L)	50	100	200	300	400
μ (h^{-1})	0.146	0.0970	0.0838	0.0734	0.0640
t_d (h) (semi theoretical)	4.74	7.15	8.27	9.44	10.8

Kinetic constants were calculated through the fitting of equation III.11 to experimental data. Results are shown in Figure III.20 for semi theoretical yield. Results for theoretical case are presented in Annex III (Figure 8; Table 3 and Table 4). Values for kinetic constants are shown in Table III.15.

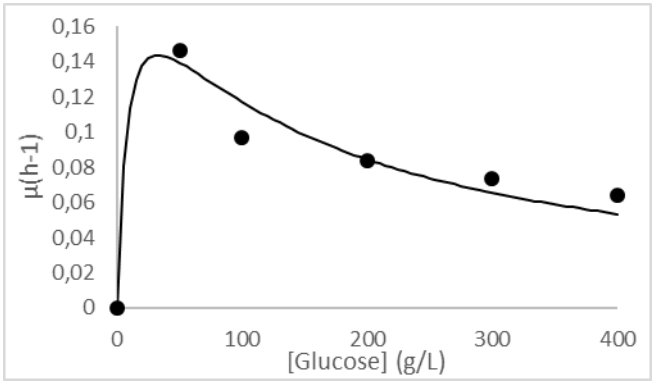

Figure III.20 – Obtained model fitted to the experimental data. Essay with immobilized yeast monitored by weight loss. $Sum\ error = 6.25 \times 10^{-4}\ h^{-1}$. $\mu_{experimental}$ (●) stands for values obtained experimentally, and μ_{model} stands for values calculated through mathematical method described by equation III.11.

Table III.15 – Values for kinetic constants obtained for for experimental data obtained using the semi-theoretical yield value

Kinetic constants	Values
K_S (g/L)	8.04
μ_{max} (h^{-1})	0.214
K_i (g/L)	134

III.4 Comparison between 1st and 2nd essays

In Table III.16 are shown all the obtained kinetic constants for all essays performed, so that a comparison between methods can be done.

Table III.16- Kinetic constants for all essays performed

	Suspended yeast			Immobilized yeast
Kinetic constants	**Weight loss**	**DNS**	**HPLC**	**Weight loss**
K_S (g/L)	4.08	1.06×10^3	56.5	8.04
μ_{max} (h^{-1})	0.0715	1.88	0.455	0.214
K_i (g/L)	994	0.797	119	134

It is possible to make a comparison between immobilized and suspended cell system. In order to accomplish this, only values obtained for the essay monitored by weight loss can be compared since they present the same error.

As one can observe, K_S is of the same order of magnitude in both cases but it is higher in the case of immobilized yeast. This was expected since when cells are immobilized, it is more difficult for them to uptake the substrate due to diffusion problems. As to μ_{max}, it was expected to be higher in the case of suspended cell system since there is no barriers to prevent cell growth. However, the opposite was observed.

In the case of the constant K_i, it is higher in the case of suspended yeast. What was expected was the exact opposite. Since cells are entrapped inside a matrix, it regulates the consumed substrate. Therefore, the inhibition effect due to high substrate concentration is attenuated. In the study of Galaction *et al.* (2010) [48], the value presented for inhibition coefficient is 117 g/L, which is of the same order of magnitude of the one obtained in the present work.

To sum up, all essays do not present kinetic constants that are in line with either literature and what was expected. The reason for this discrepancy are experimental errors and problems with equipment verified during the execution of each essay.

III.5 **Third Essay:** Bioethanol production from the Organic Fraction of Municipal Solid Wastes (OFMSW)

III.5.1 Chemical oxygen demand (COD) measurement

Chemical oxygen demand (COD) was measured in order to assess the amount of organic matter dissolved in the liquid used for fermentation at the beginning of the experiment. The value obtained was 39500±31.2 mg/L.

COD was not measured at the end of the essay since it was not expected to decrease much. The reason for this statement relies on the fact that the amount of organic matter does not vary much during fermentation, since glucose (organic matter) is transformed into ethanol (organic matter). Therefore, there is no mineralization of the initial amount of organic matter.

III.5.2 Essay with suspended yeast

In Figure III.21 are presented values obtained for biomass concentration in the case of the essay with suspended yeast.

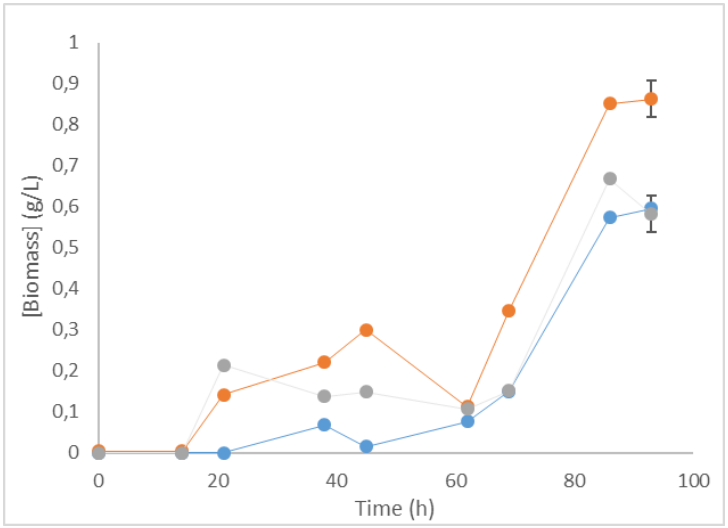

Figure III.21 – Biomass concentration during fermentation with suspended yeast and OFMSW as carbon source. Blue circles (●): pure liquid rich in organic matter; orange circles (●): liquid rich in organic matter diluted ½; grey circles (●): liquid rich in organic matter diluted ¼. Error bars represent standard deviation with n=4.

Observing data presented in Figure III.21, it is possible to conclude that in any case there is an initial lag phase, that takes around 21 hours in the essay with pure liquid, and 14 hours for the other two essays. The reason for this result relies on the fact that in the case of the pure liquid, cells take a longer time to adapt themselves to the new medium since it has higher substrate concentration than in the other two cases. Besides this, it is also evident that this is a diauxic growth since there are two phases: the first one starts at 14th hour with an exponential growth phase until 21st hour, followed by a stationary phase that took around 41 hours for all three cases,

and then a second exponential phase followed by a new stationary phase. In Figure III.22 all phases are explicit.

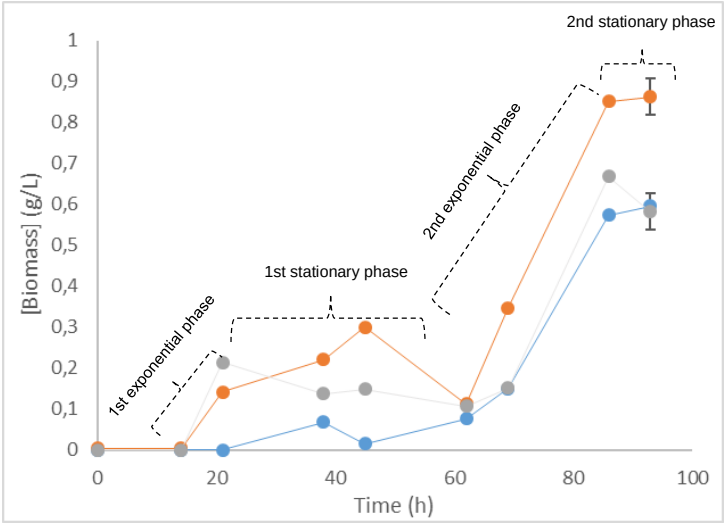

Figure III.22 – Graphical identification of the different phases of diauxic growth in the case of the fermentation with suspended yeast and OFMSW as medium.

This was expected since the medium used in this essay had different carbon sources, not only glucose and fructose, but also bigger molecules, such as starch. This diauxic growth means that yeasts firstly consume one of the substrates, and when this is exhausted, they adapt their metabolism and start to consume another. Theoretically, in this case, yeasts consumed first glucose and then fructose, since there are studies made in wine fermentation that reveal that wine yeast strains (such as the one used in this study) show preference for glucose [56].

Regarding starch, yeasts can only metabolize hexoses, thus it cannot be consumed. It needed to suffer saccharification before it could be up taken by the yeasts. This process could be done introducing amylases (enzymes that convert starch into glucose) in the fermentation system [23].

However, this statement had to be confirmed with values of glucose concentration. It should decrease until very low values at the same time biomass concentration enters into the second stationary phase. Nonetheless, glucose concentration was not measured due to technical problems.

A conclusion that can be done analysing data collected is that the best culture medium is the one with substrate diluted 1:2 since it was the one with higher biomass growth. This shows that on one hand, the culture medium with pure substrate had an inhibitory substrate concentration, and on the other hand, culture medium with substrate diluted 1:4 presented substrate concentration too low for the yeast growth.

III.5.3 Essay with immobilized yeast

III.5.3.1 Encapsulation efficiency

EE was also calculated for this essay using equation II.1 presented in subchapter II.3.2. Value obtained for $[Biomass]_{beads}$ (equation II.3 in subchapter II.3.2.) and EE were, respectively, $1.52 \times 10^{-4} \pm 4.1 \times 10^{-5}$ and 57.1±7.6. According to reference [55], this value is once again very low. In this case, the source of error is as well the approximations needed to be done for the determination of EE.

Values for final biomass concentration inside beads are presented in Table III.17.

Table III.17 – Values of $[Biomass]_{fbeads}$ for each essay (pure, diluted ½ and ¼)

Essay	Pure	1/2	1/4
$[Biomass]_{fbeads}$	$1.76 \times 10^{-3} \pm 6.5 \times 10^{-5}$	$2.23 \times 10^{-3} \pm 7.3 \times 10^{-4}$	$2.42 \times 10^{-3} \pm 8.7 \times 10^{-4}$

In this essay, samples were taken for determination of glucose and ethanol concentrations. However, due to technical problems, it was not possible to obtain these values. Nonetheless, the next step was to calculate the biomass yield, and with it and the variation of glucose concentration, calculate the variation of biomass concentration inside beads throughout fermentation time. With these data, kinetic parameters could be determined, and a comparison between essay with immobilized and suspended yeast could be done.

III.6 Scanning electron microscopy of immobilized cells

The following figures present pictures taken with scanning electron microscopy (SEM) of the *S.cerevisiae* beads before (Figure III.23) and after fermentation for the different initial glucose concentration essays (from Figure III.24 to Figure III.28).

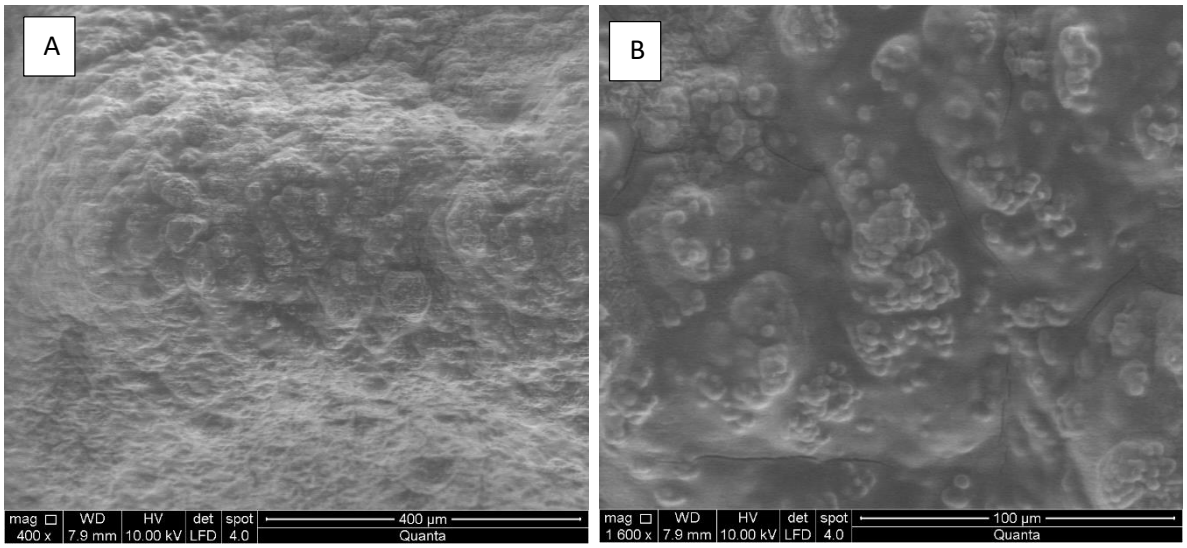

Figure III.23 – SEM images of the external surface of immobilized *S. cerevisiae* beads before fermentation. (a) Magnification 400x (b) magnification 1600x.

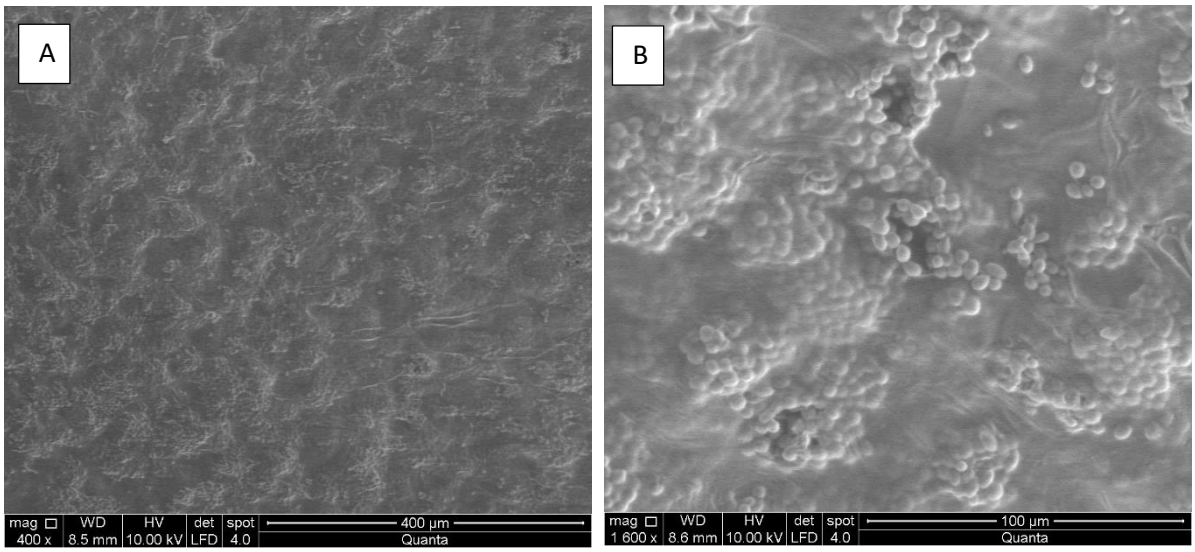

Figure III.24 – SEM images of the external surface of immobilized *S. cerevisiae* beads after fermentation with an initial glucose concentration of 50 g/L. (a) Magnification 400x (b) magnification 1600x.

