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Evaluating the potential of yeast strains to produce added value products for the food and/or pharmaceutical industries

Dissertação para obtenção do grau de mestre
em Biotecnologia

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À MINHA FAMÍLIA.

“Que os vossos esforços desafiem as impossibilidades, lembrai-vos que as grandes coisas do Homem foram conquistadas do que parecia impossível”

Charles Chaplin

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Abstract

This study focus in the valorization of the apple pomace with the main goal of obtaining added value products. For that, hot compressed water technology was used for the extraction of phenolic compounds and hydrolysis of polysaccharides presents in the lignocellulosic structure of apple pomace to obtain simple sugars. The sugars have been utilized as alternative carbon source for growth, lipid accumulation and carotenoids production by five different yeast *Yarrowia lipolytica, Rhodotorula mucilaginosa, Rhodotorula glutinis, Rhodosporidium babjevae* and *Rhodosporidium toruloides*.

Hydrolysis experiments were carried out with constant pressure of 100 bar, flow rate of 2mL/min and temperatures between 50°C and 250°C. The amount of total sugars present in apple pomace hydrolysates showed maximum values for the hydrolysis temperatures of 110°C and 190°C. In fact, these temperatures revealed the best results regarding the monosaccharides quantities. The amount of 5-HMF and furfural in each hydrolysate varied through the different temperatures. Maximum values for 5-HMF were obtained with 170°C, while furfural showed to be maximum at 210°C.

Extraction of phenolic compounds were performed in simultaneously with hydrolysis reactions. Total phenolic compounds (TPC) increased along the temperature, however with small variations between 170°C and 250°C.

Hydrolysates were then used as alternative carbon source to yeast growth. *R. mucilaginosa* shows the highest optical density, with the hydrolysate obtained at 130°C. Carotenoids produced by these yeast scored a total of 7.02µg carotenoids/g cell dry weight, while for the control assay, the same yeast scored 9.31µg caratonoides/g cell dry weight. β-carotene was quantified by HPLC, were 33% of the carotenoid production by *R. mucilaginosa* with hydrolysate as carbon source, corresponded to β-caroteno.

Key-words: apple pomace, hot compressed water (HCW), hydrolysis, oleaginous yeast.

Resumo

Este trabalho teve como objetivo a valorização do bagaço de maçã para obtenção de produtos de valor acrescentado. Para tal, foi utilizada a tecnologia de água subcrítica para extração de compostos fenólicos e hidrólise de polissacáridos presentes na estrutura lignocelulósica do bagaço da maçã para obtenção de açúcares simples. Estes foram utilizados como fonte alternativa de carbono para o crescimento de cinco diferentes leveduras *Yarrowia lipolytica, Rhodotorula mucilaginosa, Rhodotorula glutinis, Rhodosporidium babjevae e Rhodosporidium toruloides* de modo a produzirem carotenoides e acumularem lípidos.

As experiências de hidrólise foram realizadas a uma pressão constante de 100 bar, caudal de 2 mL/min e a temperaturas entre 50°C e 250°C. A quantidade de açúcares totais presente nos hidrolisados foi máxima para temperaturas de hidrólise entre 110°C e 190°C. Com estas mesmas temperaturas registou-se os melhores resultados relativamente a quantidade monossacáridos obtidos. A quantidade de 5-HMF e furfural presentes em cada hidrolisado variou ao longo das diferentes temperaturas. A quantidade máxima de 5-HMF foi obtida a 170°C, enquanto que a quantidade máxima de furfural foi obtida entre aos 210°C.

As experiências de extração foram realizadas em simultâneo com as reações de hidrólise. O total de compostos fenólicos aumentou com o aumento da temperatura aplicada no processo, não variando muito entre 170°C e 250°C.

Os hidrolisados foram posteriormente utilizados como fontes alternativas de carbono para o crescimento de leveduras. *R. mucilaginosa* obteve o melhor resultado em termos de crescimento e quando a fonte de carbono presente no meio de cultura foi originária do hidrolizado obtido a 130°C.

A quantidade de carotenoides obtida por esta levedura, crescida na presença de hidrolizado foi igual a 7.02µg carotenoides/g peso seco, enquanto que para o ensaio controlo, onde a mesma levedura cresceu em meio contendo glucose a quantidade obtida foi de 9.31µg carotenoides/g peso seco. Por HPLC foi quantificado o β-caroteno, os resultados mostraram que 33% dos carotenoides produzidos por R. mucilaginosa crescida em meio contendo hidrolisado como fonte de carbono corresponde ao β-caroteno.

Termos chave: bagaço de maçã, água subcrítica (HCW), hidrólise, leveduras oleaginosas.

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Nomenclature

AP – apple pomace
HCW – subcritical water/hot compressed water
HPLC – high performance liquid chromatography
OD – optic density
ppm – parts per million
SCF– super critical fluids
TAG – triacylglyceride
TPC – total phenolic content
YNB – yeast nitrogen based
SCO – Single Cell Oil
LD – lipid droplet

INTRODUCTION

1. Introduction

Every year, several tonnes of agro-industrial residues are generated as the result of the transformation of fruits and vegetables into food and drink products, leading to serious environmental problems if these residues undergo incorrect procedures of usage or elimination (Laufenberg, *et al.,* 2003; Schieber *et al*., 2001;).