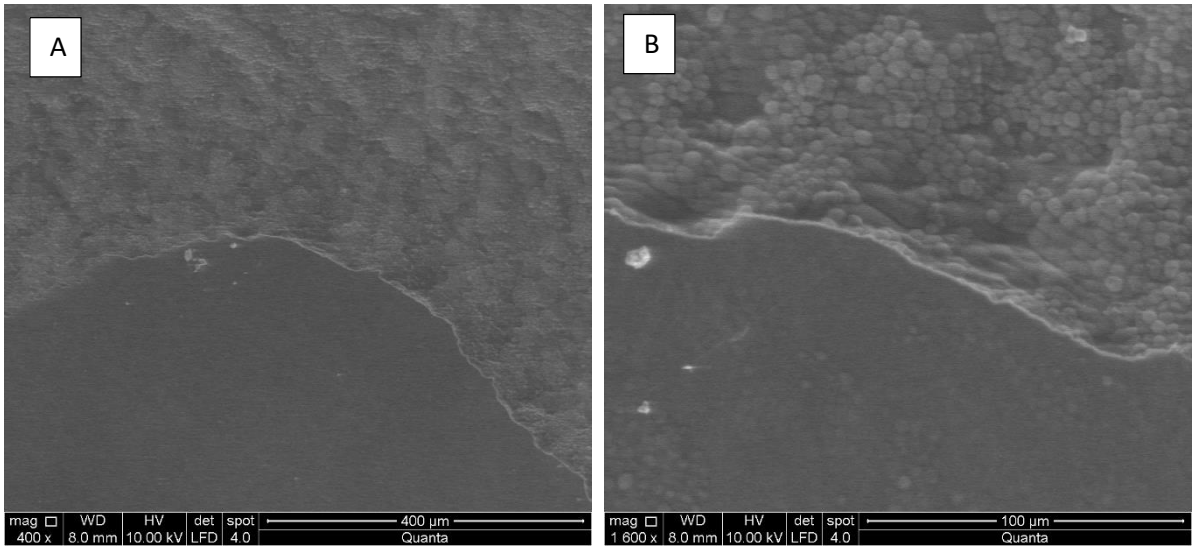

Figure III.25 – SEM images of the external surface of immobilized *S. cerevisiae* beads after fermentation with an initial glucose concentration of 100 g/L. (a) Magnification 400x (b) magnification 1600x.

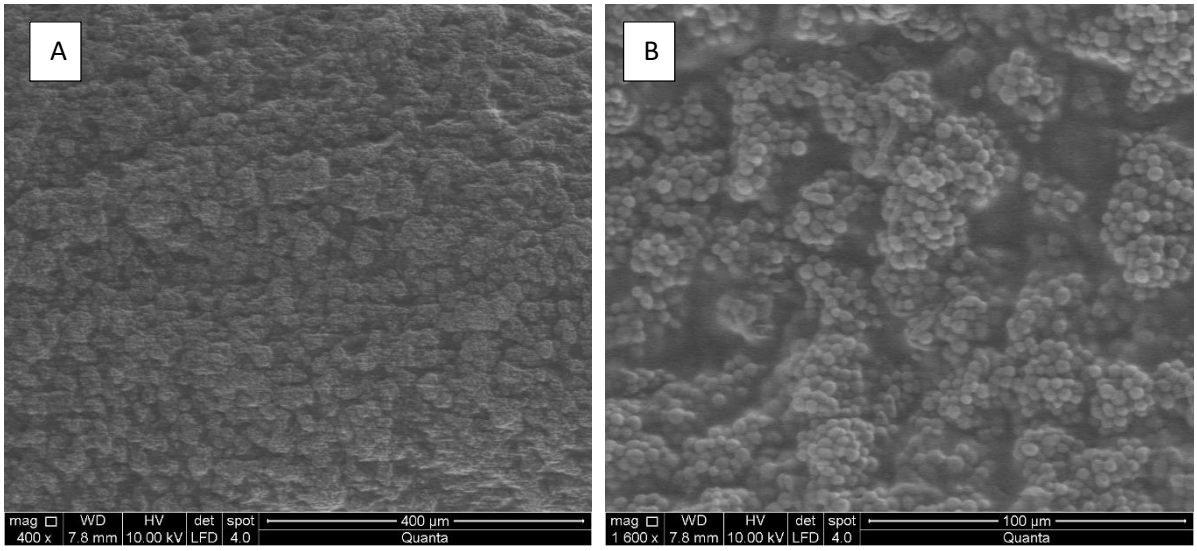

Figure III.26 – SEM images of the external surface of immobilized *S. cerevisiae* beads after fermentation with an initial glucose concentration of 200 g/L. (a) Magnification 400x (b) magnification 1600x.

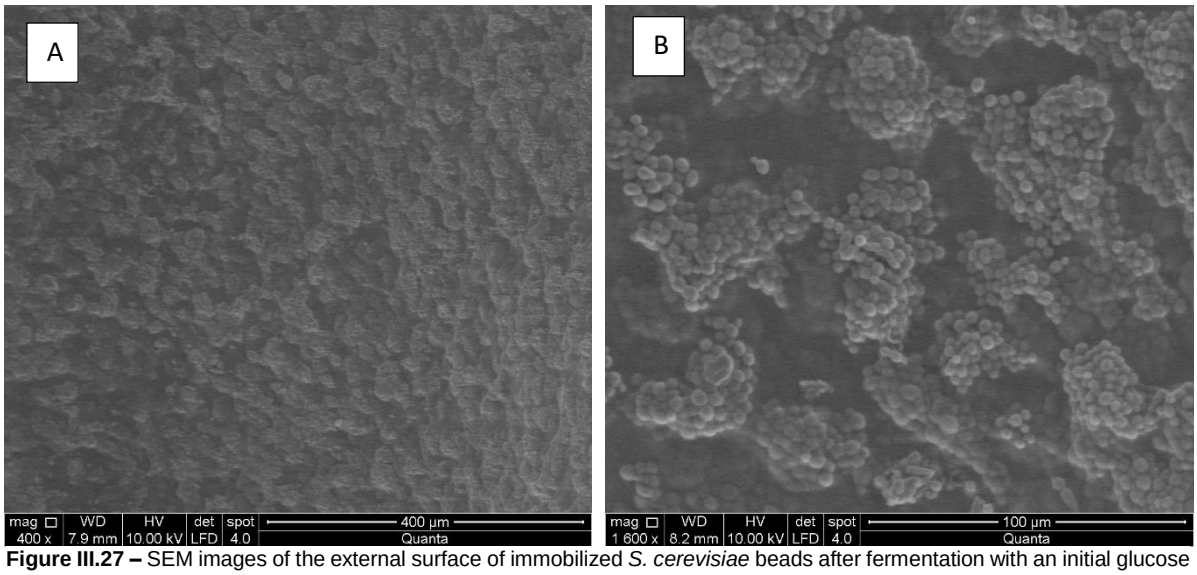

Figure III.27 – SEM images of the external surface of immobilized *S. cerevisiae* beads after fermentation with an initial glucose concentration of 300 g/L. (a) Magnification 400x (b) magnification 1600x.

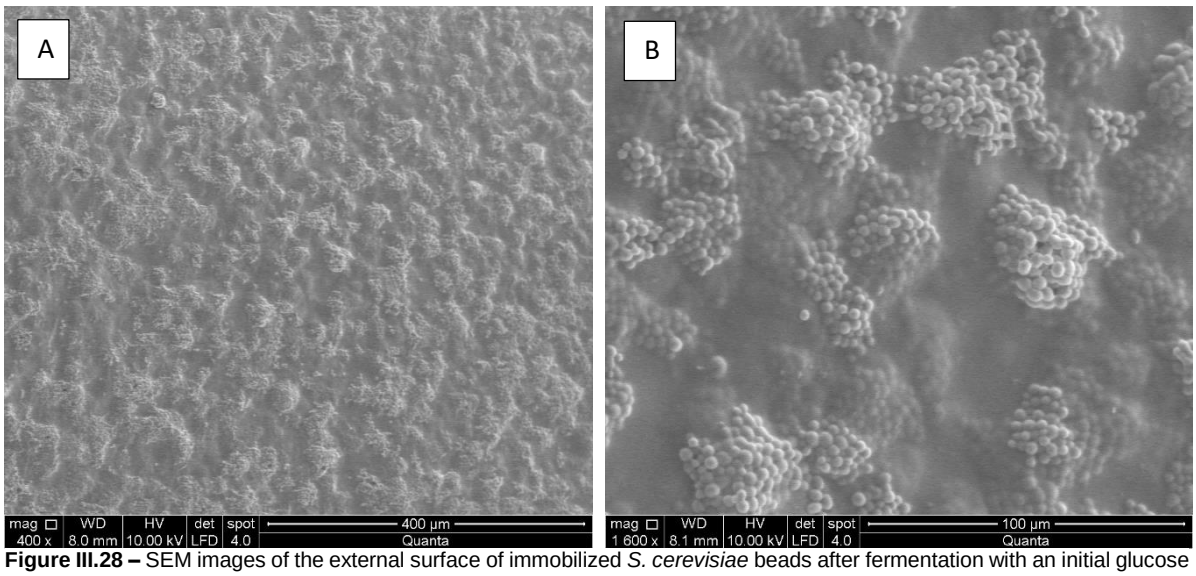

Figure III.28 – SEM images of the external surface of immobilized *S. cerevisiae* beads after fermentation with an initial glucose concentration of 400 g/L. (a) Magnification: 400x (b) magnification: 1600x.

In these images is evident the entrapment of yeasts in the calcium alginate matrix. It is notable that during each fermentation there are the formation of more prominent yeast agglomerates, which is typical in yeast growth. This difference is notable when comparing Figure III.23 with Figures III.24 to Figure III.28. In these last pictures, yeast agglomerates are more well defined than in the first one, that was taken before fermentation.

Chapter IV

Conclusions

The main objective of this work was to study bioethanol production from the Organic Fraction of Municipal Solid Wastes (OFMSW), assessing if yeast immobilization in a calcium alginate matrix brings advantages in comparison with a free-cell system. The motivation to carry out this study was to contribute to the development of the concept of biorefinery, in which renewable raw materials (such as OFMSW) are converted into energy with minimum waste production. Since OFMSW is a very complex substrate, both systems (yeast in suspension and immobilized) were first tested in a well-defined culture medium, with glucose as sole carbon source.

In general, there were technical problems of different sorts that did not allow a good comparison between essays and that prevented the achievement of a valid conclusion about the use of OFMSW as substrate for bioethanol production. The comparison between the suspended system and the immobilized one was only possible using data obtained with the method that presented more experimental uncertainty (assay monitored by weight loss). Therefore, the results obtained were inconclusive.

Nonetheless, a much higher weight loss was observed for the essay with immobilized yeast than in the case of suspended yeast for the same initial glucose concentration. This fact, suggests that there was a considerable increase in the production of bioethanol when yeasts were immobilized. Consequently, one can conclude, with inherent uncertainty, that yeast immobilization in a calcium alginate matrix promotes the production of bioethanol. This result was expected since the entrapment of the cells inside the calcium alginate matrix decreases the inhibitory effect due to high substrate concentration. However, another conclusion that can be taken from the results is that this inhibitory effect is still verified when substrate concentration is too high.

Regarding the OFSMW essay, the conclusion which can be taken from the data collected is the substrate concentration in the liquid rich in organic matter is too high, inhibiting biomass production. Therefore, this must be diluted, and the ideal dilution factor was found to be 1:2. However, it was not possible to obtain data concerning ethanol concentration. Therefore, not only was the influence of a complex substrate in bioethanol production undetermined, but also it was

not possible to assess if the immobilization brought advantages. This means that the objective set in the beginning of the experimental period was not clearly accomplished.

In order to improve the work done, the essay monitored by weight loss should be repeated in such a way, that the amount of CO_2 produced is directly measured. Besides this, all other essays should also be repeated until reproducible results are obtained. Once this assessment is completed, the next step should be the scale-up of the system in order to understand if it behaved in the same way.

Another consideration that should be taken into account is if the immobilization method used is easily implemented in an industrial facility. On one hand, in order to facilitate the application of the used method, it should be considered a way of automatizing it. This implies an initial investment that increases the total costs of the production process. On the other hand, there should be an economical evaluation to know if this initial investment would be covered by the increase in bioethanol production and possibly the reuse of the same immobilized biomass in multiple fermentation runs.

In conclusion, objectives set at the beginning of this work were not totally accomplished and all the inferences taken from the results present a considerable associated error, not discarding the evident correlation found between inhibitory effect and substrate concentration.

Chapter V

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Annex I

Table 1 – Experimental data for optical density (OD) in the case of YEPG culture medium

Sample	OD	Time	Hour	Day
0	0,076	0	12	22/02/2016
1	0,161	8	20	
2	0,212	24	12	23/02/2016
3	0,221	32	20	
4	0,225	48	12	24/02/2016

Table 2 – Experimental data for optical density (OD) and its conversion to biomass concentration. Culture media used for the first fermentation. SD stands for standard deviation

Time (h)	OD	Mean	SD	[Biomass]	Média	SD
0	0,297	0,298	0,001	0,12786	0,128254	0,001
	0,298			0,128647		
18	0,240	0,220	0,028	0,083038	0,067311	0,022
	0,200			0,051584		
24	0,309	0,302	0,010	0,137297	0,131792	0,008
	0,295			0,126288		
42	0,484	0,438	0,066	0,274908	0,238342	0,052
	0,391			0,201777		
48	0,502	0,485	0,024	0,289062	0,275694	0,019
	0,468			0,262326		
49	1,011	0,853	0,224	0,689314	0,564677	0,176
	0,694			0,440041		
72	0,696	0,708	0,016	0,441614	0,450657	0,013
	0,719			0,4597		
90	0,811	0,816	0,006	0,532044	0,535582	0,005
	0,82			0,539121		
96	0,789	0,773	0,023	0,514744	0,502162	0,018
	0,757			0,489581		
114	0,965	0,943	0,031	0,653141	0,635842	0,024
	0,921			0,618542		
120	0,989	0,9795	0,013	0,672014	0,664544	0,011
	0,97			0,657073		

Annex II

Table 2 – Stoichiometric coefficients recalculated during iterative calculation of $Y''_{x/s}$ for each initial glucose concentration essay

Stoichiometric coefficients	Initial glucose concentration (g/L)				
	50	100	200	300	400
a	0,0107	0,0107	0,0127	0,0130	0,0151
b	0,0631	0,0631	0,0746	0,0763	0,0887
c	1,98	1,98	1,97	1,97	1,97
d	1,98	1,98	1,98	1,98	1,97
e	0,0246	0,0246	0,0291	0,0297	0,0346

Table 2 – Experimental data obtained for weight loss method for the essay with suspended yeast

	A					B				
Time (h)	Concentration (g/L)					Concentration (g/L)				
	50	100	200	300	400	50	100	200	300	400
0	172,70	174,62	179,70	183,76	190,29	171,96	175,29	177,51	180,73	185,13
6	172,70	174,61	179,69	183,74	190,28	171,96	175,29	177,51	180,73	185,13
24	172,68	174,59	179,68	183,74	190,28	171,94	175,28	177,49	180,71	185,12
30	172,66	174,57	179,66	183,73	190,28	171,92	175,26	177,46	180,68	185,11
48	172,54	174,49	179,57	183,67	190,26	171,86	175,2	177,38	180,58	185,04
54	172,50	174,45	179,54	183,63	190,24	171,82	175,19	177,36	180,55	185,00
72	172,35	174,35	179,44	183,52	190,11	171,71	175,1	177,26	180,44	184,89
78	172,30	174,31	179,40	183,48	190,06	171,66	175,06	177,21	180,39	184,85
96	172,16	174,19	179,30	183,37	189,87	171,53	174,96	177,11	180,28	184,75
102	172,11	174,15	179,27	183,34	189,82	171,49	174,92	177,08	180,25	184,73
120	171,96	174,03	179,16	183,22	189,66	171,27	174,72	176,89	180,05	184,54

Annex III

Linear correlation between natural logarithm of biomass concentration (ln([Biomass])) determined with semi-theoretical yield and time for the essay with suspended yeast in the case of weight loss method is presented by Figure 1.

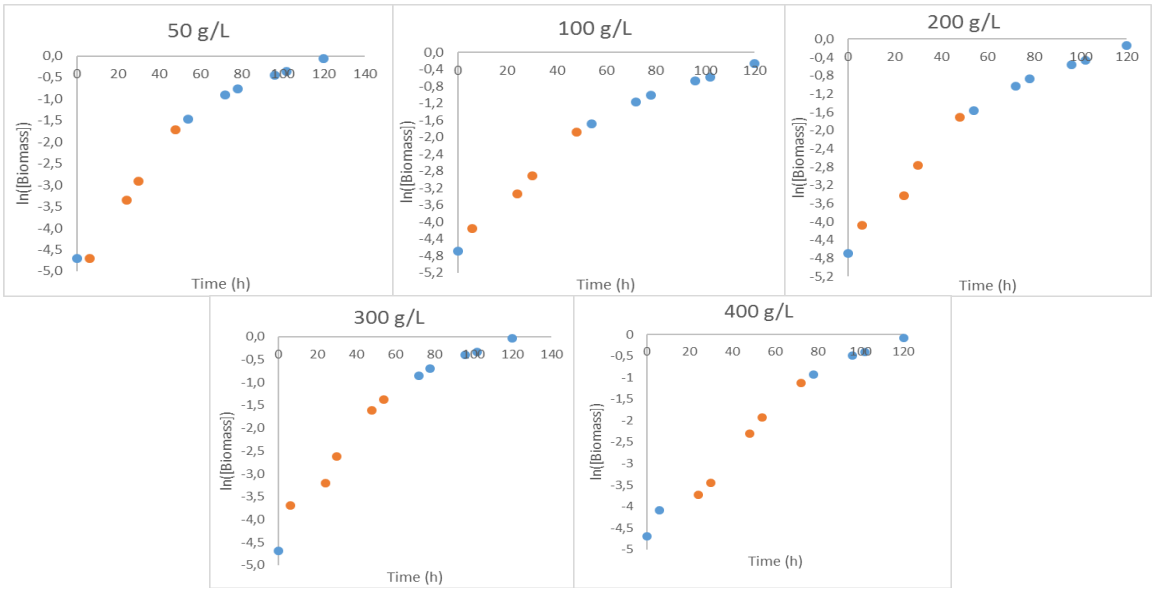

Figure 1 – ln([Biomass]) vs time for μ calculation for each essay with suspended yeast and monitoring by weight loss. Biomass concentration determine by semi-theoretical yield. Orange spots ( ) represent values that belong to exponential phase that were used for linearization. Trend line and correlation factors described by equations from 1 to 10.

$$\text{50 g/L: } \ln([Biomass]) = 0.0709t - 5.08 \quad \textbf{(1)}$$

$$R^2 = 0.9984 \quad \textbf{(2)}$$

$$\text{100 g/L: } \ln([Biomass]) = 0.0546 - 4.54 \quad \textbf{(3)}$$

$$R^2 = 0.9933 \quad \textbf{(4)}$$

$$\text{200 g/L: } \ln([Biomass]) = 0.0574t - 4.55 \quad \textbf{(5)}$$

$$R^2 = 0.9696 \quad \textbf{(6)}$$

$$\text{300 g/L: } \ln([Biomass]) = 0.0511t - 4.16 \quad \textbf{(7)}$$

$$R^2 = 0.9732 \quad \textbf{(8)}$$

$$\text{400 g/L: } \ln([Biomass]) = 0.0558t - 5.05 \quad \textbf{(9)}$$

$$R^2 = 0.9939 \quad \textbf{(10)}$$

Linear correlation between natural logarithm of biomass concentration determined with theoretical yield and time for the essay with suspended yeast in the case of weight loss method is presented by Figure 2.

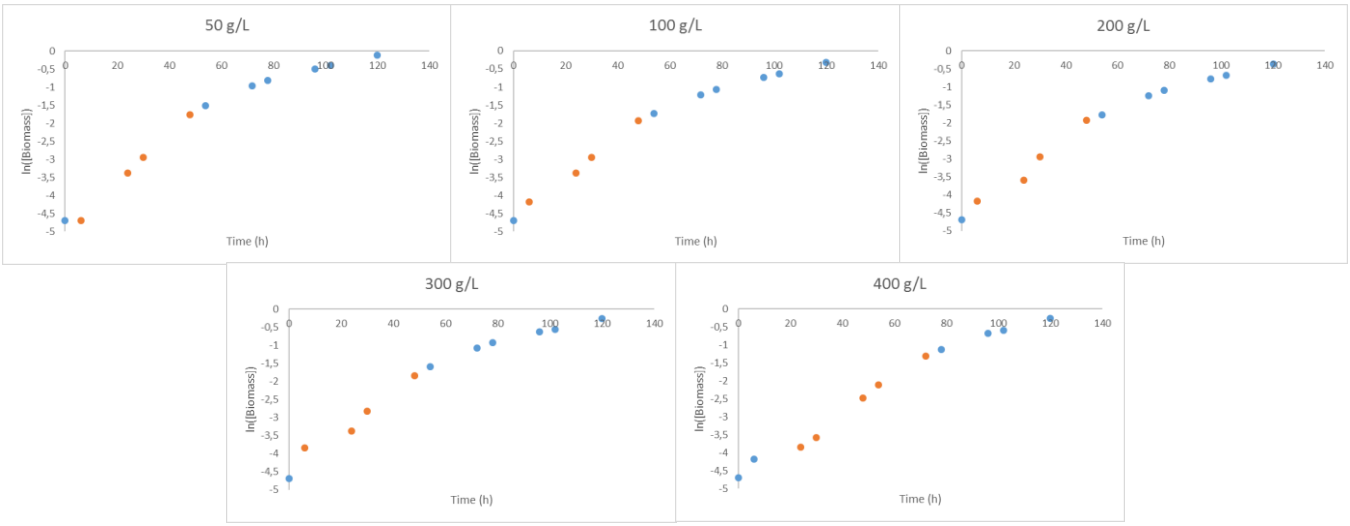

Figure 2 – ln([Biomass]) vs time for μ calculation for each essay with suspended yeast and monitoring by weight loss. Biomass concentration determine by theoretical yield. Orange spots (–●–) represent values that belong to exponential phase that were used for linearization. Trend line and correlation factors described by equations from 11 to 20.

50 g/L: $\ln([Biomass]) = 0.0697t - 5.08$ **(11)**

$R^2 = 0.9989$ **(12)**

100 g/L: $\ln([Biomass]) = 0.054t - 4.56$ **(13)**

$R^2 = 0.9927$ **(14)**

200 g/L: $\ln([Biomass]) = 0.0546t - 4.63$ **(15)**

$R^2 = 0.9659$ **(16)**

300 g/L: $\ln([Biomass]) = 0.0483t - 4.28$ **(17)**

$R^2 = 0.9522$ **(18)**

400 g/L: $\ln([Biomass]) = 0.0544t - 5.14$ **(19)**

$R^2 = 0.9947$ **(20)**

In Figure 3 is presented the adjustment to the experimental data obtained with theoretical data. Sum of error described by equation III.13.

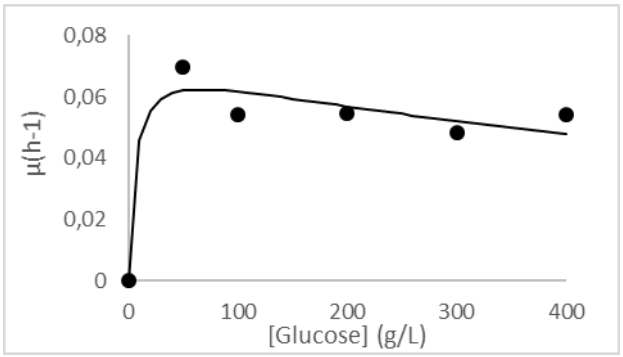

Figure 3 – Obtained model adjusted to the experimental data calculated with theoretical yield. Sum error: 1.79×10^{-4} h^{-1}. $\mu_{experimental}$ (●) stands for values obtained experimentally, and μ_{model} stands for values calculated through mathematical method described by equation III.12.

Values of μ and t_d are presented in Table 1. In Table 2 are presented all values obtained for the kinetic constants.

Table 1 - Values of specific growth rate and doubling time for each initial glucose concentration for semi theoretical case

$[Glucose]_i$ (g/L)	**50**	**100**	**200**	**300**	**400**
μ (h^{-1})	0.0697	0.0540	0.0546	0.0483	0.0543
t_d (h) (theoretical)	9.94	12.8	12.7	14.4	12.8

Table 2 – Values for kinetic constants obtained for theoretical data

Kinetic constants	**Values**
K_S (g/L)	5.74
μ_{max} (h^{-1})	0.0730
K_i (g/L)	785

Linear correlation between natural logarithm of biomass concentration and time for the essay with determination of glucose concentration with DNS is presented in Figure 4.

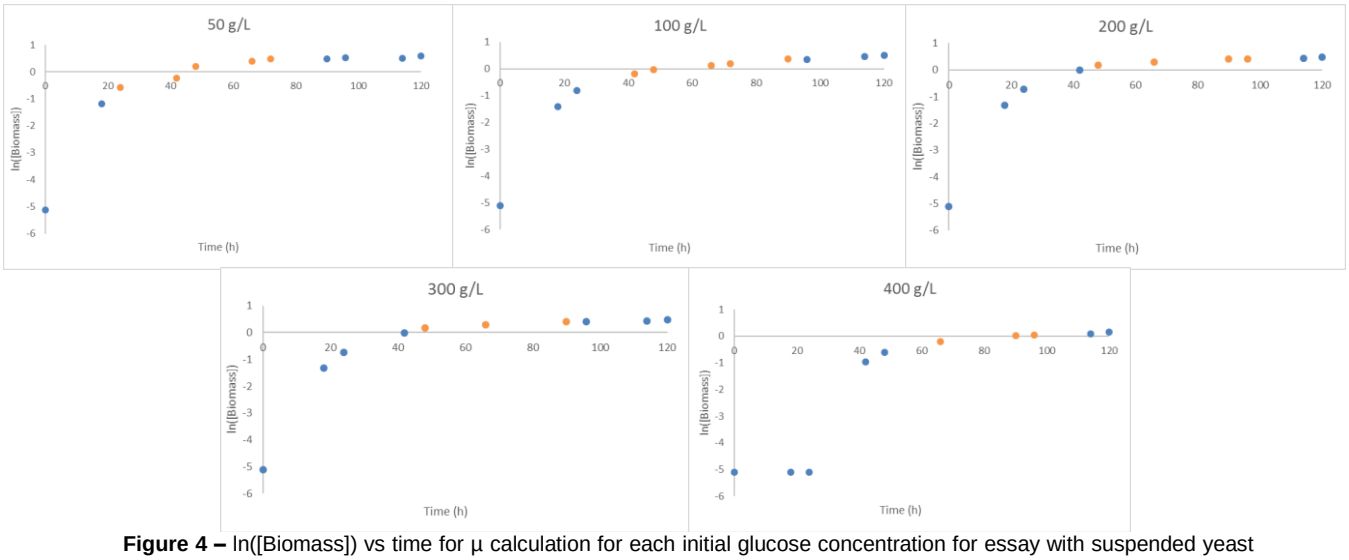

Figure 4 – ln([Biomass]) vs time for μ calculation for each initial glucose concentration for essay with suspended yeast and glucose concentration determination by DNS. Orange spots (—●—) represent values that belong to exponential phase that were used for linearization Trend line and correlation factors described by equations from 21 to 30.