In Europe, the amount of byproducts and waste produced in food processing activities accounts for approximately 2.5×10^8 tonnes per year (Federici *et al*., 2009). A substantial part of these residues still comprises important amounts of the original raw materials, since up to 75% of the original vegetables and fruits may end up as a solid residue (Kumar, 2004). Such waste streams are only partially valorized as animal feed, fertilizers, transformed into biomass fuel, or subjected to composting, whereas the main volumes are managed as wastes of environmental concern. However, in recent years there has been a growing trend for its recovery, following the evolution of environmental legislation increasingly restrictive. In this context, reuse and recycling of natural waste have been encouraged, contributing to aggregate economic value on the production line of the industry and could even be an excellent business opportunity.

In recent years new technologies have been proposed for more efficient utilization of agro-industrial residues, not only for their re-use in agriculture, but also for the production of common and novel products for other sectors and applications (Mahawar, 2012; Mussato, 2011; Reis, 2012). After the appropriate pretreatment of the raw material, followed by tailored recovery procedures, most natural residues obtained from agricultural processing can provide value-added natural antioxidants, oils, carotenoids, fragrances, biocides and other bioactive substances of enormous interest to the cosmetic, pharmaceutical, food industry etc.

The finality of the present work consists in the valorization of agro industrial residues from apple pomace through production of the value added by-products.

1.1 Agro-residues

The agro-industrial residues have a lignocellulosic structure (Figure 1) composed by carbohydrate polymers (cellulose, hemicellulose) and an aromatic polymer (lignin) which represents the major constituents of the cell walls in plant cells The primary wall is comprised of cellulose microfibrils embedded in a matrix proteins and polysaccharides such as hemicelluloses and pectin, linked by chemical bonds and for some proteins associated with carbohydrates (glycoproteins) as the extension, this structure is responsible for the development and growth of cells. The secondary is composed by cellulose chains and high amounts of lignin deposited in successive layers containing lower amounts of hemicellulose and pectic substances. The individual cells are connected to each other through the middle lamella forming a tissue. The middle lamella consists mainly of lignin (Wettstein, 2012).

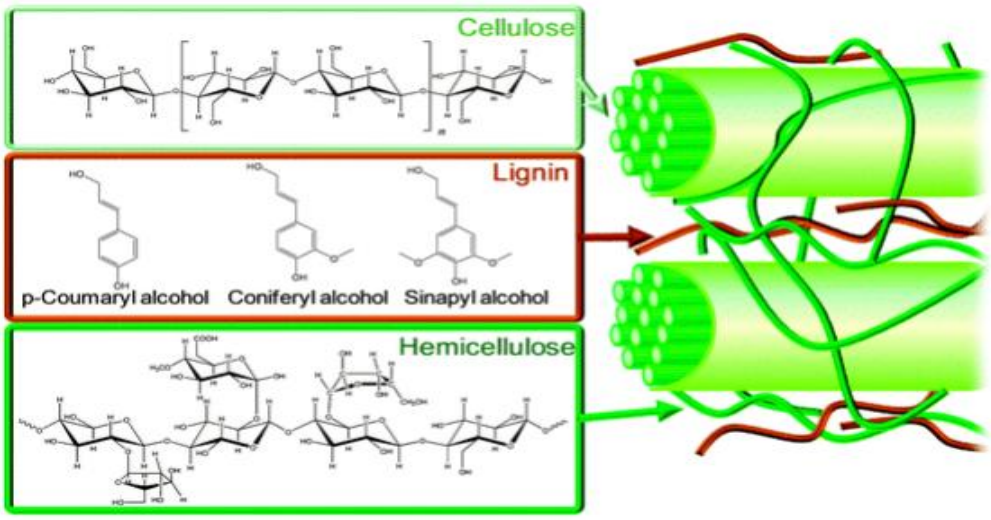


Figure 1. Schematic representation of lignocellulosic biomass (Adapted from Alonso, 2012)

1.1.1 Cellulose

Cellulose (Figure 2) is the most abundant polymer in nature and is continuously replenished by reduction of photosynthetic carbon dioxide by light. Cellulose is the main component of lignocellulosics wastes, corresponding to 40-50% of dry matter in most species and is located predominantly in the secondary cell wall (Torres *et al*., 2012)

Cellulose is composed by D-glucose units condensed through β(1→4) bonds and -glycosidic also linked by hydrogen bonds established between the multiple hydroxyl groups presented on the structure. The individual cellulose molecules associate with each other, forming fibrillar chains elements which are oriented in parallel. These fibrils are aggregated to form a beam along called microfibril which is grouped to form the cellulose fibers. The following figure represents the schematic model of the structure of cellulose (Jørgensen *et al*., 2007).

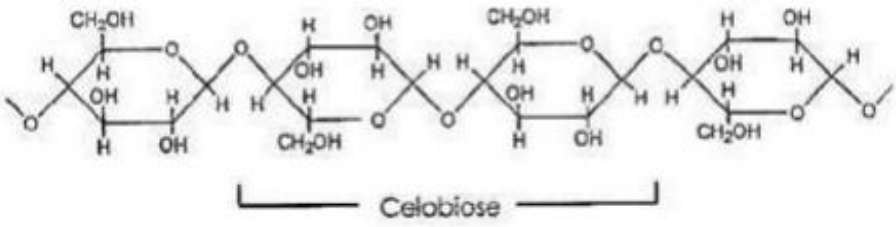


Figure 2. Schematic representation of cellulose (Fengel, 1989)

Cellulose can be found in different degrees of orientation from very crystalline regions to amorphous ones. Crystalline regions is due to the linearity of the cellulose molecules, to the intermolecular hydrogen bonds and finally to molecular parallelism.