50 g/L: $\ln([Biomass]) = 0.0227 - 1.09$ **(21)**

$R^2 = 0.9282$ **(22)**

100 g/L: $\ln([Biomass]) = 0.0109t - 0.599$ **(23)**

$R^2 = 0.9808$ **(24)**

200 g/L: $\ln([Biomass]) = 0.00520t - 0.0739$ **(25)**

$R^2 = 0.9871$ **(26)**

300 g/L: $\ln([Biomass]) = 0.00560t - 0.0953$ **(27)**

$R^2 = 0.9923$ **(28)**

400 g/L: $\ln([Biomass]) = 0.00810t - 0.734$ **(29)**

$R^2 = 0.9931$ **(30)**

Linear correlation between natural logarithm of biomass concentration and time for the essay with suspended yeast with glucose concentration monitored by HPLC was used is presented in Figure 5.

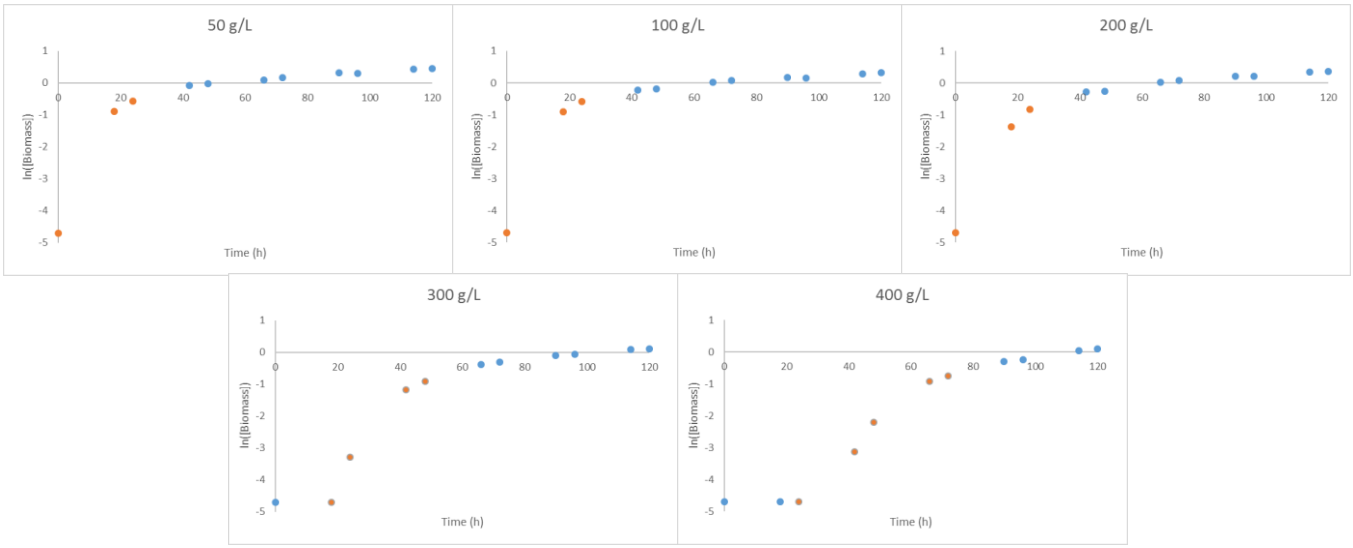

Figure 5 – ln([Biomass]) vs time for μ calculation for each initial glucose concentration. Essay with suspended yeast in which glucose concentration was monitored with HPLC. Orange spots (—●—) represent values that belong to exponential phase that were used for linearization. Trend line and correlation factors described by equations from 31 to 40.

$$\text{50 g/L: } \ln([Biomass]) = 0.181t - 4.58 \textbf{ (31)}$$

$$R^2 = 0.9704 \textbf{ (32)}$$

$$\text{100 g/L: } \ln([Biomass]) = 0.180t - 4.59 \textbf{ (33)}$$

$$R^2 = 0.9714 \textbf{ (34)}$$

$$\text{200 g/L: } \ln([Biomass]) = 0.167t - 4.63 \textbf{ (35)}$$

$$R^2 = 0.9879 \textbf{ (36)}$$

$$\text{300 g/L: } \ln([Biomass]) = 0.124t - 6.60 \textbf{ (37)}$$

$$R^2 = 0.9655 \textbf{ (38)}$$

$$\text{400 g/L: } \ln([Biomass]) = 0.0913t - 6.84 \textbf{ (39)}$$

$$R^2 = 0.9881 \textbf{ (40)}$$

Linear correlation between natural logarithm of biomass concentration and time for the essay with immobilized yeast and monitored by weight loss using data calculated with semi-theoretical and theoretical yield is presented in Figure 6 and Figure 7, respectively. Trend lines described by equation from 41 to 50 and 51 to 60, respectively.

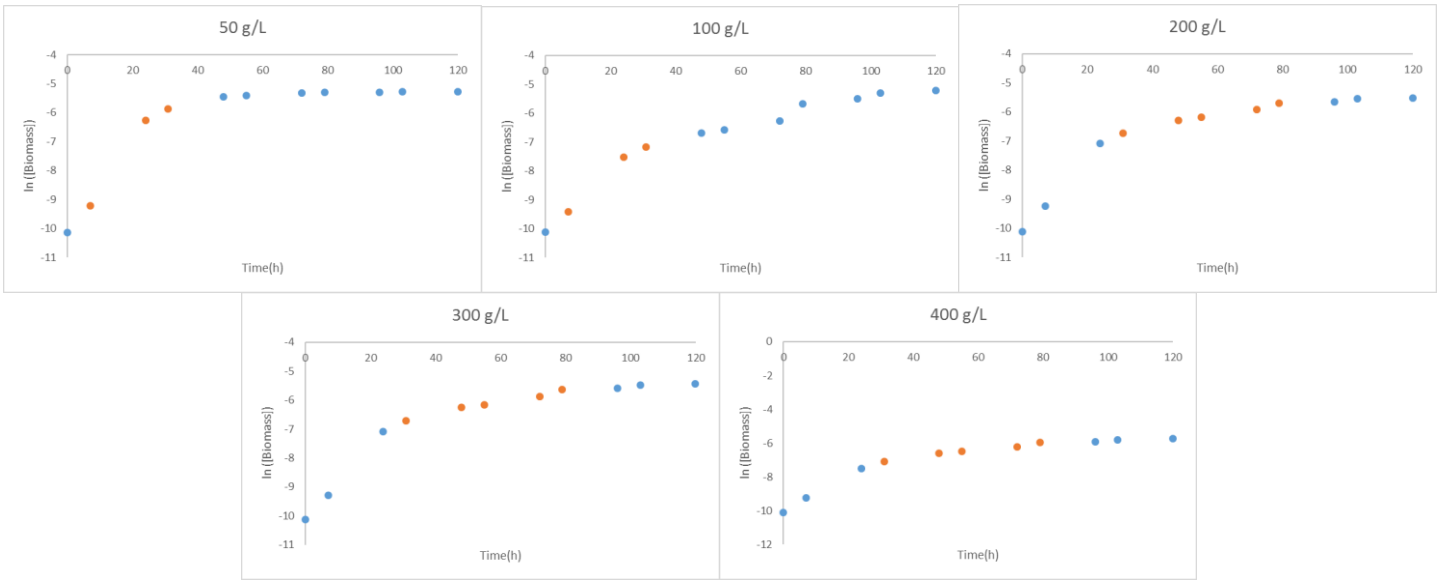

Figure 6 – ln([Biomass]) vs time for μ calculation for each initial glucose concentration. Essay with immobilized yeast and monitored by weight loss using data calculated with semi theoretical yield. Orange spots (–●–) represent values that belong to exponential phase that were used for linearization. Trend line and correlation factors described by equations from 41 to 50.

50 g/L: $\ln([Biomass]) = 0.146t - 10.1$ **(41)**

$R^2 = 0.96803$ **(42)**

100 g/L: $\ln([Biomass]) = 0.0970t - 10.0$ **(43)**

$R^2 = 0.9811$ **(44)**

200 g/L: $\ln([Biomass]) = 0.109t - 9.92$ **(45)**

$R^2 = 0.9767$ **(46)**

300 g/L: $\ln([Biomass]) = 0.0734t - 9.35$ **(47)**

$R^2 = 0.8602$ **(48)**

400 g/L: $\ln([Biomass]) = 0.0640t - 9.36$ **(49)**

$R^2 = 0.9004$ **(50)**

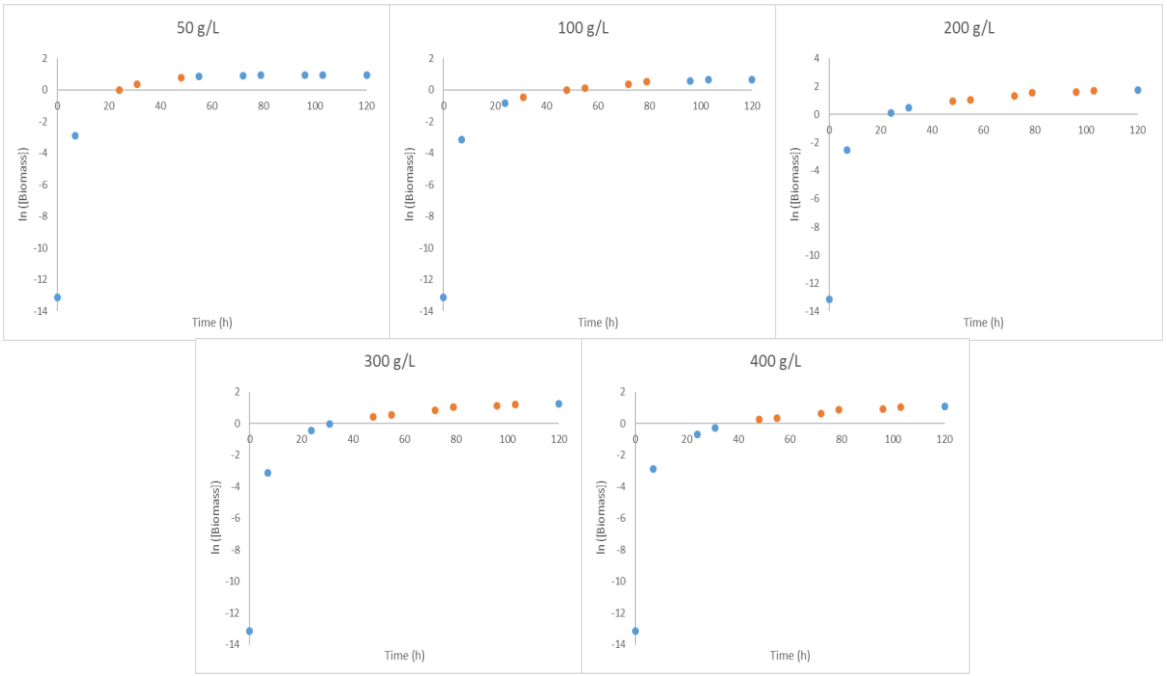

Figure 7 – ln([Biomass]) vs time for μ calculation for each initial glucose concentration. Essay with immobilized yeast and monitored by weight loss using data calculated with theoretical yield. Orange spots (●–) represent values that belong to exponential phase that were used for linearization. Trend line and correlation factors described by equations from 51 to 60.

50 g/L: $\ln([Biomass]) = 0.0318t - 0.711$ **(51)**

$R^2 = 0.9512$ **(52)**

100 g/L: $\ln([Biomass]) = 0.0192t - 1.00$ **(53)**

$R^2 = 0.9819$ **(54)**

200 g/L: $\ln([Biomass]) = 0.0137t + 0.335$ **(55)**

$R^2 = 0.9468$ **(56)**

300 g/L: $\ln([Biomass]) = 0.0145t + 0.233$ **(57)**

$R^2 = 0.9486$ **(58)**

400 g/L: $\ln([Biomass]) = 0.0148t + 0.441$ **(59)**

$R^2 = 0.9513$ **(60)**

In Figure 8 is presented the adjustment to the experimental data obtained with theoretical data. Sum of error described by equation III.13.

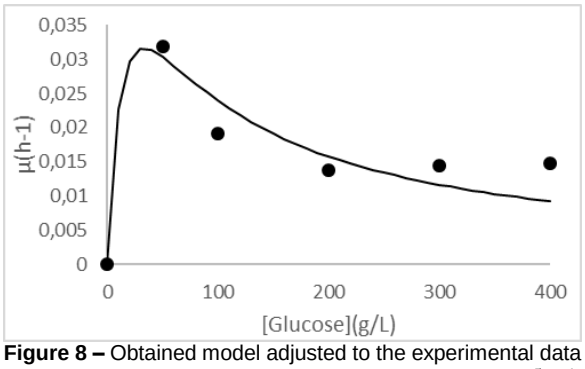

Figure 8 – Obtained model adjusted to the experimental data calculated with theoretical yield. Sum error: 6.77×10^{-5} h^{-1}. $\mu_{experimental}$ (●) stands for values obtained experimentally, and μ_{model} stands for values calculated through mathematical method described by equation III.12.

Values of μ and t_d are presented in Table 3. In Table 4 are presented all values obtained for the kinetic constants.

Table 3 - Values of specific growth rate and doubling time for each initial glucose concentration for theoretical case

$[Glucose]_i$ (g/L)	**50**	**100**	**200**	**300**	**400**
μ (h^{-1})	0.0318	0.0192	0.0137	0.0145	0.0148
t_d (h) (theoretical)	21.8	36.1	50.6	47.8	46.8

Table 4 – Values for kinetic constants obtained for theoretical data

Kinetic constants	**Values**
K_S (g/L)	15.4
μ_{max} (h^{-1})	0.0606
K_i (g/L)	72.4

Annex IV

Trend lines, sum of variance and correlation factor for each initial glucose concentration are described by equations from 1 to 10. Essay in which glucose concentration was determine by DNS method.

50 g/L: $[Glucose] = 3.00 \times 10^{-3}x^2 - 0.820x + 53.0$ **(1)**

$R^2 = 0.9111$; $\sum Var = 256$ **(2)**

100 g/L: $[Glucose] = 9.46 \times 10^{-5}x^3 - 1.55 \times 10^{-2}x^2 + 0.145x + 74.5$ **(3)**

$R^2 = 0.6110$; $\sum Var = 1662$ **(4)**

200 g/L: $[Glucose] = 1.86 \times 10^{-4}x^3 - 2.62 \times 10^{-2}x^2 - 7.11 \times 10^{-2}x + 159$ **(5)**

$R^2 = 0.6147$; $\sum Var = 4946$ **(6)**

300 g/L: $[Glucose] = 2.55 \times 10^{-4}x^3 - 4.64 \times 10^{-2}x^2 + 1.45x + 185$ **(7)**

$R^2 = 0.3164$; $\sum Var = 15272$ **(8)**

400 g/L: $[Glucose] = 8.74 \times 10^{-5}x^3 + 7.78 \times 10^{-4}x^2 - 2.47x + 350$ **(9)**

$R^2 = 0.3186$; $\sum Var = 45778$ **(10)**

Annex V

Table 1 – Experimental values of absorvence at 540 nm for each initial glucose concentration, the conversion to glucose concentration and the dilution factor

Time (h)	Concentration (g/L)	Abs (540 nm)	[G]i	Diluition	[G]f
0	50	1,002	1,878307	25	46,95767
	100	0,936	1,757891	50	87,89454
	200	0,896	1,684912	100	168,4912
	300	0,406	0,790914	150	118,6371
	400	0,915	1,719577	200	343,9153
4	50	1,018	1,907499	25	47,68747
	100	0,594	1,133917	50	56,69586
	200	0,79	1,491516	100	149,1516
	300	0,905	1,701332	150	255,1998
	400	0,994	1,863711	200	372,7422
17	50	0,873	1,642948	25	41,07371
	100	0,916	1,721401	50	86,07006
	200	0,823	1,551724	100	155,1724
	300	0,77	1,455026	150	218,254
	400	0,872	1,641124	200	328,2248
20	50	0,916	1,721401	25	43,03503
	100	0,674	1,279876	50	63,9938
	200	0,791	1,493341	100	149,3341
	300	0,837	1,577267	150	236,59
	400	0,849	1,599161	200	319,8321
24	50	0,628	1,19595	25	29,89874
	100	0,574	1,097427	50	54,87137
	200	0,682	1,294472	100	129,4472
	300	0,556	1,064587	150	159,688
	400	0,475	0,916804	200	183,3607
28	50	0,7	1,327313	25	33,18281
	100	0,802	1,51341	50	75,6705
	200	0,76	1,436782	100	143,6782
	300	0,597	1,139391	150	170,9086
	400	0,638	1,214194	200	242,8389
41	50	0,744	1,40759	25	35,18975
	100	0,875	1,646597	50	82,32987
	200	0,898	1,68856	100	168,856
	300	0,786	1,484218	150	222,6327
	400	0,869	1,63565	200	327,1301
44	50	0,48	0,925926	25	23,14815
	100	0,504	0,969714	50	48,48568
	200	0,342	0,674147	100	67,41471
	300	0,425	0,825579	150	123,8369
	400	0,499	0,960591	200	192,1182
52	50	0,657	1,24886	25	31,22149
	100	0,751	1,420361	50	71,01806
	200	0,842	1,586389	100	158,6389
	300	0,823	1,551724	150	232,7586
	400	1,16	2,166575	200	433,3151
65	50	0,151	0,32567	25	8,141762
	100	0,358	0,703339	50	35,16694
	200	0,365	0,71611	100	71,61102
	300	0,454	0,878489	150	131,7734
	400	0,609	1,161284	200	232,2569
68	50	0,011	0,070243	25	1,756066
	100	0,131	0,289181	50	14,45904
	200	0,329	0,650429	100	65,04288
	300	0,245	0,497172	150	74,57581
	400	0,155	0,332968	200	66,59369
76	50	0,228	0,466156	12,5	5,826948
	100	0,981	1,839993	25	45,99982
	200	1,138	2,126437	50	106,3218
	300	1,157	2,161102	75	162,0826
	400	0,921	1,730524	100	173,0524
89	50	0,003	0,055647	12,5	0,695585
	100	0,845	1,591863	25	39,79657
	200	0,881	1,657544	50	82,87721
	300	1,205	2,248677	75	168,6508
	400	1,412	2,626346	100	262,6346
92	50	0,002	0,053822	6,25	0,336389
	100	0,887	1,668491	12,5	20,85614
	200	1,228	2,29064	25	57,26601
	300	1,528	2,837986	37,5	106,4245
	400	1,75	3,243021	50	162,1511
100	50	0,009	0,066594	6,25	0,416211
	100	1,927	3,565955	12,5	44,57444
	200	1,911	3,536763	25	88,41908
	300	2,075	3,835979	37,5	143,8492
	400	2,286	4,220945	50	211,0473
120	50	0,021	0,088488	3,125	0,276523
	100	0,99	1,856413	12,5	23,20516
	200	2,016	3,728334	25	93,20836
	300	1,868	3,458311	37,5	129,6866
	400	2,466	4,549352	50	227,4676

Table 2 – Experimental values obtained for optical density and dry weight for the essay with suspended yeast in which glucose concentration was determined by DNS

Initial concentration (g/L)																				Time (h)
50				100				200				300				400				
OD	DW (g/L)	Mean	SD	OD	DW (g/L)	Mean	SD	OD	DW (g/L)	Mean	SD	OD	DW (g/L)	Mean	SD	OD	DW (g/L)	Mean	SD	
0,040	0,000	0,006	0,000	0,016	0,000	0,006	0,000	0,013	0,000	0,00602	0,007	0,000	0,000	0,006	0,000	0,000	0,000	0,006	0,0000	0
0,032	0,000			0,001	0,000			0,016	0,000			0,008	0,000			0,001	0,000			
0,040	0,000			0,026	0,000			0,001	0,000			0,009	0,000			0,002	0,000			
0,025	0,000			0,018	0,000			0,012	0,000			0,002	0,000			0,005	0,000			
0,528	0,310	0,309	0,061	0,463	0,258	0,243	0,150	0,412	0,218	0,269	0,072	0,197	0,049	0,041	0,033	0,038	0,000	0,006	0,0000	18
0,616	0,379			0,697	0,442			0,439	0,240			0,181	0,037			0,041	0,000			
0,427	0,230			0,373	0,188			0,479	0,271			0,235	0,079			0,055	0,000			
0,536	0,316			0,243	0,085			0,577	0,348			0,109	0,000			0,074	0,000			
0,741	0,477	0,556	0,063	0,701	0,446	0,449	0,054	0,740	0,476	0,483	0,127	0,525	0,307	0,312	0,044	0,111	0,000	0,006	0,0000	24
0,816	0,536			0,801	0,524			0,881	0,587			0,611	0,375			0,101	0,000			
0,927	0,623			0,681	0,430			0,58	0,350			0,511	0,296			0,033	0,000			
0,88	0,586			0,641	0,398			0,795	0,519			0,479	0,271			0,024	0,000			
1,134	0,786	0,786	0,078	1,218	0,852	0,818	0,048	1,449	1,034	0,987	0,274	1,127	0,781	0,775	0,004	0,611	0,375	0,383	0,0421	42
1,091	0,752			1,201	0,839			1,599	1,152			1,121	0,776			0,561	0,335			
1,27	0,893			1,084	0,747			0,989	0,672			1,119	0,774			0,622	0,383			
1,04	0,712			1,197	0,836			1,522	1,091			1,115	0,771			0,691	0,438			
1,699	1,230	1,230	0,065	1,329	0,939	0,969	0,054	1,774	1,289	1,277	0,084	1,373	0,974	0,958	0,046	0,895	0,598	0,551	0,0550	48
1,755	1,274			1,316	0,929			1,689	1,222			1,273	0,895			0,895	0,598			
1,582	1,138			1,358	0,962			1,699	1,230			1,351	0,957			0,769	0,499			
1,759	1,278			1,466	1,047			1,87	1,365			1,412	1,005			0,779	0,507			
2,06	1,514	1,482	0,028	1,551	1,114	1,122	0,049	1,876	1,370	1,332	0,096	1,512	1,083	1,130	0,040	1,137	0,788	0,820	0,0405	66
2,01	1,475			1,612	1,162			1,941	1,421			1,612	1,162			1,13	0,783			
1,974	1,447			1,477	1,056			1,756	1,275			1,546	1,110			1,235	0,865			
2,03	1,491			1,604	1,156			1,741	1,263			1,615	1,164			1,205	0,842			
2,201	1,625	1,628	0,091	1,618	1,167	1,204	0,088	1,439	1,026	1,008	0,016	1,155	0,803	0,803	0,026	1,032	0,706	0,714	0,0388	72
2,151	1,586			1,669	1,207			1,407	1,001			1,124	0,778			1,012	0,690			
2,102	1,547			1,818	1,324			1,412	1,005			1,201	0,839			1,115	0,771			
2,367	1,756			1,555	1,117			1,405	0,999			1,141	0,792			1,011	0,689			
2,289	1,694	1,619	0,120	1,985	1,455	1,458	0,145	1,989	1,458	1,498	0,059	1,632	1,178	1,256	0,064	1,52	1,090	1,011	0,1055	90
2,014	1,478			1,751	1,271			2,018	1,481			1,807	1,315			1,231	0,862			
2,348	1,741			2,199	1,623			2,024	1,486			1,789	1,301			1,511	1,082			
2,123	1,564			2,017	1,480			2,125	1,565			1,698	1,230			1,42	1,011			
2,290	1,695	1,655	0,075	1,924	1,407	1,407	0,147	2,109	1,553	1,517	0,054	1,763	1,281	1,281	0,041	1,556	1,118	1,039	0,1201	96
2,347	1,740			1,784	1,297			2,015	1,479			1,823	1,328			1,469	1,049			
2,159	1,592			2,187	1,614			2,019	1,482			1,696	1,228			1,561	1,122			
2,159	1,592			1,798	1,308			2,112	1,555			1,774	1,289			1,235	0,865			
2,520	1,876	1,657	0,151	2,029	1,490	1,568	0,075	2,116	1,558	1,536	0,060	1,713	1,241	1,320	0,056	1,407	1,001	1,080	0,0834	114
2,085	1,534			2,128	1,568			2,018	1,481			1,822	1,327			1,652	1,193			
2,161	1,594			2,098	1,544			2,156	1,590			1,881	1,373			1,518	1,088			
2,202	1,626			2,257	1,669			2,063	1,517			1,835	1,337			1,456	1,039			
2,402	1,783	1,799	0,170	2,210	1,632	1,657	0,098	2,114	1,557	1,603	0,046	1,962	1,437	1,379	0,064	1,617	1,166	1,170	0,0368	120
2,125	1,565			2,248	1,662			2,211	1,633			1,816	1,322			1,575	1,133			
2,562	1,909			2,105	1,550			2,207	1,630			1,954	1,431			1,611	1,161			
2,601	1,940			2,405	1,785			2,158	1,591			1,818	1,324			1,687	1,221			

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Table 3 – Experimental data of ethanol concentration for the essay with suspended yeast and glucose concentration determination with DNS. Peak area for each sample. SD stands for standard deviation