Cellulose reactivity depends on its physical and chemical structure. The presence of amorphous and crystalline regions results in different accessibility to reagents. Indeed, crystalline regions tend to react slowly and are chemically more resistant than amorphous regions (Delmer, 1999).

1.1.2 Hemicellulose

Hemicellulose present branched linear polymers of monosaccharide united by glycosidic bonds. These polymers are characterized by different glicosidic bonds between monomers (bonds (1,4), (1,3) and (1,6)) and for another monomer in different proportions (mainly D-glucose, D-manose, D-galactose, D-xylose, D-arabinose). Hemicelluloses have highly heterogeneous non-crystalline structures, which correspond to higher reactivity and easier hability to obtain monosaccharides. Moreover, hemicellulose serves as a connection between the lignin and the cellulose fibbers and gives more rigidity to the cellulose – hemicelluloses – lignin network (Octave, 2009; Xiao, 2011).

1.1.3 Lignin

Lignin is another component of the cellular wall acting as bonding agent in cells, and giving rigidity to the cellular wall. Because of this the wall is a structure resistant to compression and impact. It's a relatively hydrophobic and aromatic structure made of racemic units of phenylpropanoid from which sinapyl alcohol, coniferyl alcohol and p-coumaryl alcohol are most abundant. The phenolic structure of this polymer confers resistance to enzymatic digestion, as a result its disruption represents the main target of raw material pretreatments before enzymatic hydrolysis (Bebru, 2010).

1.2 Residue valorization: Added value by-products

There is a growing interest in using lignocellulosic biomass as renewable resource for several bioprocesses adding value to these byproducts. Strong research is focused in the development of new technologies and new uses for these by-products in order to reduce their environmental impact. The utilization of agro industrial residues in bioprocesses returns a wide range of low cost subtract and consequently present huge potential for usages in different biotechnological (Briones, 2012; Federici, 2009; Pandeya, 2000; Mussato, 2011).

It is estimated that more than half of the plant production is discarded and not used for human consumption (leaves, seeds, etc). Bioconversion of lignocelluloses residues has been leading to new opportunities for the energy and food sectors, searching for full utilization of the natural resources existing in the world (Villas-Bôas, 2002). In fact, this natural way of recovering useful resources has been reducing the amount of residues in the environment, normally rich in carbon (Mahawar, 2012). The biomass production throughout bioconversion of agro industrial discards is revealing that is a valuable alternative to traditional applications of residues, since these subtracts of lignocelluloses are abundant and low cost.

Apple pomace is obtained as a by-product during the processing of apple fruits for juice, cider or wine preparation. Owing to the high carbohydrate content, apple pomace is used as a substrate in a number of microbial processes for the production of organic acids, enzymes, single cell protein, ethanol, low alcoholic drinks and pigments but also to the extraction of value added

products such as dietary fibre, protein, natural antioxidants, biopolymers and pigments (Bhushan, 2008; Edwards, 2012).

This work was focused on the valorization of apple pomace with high by-products potential as extractable antioxidants that could use in pharmaceutical and food industry, and also apple pomace is rich in polysaccharides that could be hydrolysed in simple structures as used by oleaginous yeast, for lipids accumulation and carotenoids production. The growth of oleaginous yeast on the lignocellulosic biomass hydrolysates is being explored as an effective option for lipid accumulation. Oleaginous yeast are known to accumulate lipids up to 40% of their total dry weight, and under starvation conditions oil production can exceed 70% of cell dry weight (Cescut, 2014).

In order to use lignocellulosic residues as subtract for yeast growth, it is necessary that the polisaccharides present in the structure of those residues be converted in simple sugars, through hydrolysis. This process breaks the hydrogen and the glicosidic bonds, in fractions of hemicelluloses and cellulose, reducing them to the constituent sugars, pentoses and hexoses, which can be assimilated by microorganisms (Demirbas, 2005).

There are many different processes to the conversion of biomass into simple sugars. Presently, chemical and biological processes have been used with this purpose. Moreover, the chemical treatments use different agents, such as acids (acidic hydrolysis) and organic solvents (Taherzadeh and Karimi, 2007) and biological treatments use enzymes (Kumar, 2009).

Acidic Hydrolysis

The term “acidic hydrolosis” is used when the glucides present in the structure of lignocellulosic materials are extracted due to the action of diluted acidic solutions at high temperatures, or when moderated temperatures are used along with concentrated acidic solutions. The most commonly acids used are the sulfuric, hydrochloric, hydrofluoric acid and acidic solutions with sulfuric treatments or mixtures of inorganic with organic acids, like the formic acid. Furthermore, solvents with similar physic-chemical treats to methane and acetone can also be utilizes for this purpose (Sun, 2002; Kumar, 2009).

Generally, hydrolysis in the presence of acidic solutions is performed in two or more different stages. Firstly, with high concentrations of acidic solutions low temperatures are used. In this phase, occurs the hydrolysis of the hemicelluloses. Further on, the next stages aim to hydrolyze cellulose, using high temperatures with diluted acids or concentrated acids and low temperatures, due to the fact that energy is needed to promote a rupture of the organized structure of cellulose (Carvalheiro, 2008).It is agreed that hydrolysis speed increases with the concentration of the acidic solutions used.

The presence of furfural and 5-(hidroximetil)-2furaldeid (HMF) in the hydrolysates of lignocellulosic materials, result from the decomposition of pentoses and hexoses, present in the chemical structure of these materials.The concentrations that we find in the subtracts depend of the temperatures during the hydrolysis, prevents the degradation of hexoses and pentoses in furfural and hidroximetifurfural (HMF), (Saha, 2005), which as shown before to be inhibitor compounds to microorganism growth (Klinke, 2004).