	Area														
	50 g/L			100 g/L			200 g/L			300 g/L			400 g/L		
Time	Sample	Mean	SD	Sample	Mean	SD	Sample	Mean	SD	Sample	Mean	SD	Sample	Mean	SD
0	2,01E+02	1,94E+02	15	1,65E+02	1,56E+02	10	1,50E+02	1,38E+02	11	1,36E+03	1,18E+03	160	2,00E+02	2,79E+02	55
	1,84E+02			1,50E+02			1,32E+02			1,24E+03			3,00E+02		
	1,80E+02			1,46E+02			1,25E+02			1,12E+03			2,89E+02		
	2,11E+02			1,65E+02			1,44E+02			9,86E+02			3,28E+02		
18	1,69E+03	1,16E+03	358	1,14E+03	1,10E+03	65	7,43E+02	7,27E+02	39	2,97E+04	2,69E+04	2457	5,99E+02	6,07E+02	30
	9,89E+02			1,11E+03			6,69E+02			2,40E+04			5,80E+02		
	1,00E+03			1,00E+03			7,54E+02			2,60E+04			6,01E+02		
	9,48E+02			1,14E+03			7,41E+02			2,77E+04			6,50E+02		
24	4,95E+04	4,79E+04	2335	5,12E+04	4,83E+04	2914	6,44E+03	6,17E+03	196	1,71E+04	1,56E+04	1269	4,96E+04	4,55E+04	2854
	4,98E+04			4,43E+04			6,20E+03			1,55E+04			4,38E+04		
	4,47E+04			4,88E+04			6,05E+03			1,40E+04			4,51E+04		
	4,77E+04			4,89E+04			6,00E+03			1,58E+04			4,34E+04		
42	2,00E+05	1,50E+05	52069	5,79E+04	5,65E+04	3424	1,98E+05	1,87E+05	10056	3,70E+04	3,58E+04	2099	1,38E+05	1,36E+05	18228
	1,90E+05			5,60E+04			1,89E+05			3,29E+04			1,30E+05		
	1,11E+05			6,01E+04			1,89E+05			3,76E+04			1,59E+05		
	1,00E+05			5,20E+04			1,74E+05			3,58E+04			1,16E+05		
48	7,09E+05	7,72E+05	53437	6,11E+05	5,93E+05	16221	9,01E+05	9,11E+05	12849	1,14E+06	1,09E+06	121530,4	3,20E+05	2,92E+05	52406
	8,20E+05			5,99E+05			9,30E+05			9,80E+05			3,11E+05		
	7,48E+05			5,87E+05			9,11E+05			9,92E+05			3,24E+05		
	8,13E+05			5,74E+05			9,04E+05			1,23E+06			2,14E+05		
66	1,34E+06	1,17E+06	136747,078	2,99E+05	2,62E+05	42897	1,28E+06	1,17E+06	79920	8,99E+05	8,76E+05	33552	1,02E+06	9,92E+05	37289
	1,16E+06			3,00E+05			1,09E+06			8,80E+05			1,02E+06		
	1,17E+06			2,24E+05			1,17E+06			8,27E+05			9,45E+05		
	1,00E+06			2,27E+05			1,13E+06			8,98E+05			9,81E+05		
72	1,43E+06	1,27E+06	146901,565	1,01E+06	1,02E+06	51485,9743	1,30E+06	1,17E+06	191431,5	1,14E+06	1,09E+06	56426	9,15E+05	9,21E+05	20453
	1,36E+06			9,90E+05			1,37E+06			1,11E+06			9,20E+05		
	1,20E+06			1,10E+06			1,01E+06			1,01E+06			9,49E+05		
	1,10E+06			9,99E+05			1,00E+06			1,10E+06			9,00E+05		
90	2,35E+06	2,14E+06	147047,74	1,99E+06	1,67E+06	245440,54	1,88E+06	1,75E+06	141468,5	7,37E+05	7,54E+05	50802,02	1,87E+06	1,75E+06	117307,5
	2,15E+06			1,57E+06			1,55E+06			8,23E+05			1,59E+06		
	2,09E+06			1,41E+06			1,82E+06			7,02E+05			1,76E+06		
	2,00E+06			1,72E+06			1,76E+06			7,52E+05			1,79E+06		
96	1,90E+06	1,79E+06	95544	1,42E+06	1,37E+06	178735,385	1,86E+06	1,66E+06	161843,8	1,49E+06	1,52E+06	42402	4,50E+05	4,57E+05	26237
	1,78E+06			1,53E+06			1,56E+06			1,50E+06			4,41E+05		
	1,67E+06			1,11E+06			1,71E+06			1,58E+06			4,95E+05		
	1,80E+06			1,42E+06			1,50E+06			1,50E+06			4,40E+05		
114	1,98E+06	1,81E+06	130912,148	1,48E+06	1,37E+06	148393,442	1,84E+06	1,75E+06	136102,3	1,85E+06	1,75E+06	186936,4	1,59E+06	1,41E+06	208674
	1,82E+06			1,51E+06			1,63E+06			1,86E+06			1,11E+06		
	1,79E+06			1,22E+06			1,63E+06			1,80E+06			1,41E+06		
	1,66E+06			1,26E+06			1,90E+06			1,47E+06			1,51E+06		
120	1,70E+06	1,59E+06	196214,316	2,00E+06	1,90E+06	215831,723	1,90E+06	1,76E+06	255090,8	1,99E+06	1,84E+06	146243,8	2,00E+06	2,06E+06	74022
	1,66E+06			2,11E+06			1,98E+06			1,87E+06			2,06E+06		
	1,30E+06			1,90E+06			1,76E+06			1,64E+06			2,16E+06		
	1,70E+06			1,60E+06			1,40E+06			1,86E+06			2,00E+06		

N

Table 4 – Experimental data for the essay with suspended yeast and glucose concentration determination with DNS. Conversion of data presented in Table 3 to ethanol concentration (mmol/L). SD stands for standard deviation

	Ethanol concentration (mmol/L)														
	50 g/L			100 g/L			200 g/L			300 g/L			400 g/L		
Time	Sample	Mean	SD	Sample	Mean	SD	Sample	Mean	SD	Sample	Mean	SD	Sample	Mean	SD
0	6,23	6,23	0,00	6,23	6,23	0,000746	6,23	6,22	0,000804	6,31	6,30E+00	0,011557	6,23	6,23	0,003994
	6,23			6,23			6,22			6,30			6,24		
	6,23			6,23			6,22			6,30			6,24		
	6,23			6,23			6,23			6,29			6,24		
18	6,34	6,30	0,03	6,30	6,29	0,004698	6,27	6,27	0,002803	8,36	8,15E+00	0,177326	6,26	6,26	0,002148
	6,29			6,30			6,26			7,95			6,26		
	6,29			6,29			6,27			8,09			6,26		
	6,28			6,30			6,27			8,22			6,26		
24	9,79	9,67	0,17	9,91	9,70	0,21	6,68	6,66	0,014137	7,45	7,34E+00	0,091614	9,80	9,50	0,206018
	9,81			9,41			6,66			7,34			9,38		
	9,44			9,73			6,65			7,22			9,47		
	9,66			9,74			6,65			7,36			9,35		
42	20,66	17,1	3,8	10,39	1,03E+01	0,2	20,47	1,97E+01	0,725863	8,88	8,80E+00	0,151495	16,15	1,60E+01	1,315738
	19,96			10,26			19,88			8,59			15,59		
	14,25			10,55			19,89			8,93			17,73		
	13,44			9,97			18,74			8,80			14,58		
48	57,37	62,0	3,9	50,35	4,90E+01	1,2	71,28	7,20E+01	0,927458	88,28	8,46E+01	8,772228	29,35	2,73E+01	3,78274
	65,39			49,47			73,32			76,93			28,67		
	60,20			48,58			71,98			77,82			29,58		
	64,92			47,63			71,44			95,20			21,67		
66	102,64	90,3	9,9	27,79	2,51E+01	3,1	98,52	9,06E+01	5,768702	71,13	6,94E+01	2,42186	79,84	7,78E+01	2,691585
	89,65			27,85			85,18			69,71			80,13		
	90,52			22,35			90,79			65,94			74,41		
	78,49			22,57			87,82			71,00			76,99		
72	109,14	98,1	10,6	78,78	8,01E+01	3,7	99,95	9,07E+01	13,81778	88,49	8,47E+01	4,072908	72,30	7,27E+01	1,47634
	104,37			77,69			105,06			86,02			72,66		
	93,10			85,65			79,29			78,94			74,73		
	85,81			78,35			78,42			85,35			71,19		
90	175,54	161	11	149,82	1,27E+02	18	141,73	1,33E+02	10,21138	59,41	6,06E+01	3,666957	141,51	1,33E+02	8,467409
	161,16			119,53			118,40			65,63			121,25		
	156,96			108,16			137,84			56,91			133,05		
	150,50			130,21			133,23			60,52			135,20		
96	143,26	135	7	108,70	1,05E+02	13	140,75	1,26E+02	11,6821	114,05	1,16E+02	3,060613	38,66	3,92E+01	1,89384
	135,05			116,46			118,80			114,74			38,07		
	126,43			86,50			129,91			120,61			41,97		
	136,14			108,45			114,83			114,77			37,97		
114	148,96	137	9	113,03	1,05E+02	11	138,78	1,33E+02	9,824046	140,04	1,32E+02	13,49331	120,80	1,08E+02	15,06237
	137,41			115,38			124,22			140,79			86,53		
	135,32			94,56			124,22			136,13			108,29		
	125,96			97,07			143,13			112,31			115,48		
120	128,83	121	14	150,60	1,44E+02	16	143,10	1,33E+02	18,41279	149,59	1,39E+02	10,55607	150,57	1,55E+02	5,343014
	126,25			158,21			148,88			141,52			155,17		
	99,74			143,48			133,25			124,37			161,89		
	128,81			122,02			107,25			140,35			150,61		

O

Table 5 – Experimental data for the essay with suspended yeast and glucose concentration determination with DNS. Conversion of data presented in Table 4 to ethanol concentration (g/L). SD stands for standard deviation

	Ethanol concentration (g/L)														
	50 g/L			100 g/L			200 g/L			300 g/L			400 g/L		
Time	Sample	Mean	SD	Sample	Mean	SD	Sample	Mean	SD	Sample	Mean	SD	Sample	Mean	SD
0	0,29	0,287	0,000	0,29	0,29	0,00	0,29	0,286712	3,7E-05	0,29	0,29	0,000532	0,29	0,287	0,000184
	0,29			0,29			0,29			0,29			0,29		
	0,29			0,29			0,29			0,29			0,29		
	0,29			0,29			0,29			0,29			0,29		
18	0,29	0,290	0,001	0,29	0,29	0,00	0,29	0,288671	0,000129	0,39	0,38	0,008168	0,29	0,288	9,89E-05
	0,29			0,29			0,29			0,37			0,29		
	0,29			0,29			0,29			0,37			0,29		
	0,29			0,29			0,29			0,38			0,29		
24	0,45	0,446	0,008	0,46	0,45	0,01	0,31	0,306773	0,000651	0,34	0,34	0,00422	0,45	0,438	0,009489
	0,45			0,43			0,31			0,34			0,43		
	0,43			0,45			0,31			0,33			0,44		
	0,44			0,45			0,31			0,34			0,43		
42	0,95	0,787	0,173	0,48	0,474132	0,01	0,94	0,90956	0,033433	0,41	0,41	0,006978	0,74	0,737	0,060603
	0,92			0,47			0,92			0,40			0,72		
	0,66			0,49			0,92			0,41			0,82		
	0,62			0,46			0,86			0,41			0,67		
48	2,64	2,854	0,178	2,32	2,257343	0,05	3,28	3,316541	0,042719	4,07	3,89	0,404049	1,35	1,258	0,174233
	3,01			2,28			3,38			3,54			1,32		
	2,77			2,24			3,32			3,58			1,36		
	2,99			2,19			3,29			4,39			1,00		
66	4,73	4,160	0,455	1,28	1,158025	0,14	4,54	4,17197	0,265706	3,28	3,20	0,111551	3,68	3,585	0,123974
	4,13			1,28			3,92			3,21			3,69		
	4,17			1,03			4,18			3,04			3,43		
	3,62			1,04			4,04			3,27			3,55		
72	5,03	4,519	0,488	3,63	3,690331	0,17	4,60	4,176783	0,636447	4,08	3,90	0,187598	3,33	3,349	0,068
	4,81			3,58			4,84			3,96			3,35		
	4,29			3,95			3,65			3,64			3,44		
	3,95			3,61			3,61			3,93			3,28		
90	8,09	7,417	0,489	6,90	5,846381	0,82	6,53	6,116801	0,470336	2,74	2,79	0,1689	6,52	6,115	0,390009
	7,42			5,51			5,45			3,02			5,58		
	7,23			4,98			6,35			2,62			6,13		
	6,93			6,00			6,14			2,79			6,23		
96	6,60	6,228	0,318	5,01	4,837604	0,59	6,48	5,806814	0,538078	5,25	5,34	0,140972	1,78	1,804	0,08723
	6,22			5,36			5,47			5,28			1,75		
	5,82			3,98			5,98			5,56			1,93		
	6,27			5,00			5,29			5,29			1,75		
114	6,86	6,306	0,435	5,21	4,836763	0,49	6,39	6,106834	0,452496	6,45	6,09	0,621502	5,56	4,964	0,693773
	6,33			5,31			5,72			6,48			3,99		
	6,23			4,36			5,72			6,27			4,99		
	5,80			4,47			6,59			5,17			5,32		
120	5,93	5,569	0,652	6,94	6,613169	0,72	6,59	6,131496	0,848093	6,89	6,40	0,486213	6,94	7,119	0,246099
	5,82			7,29			6,86			6,52			7,15		
	4,59			6,61			6,14			5,73			7,46		
	5,93			5,62			4,94			6,46			6,94		

Annex VI

Table 5 – Experimental data of optical density (OD) and dry weight (DW) for the essay with suspended yeast in the case that glucose concentration was measured with HPLC

				Concentration (g/L)																		
				50				100				200				300				400				
Day	Hour	Sample		OD	DW (g/L)	Mean	SD	OD	DW (g/L)	Mean	SD	OD	DW (g/L)	Mean	SD	OD	DW (g/L)	Mean	SD	OD	DW (g/L)	Mean	SD	Time (h)
14/04/2016	16h00	A	1	0,005	0,000	0,000	0,000	0,007	0,000	0,000	0,000	0,006	0,000	0,000	0,000	0,003	0,000	0,000	0,000	0,002	0,000	0,000	0,000	0
			2	0,006	0,000			0,010	0,000			0,004	0,000			0,002	0,000			0,004	0,000			
		B	1	0,002	0,000			0,003	0,000			0,006	0,000			0,008	0,000			0,002	0,000			
			2	0,002	0,000			0,007	0,000			0,006	0,000			0,020	0,000			0,001	0,000			
15/04/2016	10h00	A	1	0,725	0,464	0,414	0,061	0,596	0,363	0,401	0,086	0,558	0,333	0,252	0,083	0,102	0,000	0,000	0,000	0,027	0,000	0,000	0,000	18
			2	0,728	0,467			0,807	0,529			0,526	0,308			0,099	0,000			0,028	0,000			
		B	1	0,610	0,374			0,602	0,368			0,330	0,154			0,110	0,000			0,027	0,000			
			2	0,578	0,349			0,572	0,344			0,407	0,214			0,113	0,000			0,028	0,000			
	16h00	A	1	0,954	0,644	0,569	0,075	0,789	0,515	0,558	0,060	0,903	0,604	0,439	0,190	0,180	0,036	0,037	0,010	0,053	0,000	0,000	0,000	24
			2	0,927	0,623			0,785	0,512			0,900	0,602			0,171	0,029			0,057	0,000			
		B	1	0,786	0,512			0,856	0,567			0,482	0,273			0,177	0,033			0,051	0,000			
			2	0,766	0,497			0,948	0,640			0,484	0,275			0,200	0,052			0,052	0,000			
16/04/2016	10h00	A	1	1,330	0,940	0,920	0,026	1,262	0,887	0,803	0,071	1,220	0,854	0,748	0,153	0,562	0,336	0,309	0,022	0,182	0,037	0,044	0,006	42
			2	1,258	0,884			1,168	0,813			1,284	0,904			0,539	0,318			0,186	0,041			
		B	1	1,326	0,937			1,152	0,800			0,905	0,606			0,507	0,293			0,199	0,051			
			2	1,302	0,918			1,041	0,713			0,933	0,628			0,502	0,289			0,195	0,048			
	16h00	A	1	1,467	1,048	0,969	0,095	1,227	0,859	0,827	0,046	1,231	0,862	0,770	0,122	0,657	0,411	0,404	0,027	0,241	0,084	0,111	0,029	48
			2	1,475	1,054			1,245	0,873			1,265	0,889			0,632	0,391			0,245	0,087			
		B	1	1,261	0,886			1,145	0,795			0,983	0,667			0,612	0,376			0,306	0,135			
			2	1,262	0,887			1,129	0,782			0,977	0,663			0,691	0,438			0,308	0,137			
17/04/2016	10h00	A	1	1,536	1,102	1,105	0,024	1,430	1,019	1,025	0,061	1,590	1,145	1,029	0,149	1,056	0,725	0,678	0,040	0,549	0,326	0,401	0,092	66
			2	1,554	1,116			1,463	1,045			1,624	1,171			1,017	0,694			0,538	0,317			
		B	1	1,568	1,127			1,338	0,946			1,279	0,900			0,936	0,630			0,739	0,475			
			2	1,498	1,072			1,523	1,092			1,281	0,902			0,979	0,664			0,753	0,486			
	16h00	A	1	1,678	1,214	1,170	0,029	1,498	1,072	1,085	0,072	1,702	1,233	1,080	0,175	1,122	0,777	0,730	0,039	0,619	0,381	0,470	0,099	72
			2	1,599	1,152			1,579	1,136			1,699	1,230			1,083	0,746			0,629	0,389			
		B	1	1,608	1,159			1,391	0,988			1,314	0,928			1,013	0,691			0,818	0,538			
			2	1,606	1,157			1,590	1,145			1,314	0,928			1,032	0,706			0,863	0,573			
18/04/2016	10h00	A	1	1,977	1,449	1,380	0,077	1,713	1,241	1,185	0,056	1,875	1,369	1,235	0,130	1,285	0,905	0,907	0,007	0,946	0,638	0,742	0,119	90
			2	1,971	1,444			1,691	1,224			1,571	1,130			1,296	0,913			0,948	0,640			
		B	1	1,804	1,313			1,585	1,141			1,556	1,118			1,276	0,898			1,203	0,840			
			2	1,806	1,314			1,574	1,132			1,817	1,323			1,295	0,913			1,217	0,851			
	16h00	A	1	1,995	1,463	1,343	0,144	1,635	1,180	1,169	0,030	1,888	1,379	1,243	0,150	1,325	0,936	0,939	0,017	0,953	0,644	0,780	0,160	96
			2	2,005	1,471			1,664	1,203			1,872	1,366			1,299	0,916			0,948	0,640			
		B	1	1,706	1,236			1,610	1,160			1,540	1,105			1,347	0,954			1,290	0,909			
			2	1,661	1,200			1,574	1,132			1,560	1,121			1,344	0,951			1,313	0,927			
19/04/2016	10h00	A	1	2,205	1,628	1,549	0,104	1,898	1,387	1,334	0,063	2,101	1,546	1,393	0,176	1,528	1,096	1,092	0,007	1,223	0,856	1,047	0,222	114
			2	2,232	1,649			1,902	1,390			2,097	1,543			1,510	1,082			1,221	0,854			
		B	1	1,971	1,444			1,760	1,278			1,700	1,231			1,526	1,094			1,710	1,239			
			2	2,011	1,476			1,762	1,280			1,724	1,250			1,528	1,096			1,711	1,240			
	16h00	A	1	2,241	1,657	1,578	0,083	1,940	1,420	1,377	0,045	2,135	1,573	1,432	0,149	1,571	1,130	1,122	0,013	1,273	0,895	1,100	0,236	120
			2	2,223	1,642			1,931	1,413			2,104	1,549			1,578	1,135			1,273	0,895			
		B	1	2,051	1,507			1,832	1,335			1,790	1,302			1,542	1,107			1,795	1,306			
			2	2,048	1,505			1,841	1,342			1,793	1,304			1,554	1,116			1,790	1,302			

Q

Table 2 – Experimental data obtained for glucose concentration measured by HPLC in the essay with suspended yeast. Orange squares stand for samples not measured. SD stand for standard deviation with n=4

Sample A	Area		Sample B	Area		Mean	SD
50A1.1			**50B1.1**	6449,5	66,13643	53,8479	22,53035
50A1.2	6579,9	67,56216	**50B1.2**	2947,3	27,84512		
100A1.1	15858,8	120,354	**100B1.1**	9430,1	98,72483	100,9273	23,04742
100A1.2	16208,2	115,369	**100B1.2**	6735,3	69,26122		
200A1.1	27214,5	197,248	**200B1.1**	20243,5	200,574	216,4802	24,75696
200A1.2	28499,7	251,348	**200B1.2**	20225	216,7509		
300A1.1	12792,3	301,452	**300B1.1**	33091,4	357,4257	314,1521	50,35503
300A1.2	12254,3	248,34	**300B1.2**	32356,5	349,3907		
400A1.1	36949,9	399,6126	**400B1.1**	31256	423,658	389,1731	28,294
400A1.2	34568,9	373,58	**400B1.2**	28665,2	359,842		
50A2.1	5897,9	60,10551	**50B2.1**	5945,8	60,62922	56,84678	13,56899
50A2.2	6727,8	69,17922	**50B2.2**	3827,9	37,47316		
100A2.1	11872,6	125,4299	**100B2.1**	9110,8	95,23376	113,8303	13,03674
100A2.2	10986,9	115,7461	**100B2.2**	11276,4	118,9114		
200A2.1	23006,6	247,1635	**200B2.1**	22200,9	238,3544	225,372	21,48518
200A2.2	18666,8	199,7143	**200B2.2**	20179,7	216,2556		
300A2.1	34428	372,0394	**300B2.1**	30276,6	326,6501	333,7052	26,54019
300A2.2	28829,8	310,8315	**300B2.2**	30153,1	325,2998		
400A2.1	41820,4	452,8642	**400B2.1**	32738,5	353,5673	356,762	67,46854
400A2.2	28154	303,4426	**400B2.2**	29409,9	317,174		
50A3.1	4294,9	42,5791	**50B3.1**	833,8849	45,7995	43,72058	1,681803
50A3.2	4255,8	42,1516	**50B3.2**	820,1896	44,3521		
100A3.1	10771,7	113,3932	**100B3.1**			96,18935	24,32994
100A3.2	7624,7	78,98548	**100B3.2**				
200A3.1	16842,9	179,7727	**200B3.1**			192,7504	14,96866
200A3.2	17719,1	189,3526	**200B3.2**	19527,6	209,1259		
300A3.1	33665	363,6972	**300B3.1**	5972,5	60,92115	205,9256	165,5119
300A3.2	30953,1	334,0466	**300B3.2**	6349	65,03761		
400A3.1	42986	465,6083	**400B3.1**	22822,1	245,1463	383,6101	97,47007
400A3.2	40125	434,3276	**400B3.2**	36012	389,3581		
50A4.1	6075,2	62,04402	**50B4.1**	4172,2	41,23756	46,74313	10,27733
50A4.2	4364,5	43,34008	**50B4.2**	4091,1	40,35086		
100A4.1			**100B4.1**	9952,3	104,4343	95,43202	10,97377
100A4.2	9423,6	98,65376	**100B4.2**	8010,9	83,208		
200A4.1	19802,3	212,1293	**200B4.1**	17141,8	183,0407	206,9701	20,33648
200A4.2	18793,7	201,1018	**200B4.2**	21583,9	231,6084		
300A4.1	31194,2	336,6827	**300B4.1**	12753	135,0558	268,2433	104,3976
300A4.2	33744	364,5609	**300B4.2**	22047,2	236,6739		
400A4.1	28143	303,3224	**400B4.1**	15914,8	169,6253	257,8905	70,73211
400A4.2	30156,23	325,334	**400B4.2**	21736,8	233,2802		
50A5.1	4680	46,7896	**50B5.1**	5690,3	57,83571	49,90865	5,52033
50A5.2	4570,7	45,59456	**50B5.2**	4920,1	49,41473		
100A5.1	9458,5	99,03534	**100B5.1**	10424,1	109,5927	113,5097	14,59526
100A5.2	12635,9	133,7754	**100B5.2**	10610,9	111,6351		
200A5.1	8721,9	90,98172	**200B5.1**	21731,2	233,2189	156,738	83,20796
200A5.2	7606	78,78102	**200B5.2**	20885,3	223,9703		
300A5.1	33248,3	359,1412	**300B5.1**	23699,4	254,7383	297,0534	52,70246
300A5.2	29893,7	322,4636	**300B5.2**	23437,1	251,8704		
400A5.1	29893,6	322,4626	**400B5.1**	26089,2	280,8671	312,905	90,45905
400A5.2	39893,6	431,7976	**400B5.2**	20201,4	216,4929		

R

Table 3 – Continuation of Table 1

Sample A	Area		Sample B	Area		Mean	SD
50A6.1	3530,7	34,22372	**50B6.1**	4946,8	49,70665	37,14761	9,164261
50A6.2	3762,7	36,76029	**50B6.2**	2952,3	27,89978		
100A6.1	10654,3	112,1096	**100B6.1**	9283,7	97,12416	107,5744	14,12829
100A6.2	11884,3	125,5578	**100B6.2**	9135,7	95,506		
200A6.1	20050,9	214,8474	**200B6.1**	18946,8	202,7757	206,372	6,065805
200A6.2	18813,1	201,3139	**200B6.2**	19292,1	206,551		
300A6.1	28246,6	304,4551	**300B6.1**	20430	218,9923	238,3167	110,6302
300A6.2	31420,3	339,1547	**300B6.2**	8692,9	90,66465		
400A6.1	41041,5	444,3481	**400B6.1**	12486,3	132,1398	338,4971	150,5632
400A6.2	42149,3	456,4603	**400B6.2**	29763,5	321,0401		
50A7.1	1749,6	14,75006	**50B7.1**	4181,1	41,33487	27,87546	12,29971
50A7.2	2286,9	20,62463	**50B7.2**	3582,7	34,79226		
100A7.1	2483,7	22,77634	**100B7.1**	9186,7	96,06361	62,14488	40,31515
100A7.2	3327,4	32,00094	**100B7.2**	9339,9	97,73862		
200A7.1	20546,3	220,2638	**200B7.1**	21228,5	227,7227	215,0611	18,66869
200A7.2	17544,1	187,4393	**200B7.2**	20962,9	224,8187		
300A7.1	41933,3	454,0986	**300B7.1**	21170,7	227,0907	327,5021	115,5554
300A7.2	36701,2	396,8935	**300B7.2**	21612,9	231,9255		
400A7.1	30469,9	328,7635	**400B7.1**	21199,8	227,4089	310,7049	105,1299
400A7.2	41771,5	452,3296	**400B7.2**	21831,7	234,3177		
50A8.1	2030	17,81581	**50B8.1**	3991,6	39,26297	25,33533	11,51973
50A8.2	1695,7	14,16074	**50B8.2**	3153,7	30,10179		
100A8.1	9081,4	94,91231	**100B8.1**	8861,6	92,50913	101,3899	12,55082
100A8.2	11365,1	119,8812	**100B8.2**	9387,3	98,25687		
200A8.1	20194,7	216,4196	**200B8.1**	19295,8	206,5915	207,4869	8,332319
200A8.2	18378,5	196,5622	**200B8.2**	19641,8	210,3745		
300A8.1	31362,8	338,5261	**300B8.1**	23261,9	249,9548	271,4485	86,34798
300A8.2	31417,2	339,1208	**300B8.2**	14869,1	158,1921		
400A8.1	43714,2	473,5701	**400B8.1**	29814,1	321,5933	400,7565	79,90423
400A8.2	42970,1	465,4345	**400B8.2**	31719,7	342,4282		
50A9.1	1079,5	7,42352	**50B9.1**	2333,1	21,12976	12,64946	11,13955
50A9.2	350,4	-0,5481	**50B9.2**	2466,9	22,59266		
100A9.1	7467,2	77,26345	**100B9.1**	7977,4	82,84173	80,5077	4,210745
100A9.2	7413,2	76,67304	**100B9.2**	8197,9	85,25256		
200A9.1	16563,7	176,7201	**200B91.1**	17081,3	182,3792	185,591	9,884954
200A9.2	18668,2	199,7296	**200B9.2**	17187	183,5349		
300A9.1	27406,8	295,2731	**300B9.1**	17828,8	190,552	243,1498	59,17098
300A9.2	27243,5	293,4877	**300B9.2**	18078,9	193,2865		
400A9.1	20531	220,0965	**400B9.1**	10876,6	114,5401	218,5366	93,40282
400A9.2	18570,7	198,6636	**400B9.2**	31575	340,8461		
50A10.1	351,6	-0,53498	**50B10.1**	1513,2	12,16538	5,805088	7,055738
50A10.2	394,5	-0,06593	**50B10.2**	1466,6	11,65588		
100A10.1	7077,3	73,00048	**100B10.1**	7158	73,88281	73,56957	0,431913
100A10.2	7120,1	73,46843	**100B10.2**	7162	73,92655		
200A10.1	16024,4	170,8236	**200B10.1**	2313,6	20,91656	91,38681	84,98607
200A10.2	14928,9	158,846	**200B10.2**	1768,9	14,96108		
300A10.1	26227	282,3738	**300B10.1**	17029	181,8074	228,8272	51,01145
300A10.2	24396	262,3545	**300B10.2**	17666,1	188,7732		
400A10.1	22127	237,5464	**400B10.1**	19054,5	203,9532	216,1506	46,33158
400A10.2	24671	265,3612	**400B10.2**	14827,9	157,7417		
50A11.1			**50B11.1**	919,6	5,675253	5,05915	0,871301
50A11.2			**50B11.2**	806,9	4,443047		
100A11.1	6605,6	67,84315	**100B11.1**	6030,9	61,55966	69,21503	8,471988
100A11.2	7834,7	81,28152	**100B11.2**	6453,1	66,17579		
200A11.1	13534	143,5948	**200B11.1**	13731,5	145,7542	110,4865	69,59124
200A11.2	13798	146,4813	**200B11.2**	959,9	6,115873		
300A11.1	22003,1	236,1918	**300B11.1**	13733,2	145,7728	195,2463	43,85707
300A11.2	21243	227,8812	**300B11.2**	16053,3	171,1396		
400A11.1	22462,1	241,2102	**400B11.1**	14978,8	159,3916	203,4558	51,13573
400A11.2	23615,5	253,8209	**400B11.2**	14979,6	159,4003		