The main challenge of acidic hydrolysis is to enhance the recovery rates of glucose up to values bigger than 70% in a industrialized process economically viable, maintaining at the same time high rates for the cellulose hydrolysis and minimizing the decomposition of glucose (Balat, 2011).

Enzymatic Hidrolysis

Enzymes are natural proteins that catalyze certain chemical reactions. To achieve effective function of enzymes, these proteins should have access to the molecules which are going to be hydrolyzed. In fact this requires a previous treatment of the lignocellulosic biomass, in order to remove lignin and exposing the hemicelluloses and cellulose molecules, and simultaneously breaking the crystalline structure of cellulose (Demirbas, 2005).In fact, enzymatic hidrolysis of lignocellulosic materials is a slow process, because the hydrolysis of cellulose is damaged due to structural parameters of the subtract, like the lignin and hemicelluloses content, surface area and cellulose crystallinity (Balat *et al*., 2008).

Cellulose is hydrolyzed by enzymes denominated cellulases. They can be produced by several microorganisms, normally by fungi and bacteria. An efficient hydrolysis requires a certain number of enzymes. According to the traditional classification, cellulosic enzymes are divided in three classes: exo-1,4-β-D-glucanases or celobiohidrolases (CBH), that move along the cellulose chain, producing celobiose units in the endings (disaccharide with two glucose molecules); endo-1,4-β-D-glucanases that hydrolyse internal glicosidic bonds β-1,4, randomLy in cellulose chain; 1,4-β-glucosidases, that hydrolyze cellobioses in glucose. All of these enzymes work synergistically to hydrolyze cellulose, creating new accessible bonds one to another (Jørgensen *et al*., 2007).

Equipment costs to perform enzymatic hydrolysis are inferior to the ones of chemical hydrolysis, once enzymatic hydrolysis is normally carried out in undamaging conditions (pH of 4,8 and temperatures between 46 and 51°C) not featuring corrosion problems (Balat *et al*., 2008).

The central dogma of agro residues valorization is still the stability, safety and economic feasibility of the processes/products development. Besides the associated effectiveness and advantages in the hydrolysis processes previously described, they also show some down sides. Enzymatic hydrolysis of lignocellusosic products requires application of pre-specific treatments to the raw material, in order to increase the accessibility of microorganisms to cellulose, this raises the duration and the cost of the process, so that economic viability of the process involves application of procedures for the recycling of the enzymes.

On the other hand, in acidic hydrolysis processes the decomposition of some of the glucids present in the structure of the material, generate a significant number of inhibitors of microbial growth (furfural, organic acids, etc.) with consequent reduction of the process efficacy (production of lipids and carotenoids). Acidic hydrolysis requires the utilization of equipments with specific anticorrosive protection, increasing the process cost. Economic viability of processes of acidic hydrolysis is associated to the usage of efficient technologies for the recovery of the used acids and to the existence of markets to commercialize the sub products resulting from the process.

Thus, to face these process problems, more and more research lies on studies that present viable economic alternatives. More recently, supercritical fluids has been reported to be one of the alternative and promising treatment methods to extraction of value added compounds and to break lignocellulosic structure (Binder andRaines, 2009).

In this work apple pomace was treated by the Hot Compressed Water technology (HCW). This is an environmentally benign alternative to conventional treatments. Water is considered a “green” solvent, highly available and environmentally benign.

1.3 Apple

The apple, genus *Malus*, family rosettes can be found in vast areas around the globe, with special incidence in the temperate regions of the world (Shalini, 2009). There are 25 species and more than 7.500 varieties of farmed apples, which vary in size, shape, color, firmness, texture, juiciness, flavor (sweet, sour, salty and biter sensations), aroma (volatile composts) and nutritional value (Bhatti and Jha, 2010).

Apples are the fourth most widely produced fruit in the world after bananas, oranges and grapes (Mahawar, 2012). The biggest apple producers are located at the North hemisphere and are ruled by China (Figure 3) that in 2013 registered a production of about 35 billion tones, in other words, 48% of the worldwide production. The United States of America (USA) come up in second place, with significant displacement of a production over the 4 billion tones (6%). Turkey is the country with the highest production of apple in Europe with 3,7%, while Portugal contribute with 0,24% of the production (FAOSTAT, 2014).

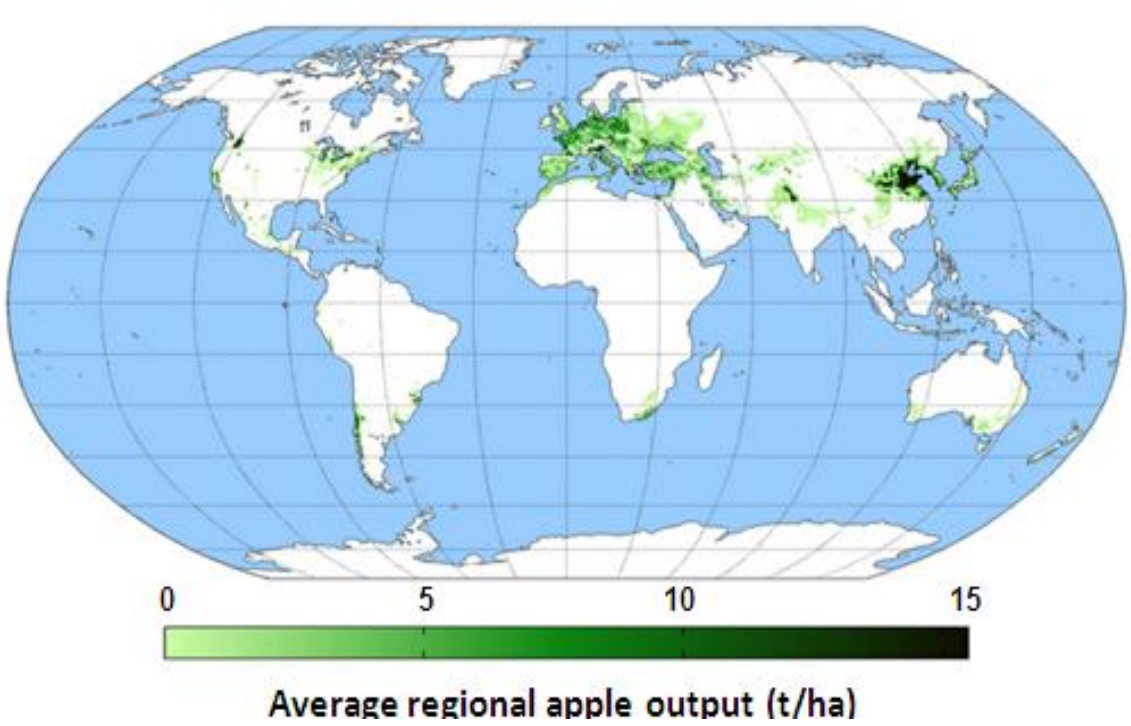


Figure 3. Map of world apple production regions

1.3.1 Apple pomace

From the entire production of apple across the world, 71% of the fruit is consumed for table purposes as fresh apple, while about 20% is processed into value added products, of which 65% are processed into apple juice concentrate and the balance into apple juice, apple wine, cider, apple purees, jams and dried apple products, etc. (Shalini, 2009; Vendruscolo *et al*., 2008).

After the apple harvest, these go through cleaning and selection stages. The fruits that are not in standards for commercialization are descarted of industrial use, being later on cleaned and sanitized externally. Further on the extraction of the juice by pressing. The juice is divided and one part is clarified for removal of suspense particles, accounted for turbidity (e.g. pectin). In addition, the clarified juice is deflavored, where the aroma is commercialized and the stock remaining is concentrated, producing concentrated apple juice. The reaming part that was not claryfied is transformed in *mosto*, which is going to be fermented for production of beverages such as cider, apple wines, brandies and vinegars (Villas-Bôas, 2001).

Apple pomace (Figure 4) is the solid residue that remains after the extraction of juice from apple and builds of a heterogeneous mixture of pulp, seeds and peels. Conventional process of juice recovery removes 75% of fresh weight as juice and 25% as pomace (Vendruscolo *et al*., 2008). It is characterized for presenting high quantities of fibers, from biopolymers of high molecular weight, likehemicelluloses, pectin and lignin and other mono and disaccharides (Joshi and Sandhu, 1996; Shantali, 2009). Table 1 shows the physical and chemical composition of the apple pomace.

Figure 4. Apple pomace

Table 1. Chemical composition of apple pomace (Sato, 2010)

Composition	Content
Apple pomace	
Moisture (%)	11.43
Ash (%)	1.80
Lipids (%)	1.53
Proteins (%)	2.74
Total polyphenols g Kg^{-1}	4.61
Total sugar g $100g^{-1}$	39.35
Dietary fiber g $100g^{-1}$	43.63

Apple pomace is traditionally intended for feeding cattle or soil uses, since it reveals a low commercial value due to the low levels of protein. But, because of high concentrations in sugar, the apple pomace has big potential for biotechnological procedures, acting as a source of carbon to microorganisms; the conversion of pomace into noble products has increased within the last years (Vendruscolo *et al*., 2008).

Several studies used until now the apple pomace as carbon source for microorganisms, producing citric acids (Shojaosadati and Babaeipour, 2002), SCP (Joshi and Sandhu, 1996; Rajoka *et al*., 2006; Vendruscolo *et al*., 2007), eatable fungi (Zheng and Shetty, 1998), enzymes (Zheng and Shetty, 2000) and ethanol (Ngadi and Correia, 1992).

In our study, through hot compressed water technology, antioxidants from pomace will be extracted and the lignocellulosic material will be hydrolysed to obtain simple sugar, which will be used as substract for the growth of oleaginosas yeast that will produce added value products, lipids and carotenoids.

1.4 Supercritical Fluids

Supercritical fluids (SCFs) are substances at pressures and temperatures above their critical values (Figure 5). A pure component is considered to be supercritical if its temperature and pressure are higher than the critical values (T_c and p_c, respectively). When the fluid enters the supercritical region, it adopts an array of gas and liquid properties, with low viscosity, high diffusibility and almost no surface tension (Brunner, 2005).