S

Table 4 – Experimental data obtained for ethanol concentration in the essay with suspended yeast and glucose monitored by HPLC. Orange squares stand for samples not measured. SD stands for standard deviation

Sample A	g/L	Sample B	g/L	Mean	SD
50A1.1	0,000	**50B1.1**		0,000	0
50A1.2	0,000	**50B1.2**			
100A1.1	0,000	**100B1.1**		0,000	0
100A1.2	0,000	**100B1.2**			
200A1.1	0,000	**200B1.1**		0,000	0
200A1.2	0,000	**200B1.2**			
300A1.1	0,000	**300B1.1**		0,000	0
300A1.2	0,000	**300B1.2**			
400A1.1	0,000	**400B1.1**		0,000	0
400A1.2	0,000	**400B1.2**			
50A2.1	1,36	**50B2.1**		1,775	0,5868986
50A2.2	2,19	**50B2.2**			
100A2.1		**100B2.1**		2,290	#DIV/0!
100A2.2	2,29	**100B2.2**			
200A2.1	1,73	**200B2.1**		1,730	#DIV/0!
200A2.2		**200B2.2**			
300A2.1		**300B2.1**		#DIV/0!	#DIV/0!
300A2.2		**300B2.2**			
400A2.1		**400B2.1**		0,240	#DIV/0!
400A2.2	0,24	**400B2.2**			
50A3.1	1,17	**50B3.1**		1,170	#DIV/0!
50A3.2		**50B3.2**			
100A3.1		**100B3.1**		1,470	#DIV/0!
100A3.2	1,47	**100B3.2**			
200A3.1	1,87	**200B3.1**		1,745	0,1767767
200A3.2	1,62	**200B3.2**			
300A3.1	0,34	**300B3.1**		0,340	#DIV/0!
300A3.2		**300B3.2**			
400A3.1		**400B3.1**		#DIV/0!	#DIV/0!
400A3.2		**400B3.2**			
50A4.1	13,05	**50B4.1**		22,265	13,031978
50A4.2	31,48	**50B43.2**			
100A4.1	14,04	**100B4.1**		20,115	8,5913474
100A4.2	26,19	**100B4.2**			
200A4.1	10,21	**200B4.1**		10,210	#DIV/0!
200A4.2		**200B4.2**			
300A4.1	18,09	**300B4.1**		29,155	15,648273
300A4.2	40,22	**300B4.2**			
400A4.1	8,29	**400B4.1**		9,980	2,3900209
400A4.2	11,67	**400B4.2**			
50A5.1	5,59	**50B5.1**		5,590	#DIV/0!
50A5.2		**50B5.2**			
100A5.1		**100B5.1**		3,680	#DIV/0!
100A5.2	3,68	**100B5.2**			
200A5.1	4,15	**200B5.1**	14,04	11,207	6,150645
200A5.2	15,43	**200B5.2**			
300A5.1	3,01	**300B5.1**		3,300	0,4101219
300A5.2	3,59	**300B5.2**			
400A5.1	4,19	**400B5.1**		2,485	2,4112341
400A5.2	0,78	**400B5.2**			

T

Table 5 – Continuation of Table 3

Sample A	g/L	Sample B	g/L	Mean	SD
50A6.1	12,05	**50B6.1**		11,730	0,452548
50A6.2	11,41	**50B6.2**			
100A6.1	7,61	**100B6.1**		7,195	0,586899
100A6.2	6,78	**100B6.2**			
200A6.1	7,69	**200B6.1**		7,020	0,947523
200A6.2	6,35	**200B6.2**			
300A6.1	6,14	**300B6.1**		6,555	0,586899
300A6.2	6,97	**300B6.2**			
400A6.1	2,32	**400B6.1**		3,265	1,336432
400A6.2	4,21	**400B6.2**			
50A7.1	12,33	**50B7.1**		11,730	0,848528
50A7.2	11,13	**50B7.2**			
100A7.1	9,86	**100B7.1**		13,385	4,985103
100A7.2	16,91	**100B7.2**			
200A7.1	11,44	**200B7.1**		22,655	15,86041
200A7.2	33,87	**200B7.2**			
300A7.1	7,26	**300B7.1**		10,815	5,027529
300A7.2	14,37	**300B7.2**			
400A7.1	7,96	**400B7.1**		5,230	3,860803
400A7.2	2,5	**400B7.2**			
50A8.1	17,15	**50B8.1**		25,710	12,10567
50A8.2	34,27	**50B8.2**			
100A8.1	16,01	**100B8.1**		13,750	3,196123
100A8.2	11,49	**100B8.2**			
200A8.1	19,99	**200B8.1**		21,220	1,739483
200A8.2	22,45	**200B8.2**			
300A8.1	6,96	**300B8.1**	15,13	11,783	3,901926
300A8.2	14,77	**300B8.2**	10,27		
400A8.1	10,31	**400B8.1**	20,39	12,405	5,400472
400A8.2	8,46	**400B8.2**	10,46		
50A9.1	16,34	**50B9.1**	18,32	16,653	1,534188
50A9.2		**50B9.2**	15,3		
100A9.1	10,27	**100B9.1**	13,22	11,745	2,085965
100A9.2		**100B9.2**			
200A9.1	18,39	**200B9.1**	11,96	16,705	6,486512
200A9.2	25,17	**200B9.2**	11,3		
300A9.1	11,58	**300B9.1**	8,5	10,695	1,510684
300A9.2	10,91	**300B9.2**	11,79		
400A9.1	7,56	**400B9.1**	9,73	10,083	3,61464
400A9.2	15,3	**400B9.2**	7,74		
50A10.1	46,62	**50B10.1**	21,26	35,718	18,90886
50A10.2	56,71	**50B10.2**	18,28		
100A10.1	15,06	**100B10.1**	14,16	13,970	1,991666
100A10.2	15,55	**100B10.2**	11,11		
200A10.1		**200B10.1**	14,47	18,847	6,849338
200A10.2	26,74	**200B10.2**	15,33		
300A10.1		**300B10.1**	14,19	15,205	1,435427
300A10.2		**300B10.2**	16,22		
400A10.1		**400B10.1**	9,91	13,540	5,133595
400A10.2		**400B10.2**	17,17		
50A11.1		**50B11.1**	20,13	21,005	1,237437
50A11.2		**50B11.2**	21,88		
100A11.1		**100B11.1**	16,03	16,300	0,381838
100A11.2		**100B11.2**	16,57		
200A11.1		**200B11.1**	25,21	20,500	6,660946
200A11.2		**200B11.2**	15,79		
300A11.1		**300B11.1**	18,02	27,110	12,8552
300A11.2		**300B11.2**	36,2		
400A11.1		**400B11.1**	15,13	16,070	1,329361
400A11.2		**400B11.2**	17,01		

U

Annex VII

Table 1 – Experimental data obtained for optical density (OD), OD minus alginate OD (OD-alginate), beads weight (weight), conversion to total suspended solids (TSS beads) and final biomass inside beads ([Biomass]beads) for each initial glucose concentration for the essay with analytical method

50 g/L							
OD	**OD-alginate**	**Weight**	**VsusC**	**TSS beads**	**[Biomass]beads (g/L)**	**Mean**	**SD**
-	-	2,135	32,135	-	-	0	0,000811417
0,159	0,131			0	0		
0,012	0			0	0		
0,442	0,414	3,0523	33,0523	0,218950666	0,002370941	0,001661	
0,379	0,351			0,169616288	0,001836716		
0,254	0,226			0,071730619	0,000776746		
100 g/L							
OD	**OD-alginate**	**Weight**	**VsusC**	**TSS beads**	**[Biomass]beads (g/L)**	**Mean**	**SD**
0,103	0,075	3,031	33,031	0	0	0,00170	0,000812266
0,374	0,346			0,165700861	0,001805762		
1,111	1,083			0,742834769	0,008095208		
0,481	0,453	3,0523	33,0523	0,249490995	0,002701652		
0,348	0,32			0,145340642	0,001573843		
0,248	0,22			0,067032106	0,000725867		
200 g/L							
OD	**OD-alginate**	**Weight**	**VsusC**	**TSS beads**	**[Biomass]beads (g/L)**	**Mean**	**SD**
0,477	0,449	3,031	33,031	0,246358653	0,002684748	0,002772	0,000706408
0,424	0,396			0,204855129	0,002232455		
0,56	0,532			0,311354738	0,003393058		
0,585	0,557	3,0523	33,0523	0,330931872	0,003583547		
0,394	0,366			0,181362569	0,001963912		
-	#VALOR!			-	-		
300 g/L							
OD	**OD-alginate**	**Weight**	**VsusC**	**TSS beads**	**[Biomass]beads (g/L)**	**Mean**	**SD**
0,04	0,012	3,031	33,031	0	0	0,00117	0,000579769
0,071	0,043			0	0		
0,396	0,368			0,182928739	0,001993507		
0,246	0,218	3,0523	33,0523	0,065465936	0,000708908		
0,26	0,232			0,076429131	0,000827625		
0,298	0,27			0,106186374	0,001149855		
400 g/L							
OD	**OD-alginate**	**Weight**	**VsusC**	**TSS beads**	**[Biomass]beads (g/L)**	**Mean**	**SD**
0,137	0,109	3,031	33,031	0	0	0,001062	0,000457692
0,174	0,146			0,00908379	9,89926E-05		
0,239	0,211			0,059984338	0,000653693		
0,299	0,271	3,0523	33,0523	0,10696946	0,001158335		
0,253	0,225			0,070947533	0,000768266		
0,359	0,331			0,153954581	0,001667121		

Table 2 – Experimental data of optical density (OD), beads weight, volume of suspension C (VsusC), conversion to total suspended solids (TSS beads) and final biomass concentration inside beads ([Biomass]beads) for the case with weight loss method

50 g/L							
OD	**OD-alginate**	**Weight**	**VsusC**	**TSS beads**	**[Biomass]beads (g/g)**	**Mean**	**SD**
0,719	0,691	3,031	33,031	0,435865309	0,00474994	0,005088156	0,000583766
0,722	0,694			0,438214565	0,004775541		
0,71	0,682			0,428817541	0,004673135		
0,774	0,746	3,0523	33,0523	0,478935004	0,005186221		
0,896	0,868			0,574471417	0,006220752		
0,743	0,715			0,454659358	0,004923349		
100 g/L							
OD	**OD-alginate**	**Weight**	**VsusC**	**TSS beads**	**[Biomass]beads (g/g)**	**Mean**	**SD**
0,734	0,706	3,0188	33,0188	0,44761159	0,004895852	0,005467705	0,000642292
0,796	0,768			0,496162882	0,005426892		
0,691	0,663			0,413938919	0,004527549		
0,891	0,863	3,0623	33,0623	0,570555991	0,006160041		
0,862	0,834			0,547846515	0,005914857		
0,858	0,83			0,544714174	0,005881038		
200 g/L							
OD	**OD-alginate**	**Weight**	**VsusC**	**TSS beads**	**[Biomass]beads (g/g)**	**Mean**	**SD**
0,637	0,609	3,0713	33,0713	0,37165231	0,004001897	0,004000596	0,0001120
0,651	0,623			0,382615505	0,004119947		
	0			0	-		
0,613	0,585	3,0265	33,0265	0,352858262	0,003850545		
0,634	0,606			0,369303054	0,004029997		
	0			0	-		
300 g/L							
OD	**OD-alginate**	**Weight**	**VsusC**	**TSS beads**	**[Biomass]beads (g/g)**	**Mean**	**SD**
0,645	0,617	3,0808	33,0808	0,377916993	0,004057971	0,00432023	0,000391981
0,704	0,676			0,424119029	0,004554076		
0,666	0,638			0,394361785	0,004234551		
0,753	0,725	3,0805	33,0805	0,462490211	0,004966534		
0,621	0,593			0,359122944	0,003856506		
0,668	0,64			0,395927956	0,004251743		
400 g/L							
OD	**OD-alginate**	**Weight**	**VsusC**	**TSS beads**	**[Biomass]beads (g/g)**	**Mean**	**SD**
0,498	0,47	3,0608	33,0608	0,262803446	0,002838634	0,003109216	0,000403525
0,588	0,56			0,333281128	0,003599889		
0,484	0,456			0,251840251	0,002720217		
0,515	0,487	3,004	33,004	0,276115897	0,003033598		
0,492	0,464			0,258104933	0,002835717		
0,584	0,556			0,330148786	0,003627241		

W

Table 3 – Experimental data obtained for weight loss method in the case of the essay with immobilized yeast

		Initial concentration (g/L)				
		50	100	200	300	400
0	A	171,68	174,61	179,98	182,49	187,61
	B	175,37	179,30	180,59	184,10	179,39
7	A	171,63	174,54	179,93	182,46	187,54
	B	175,33	179,25	180,55	184,06	179,37
24	A	170,80	173,92	179,33	181,99	187,18
	B	174,67	178,54	180,00	183,56	179,02
31	A	170,38	173,59	179,03	181,73	186,97
	B	174,33	178,19	179,73	183,30	178,79
48	A	169,76	173,03	178,52	181,28	186,58
	B	173,76	177,39	179,22	182,82	178,37
55	A	169,74	172,83	178,36	181,17	186,48
	B	173,60	177,09	179,06	182,70	178,25
72	A	169,67	172,35	177,83	180,71	186,11
	B	173,36	176,18	178,59	182,22	177,86
79	A	169,62	171,91	177,33	180,24	185,72
	B	173,31	172,00	178,09	181,72	177,41
96	A	169,61	171,80	177,20	180,12	185,62
	B	173,30	170,37	177,96	181,59	177,28
103	A	169,59	171,57	176,91	179,82	185,38
	B	173,27	167,73	177,68	181,30	177,01
120	A	169,59	171,50	176,82	179,74	185,30
	B	173,27	166,56	177,60	181,21	176,93