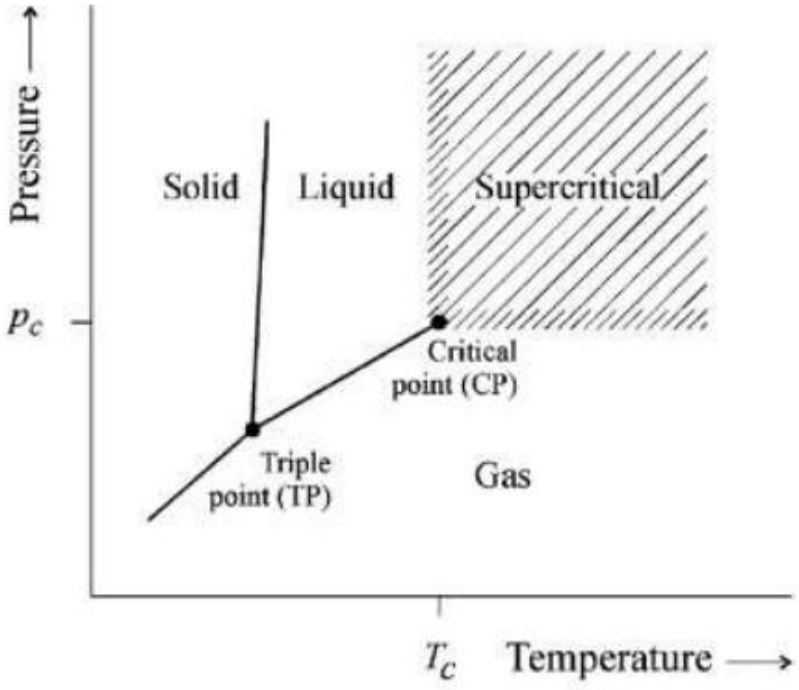


Figure 5. General diagram of supercritical state for a pure component (Adapted from Brunner, 2005)

The principal properties of the supercritical fluids are mainly their liquid-like density, high compressibility (offering large variability of solvency by small changes in temperature and pressure) viscosity, diffusivity and solvent strength. In a supercritical fluid the salvation power (dissolving capacity) is sensitive to the temperature and pressure changes in the supercritical region. In this region, there is only one phase, which possesses properties of both gas and liquid, making the salvation power higher due to high, liquid-like, density. The mass transfer rates also increase because of a high, gas-like diffusion coefficients and low viscosity values. These are the main characteristics of supercritical fluids that make them an attractive agent. As a result, supercritical fluids are ideal alternatives to organic solvents, and with these properties, mixing compounds can be improved, resulting in a better heat and mass transfer (Brunner, 2005).

In order to choose a supercritical fluid as solvent, a number of aspects must e considered, such as price, toxicity and good solvent properties (solubility of the solute in the solvent; viscosity of the fluid in the supercritical region and diffusivity of the supercritical solvent).

The most common SCF are water, CO2, acetone, alcohols as methanol and ethanol, alkanes like methane, ethane and buthane and also some unsaturated hydrocarbons such as ethylene and propylene, among others (Table 2) (Wen, 2009).

Table 2. Supercritical points of the most common SCF (Wen, 2009)

Fuid	Temperature (K)	Pressure (MPa)
Water	647.0	22.1
CO_2	304.1	7.4
Methanol	512.6	8.1
Methane	190.7	4.6
Propane	369.6	4.3
Acetone	508.1	4.7

In this work it was used water as the supercritical fluid. The choice of this particularly solvent was based on the many advantages it presents. It is an inexpensive, nontoxic compound, is considered a "green" solvent, highly available and environmentally benign. It is also largely used, at its supercritical or subcritical state, in industry mostly for extraction of value added compounds and to promote hydrolysis reaction (Brunner 2009).

However the use of supercritical water needs high additional cost due to it corrosivity and also due to higher temperatures and pressure values are required. Subcritical water reveals more promising candidate to industrial uses because it represents similar characteristics that a supercritical water but is economically advantageous due to amenable conditions needed.

1.4.1 Hot compressed water

Hot compressed water is used nowadays to many chemical reaction and it consists of the liquid water below its supercritical temperature and pressure condition.

In the supercritical conditions water changes its character from a solvent for ionic species to a solvent for non-ionic species. Below the critical temperature and at high pressures, the ionic product increase, the pH-value decreases and the water is an acid/base catalyst precursor capable to catalyzed reactions. HCW also exhibits the properties of a nonpolar solvent from the macroscopic point of view and exhibits the properties of a nonpolar solvent.

Near of the critical temperature the solubility behavior of water changes. The solubility of salts decrease to practically zero at low pressures and with increasing temperature. In this conditions the salts dissolved in the feed could precipitate and block the reactor preventing reaction and extraction processes occur making this effect is one of the major drawbacks of processing waste. The solubility remains constant or is even increasing at moderate and high pressures. The solubility of water for gases is high in the critical region, the viscosity is of the order of a normal gas and the diffusion coefficient is at least one order of magnitude higher than that of a liquid and at near critical and supercritical conditions water and gases like O2, N2, NH3, CO, CO2 are completely miscible (Brunner, 2009).

Beyond the characteristics referred, the water is considered a green solvent since it has no risks to the environment, in addition the use of subcritical water is economically advantageous comparing with the supercritical water, since this required higher temperatures and pressure values and reactors whose material is more resistant due to it corrosivity of supercritical water.

1.5 Oleaginous Yeast

Nowadays with the constant advances in the industry field and a growing worry about environment conditions the use of microorganisms to convert waste materials, predominantly residues (such as agro-industrial residues) into valuable products has been extensively studied. Yeast have presented relevant biotechnological interest, as they are important agents in the production of various compounds with industrial utility.

Due to finality of the present work, that consists in the valorization of agro industrial residue (apple pomace) through production of the value added by-products, more attention was given to a restrict group of yeast named oleaginous yeast.

Oleaginous yeast has the ability to accumulate lipids under specific cultivation conditions. Most oleaginous yeast can accumulate lipids at levels of more than 40% of their dry weight and as much as 70% under nutrient-limiting conditions and are able to utilize several different carbon sources for the production of cell mass and lipids (Beopoulos *et al*., 2009). These lipids have similar composition and energy value to plant and animal oils, but their production do not compete for food resources, in particular if it is based on inexpensive carbon sources, such as lignocellulosic residues (Zhao *et al*., 2012).

The study of oleaginous yeast has a long history: their ability to accumulate lipids has been known from the 70s, but only in the last years the attention has been focused on exploitation of SCO (single-cell oil) production (Breuer and Harms, 2006; Chen *et al*., 2009). Table 3 shows the typical oleaginous yeast species used to SCO production.

Table 3. Common oleaginous yeast species for single-cell oil production from cellulosic sugars (Abghari, 2014)

Oleaginous yeast species	Substrate
Cryptococcus sp.	Glucose and corncob hydrolysate
Cryptococcus curvatus	Oligocelluloses and oligoxyloses
Lipomyces starkeyi	Co-fermentation of cellobiose and xylose
Rhodosporidium toruloides	Jerusalem artichoke
Rhodotorula graminis	Corn stover hydrolysate
Trichosporon coremiiforme and Trichosporon dermatis	Corncob acid hydrolysate
Trichosporon cutaneum	Corn stover
Yarrowia lipolytica	Sugarcane bagasse and rice bran hydrolysate

The yeast represent a part of the microbiota in all natural ecosystems, such as soils, freshwaters and marine waters, from the ocean surface to the deep sea. Approximately 1500 species of yeast belonging to over 100 genera have been described so far (Butinar *et al*., 2007). Among the huge number of species that have been described, only 30 are able to accumulate more than 25% of their dry weight as lipids (Beopoulos *et al*., 2009). The most deeply investigated oleaginous yeast belong to the genera *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodosporodium*, *Cryptococcus*, and *Lypomyces* (Ageitos *et al*., 2011; Li *et al*., 2008; Rossi *et al*., 2009). In this work oleaginous yeast belong to the genera *Yarrowia* (Figure 6), *Rhodotorula* (Figure 7), and *Rhodosporodium* (Figure 8) were used.

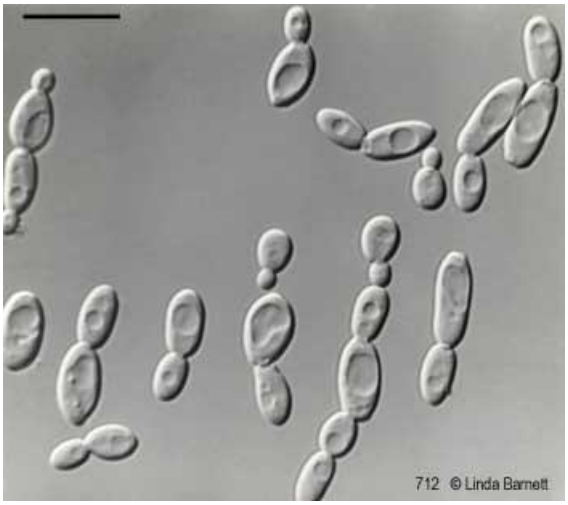


Figure 6. *Yarrowia lipolytica (Adapted from NCYC, 2014)*

Figure 7. *Rhodotorula glutinis (Adapted from NCYC, 2014)*

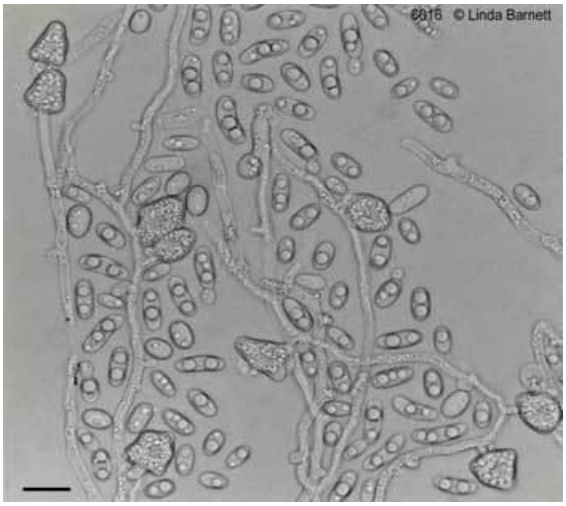


Figure 8. *Rhodosporidium toruloides (Adapted from NCYC, 2014)*

1.5.1 Lipid accumulation in oleaginous yeast

Oleaginous yeast accumulate various types of lipids, including triacylglyceride (TAGs), diacylglyceride (DAG), monoglycerides, fatty acids, sterol esters, free sterols and others. Lipids are found mainly in form of neutral lipids, glycolipids, phospholipids and free fatty acids. The fractions of neutral lipids in general, account for more than 90% of total lipids (Huang *et al*., 2013). The classes of lipids present, as well as the ratio of membrane lipid and storage, vary among species and strains, culture conditions and culture growth phase (Sitepu *et al*., 2014). The triacylglycerides (TAG) and sterol esters are the major neutral lipid and is synthesized from sterol, glycerol-3-phosphate and acyl-CoA. The composition of fatty acids which form intracellular TAG is variable, and depends on the yeast species, growth stage, environmental conditions and substrates of the medium (Abghari *et al*., 2014).

The sterols and fatty acids are important building blocks of cell membranes and to maintain balanced cellular levels. However, as both molecules are insoluble in the cytoplasm, they must be stored in cellular compartments are known as lipid droplet (LD) (Czabany *et al*., 2008). The structure of these compartments (Figure 9) is similar in all cell types. LD consists of a core formed by the neutral lipids, which are surrounded by a monolayer of phospholipids with some added protein. Figure 9 represent the schematic representation of a lipid droplet.

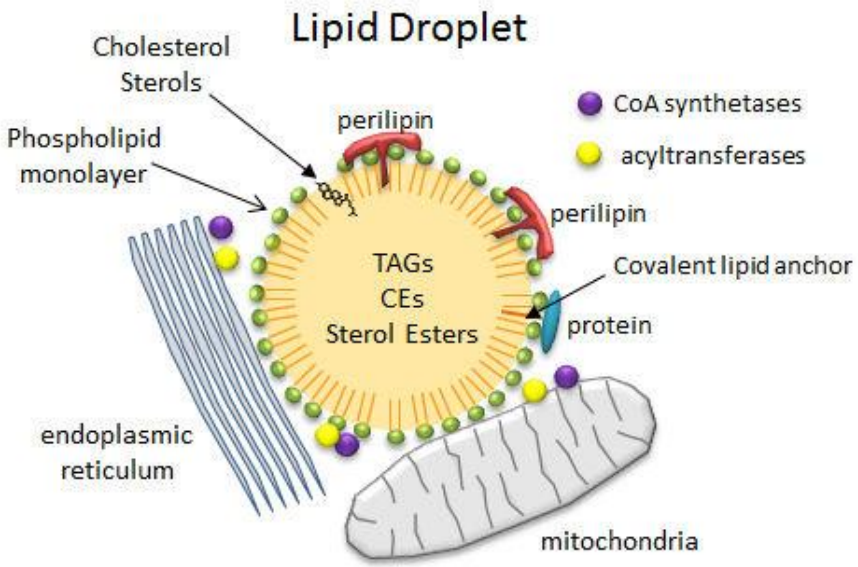


Figure 9. Schematic representation of a LD.

1.5.1.1Lipid accumulation pathways in yeast

All microorganisms are able to synthesize lipids, however, only the oleaginous strains can accumulate significant quantities within their cells (Papanikolaou *et al*., 2011). Lipids can be accumulated within the cell from two different ways, as follows (Beopoulos *et al*. 2012):

1. De novo synthesis: involving the production of precursors of fatty acids, acetyl-CoA and malonyl-CoA, and its integration with the lipid biosynthesis. Generally this approach is used when the microorganism growth grows under nitrogen limitation;

2. Ex novo synthesis: Involving the accumulation of fatty acids, oils and TAGs from the culture medium and its storage without modification within the cell. This approach requires that free fatty acids are transported into the cell.

A major difference between the *de novo* and *ex novo* synthesis is that in the *ex novo* synthesis, lipid accumulation occurs simultaneously with the growth cell, being completely independent of the nitrogen depletion in the middle cultivation (Papanikolaou *et al*., 2011).

During the *de novo* synthesis, the beginning of lipid accumulation is induced by exhaustion or limitation of an essential nutrient in the culture medium. Usually, nitrogen is used for this purpose because its addition is easy to control. When nitrogen becomes unavailable cell division decreases because nitrogen is crucial to the synthesis of proteins and nucleic acids. However, the

microorganism continues to assimilate the carbon source (sugars or glycerol) of the medium, which is now directed to the synthesis of lipids, leading to TAGs accumulation within the cell and the formation of LD.

The lipids accumulation by oleaginous yeast is associated with the presence of citric acid in the cell cytoplasm due to a decrease in the activity of the enzyme isocitrate dehydrogenase, belonging to the set of enzymes present in the Krebs Cycle (KC). The enzyme isocitrate dehydrogenase is allosterically activated by intracellular adenosine monophosphate (AMP) which, in the absence of nitrogen, is deaminated to IMP (inosine monophosphate) by the enzyme AMP deaminase. The large decrease in AMP concentration reduces the activity of isocitrase dehydrogenase, responsible for the transformation of isocitric acid to α-ketoglutaric. So, isocitric acid and also citric acid accumulats in the mitochondria. When the concentration of citric acid in the mitochondria reaches critical values, it is transported to the cytosol through the citrate-malate transport and is cleaved by the ATP citrate lyase (ACL) forming acetyl-CoA and oxaloacetate. The acetyl-CoA, the basic unit of fatty acid biosynthesis, is the precursor of *de novo* lipid synthesis in oleaginous yeast (Papanikolaou *et al*., 2011).

The general mechanism of lipid accumulation in oleaginous yeast has not yet been fully clarified (the fact that a number of synthetic steps are performed in different cellular compartments creates a higher complexity. The accumulation of lipids reaches a peak in the early stationary phase, when cells depleted nitrogen, but still have excess carbon in the medium (Zhang *et al*., 2012).

1.5.2 Carotenoids: Properties and synthesis by yeast

The yeast used in this work (excepted *Yarrowia lipolytica*) also produce carotenoids pigments and might be considered as a valuable source of both carotenoids and lipids.

Carotenoids are natural pigments responsible for yellow, orange and red of many foods such as fruits, vegetables, egg yolk, some fish such as salmon and trout, and crustaceous. In addition to coloring, carotenoids have important biological activities in diseases in which free radicals have a fundamental role, such as arteriosclerosis, cataracts, macular degeneration, multiple sclerosis, cancer and degenerative and cardiovascular diseases (Krinsky, *et al*. 2006).

In the food industries, carotenoids are mainly used as colorants, aiming to restore the color lost during processing and storage, coloring colorless food and standardize the color of some food products. More recently, with the growing interest in healthy products, carotenoids have also been added to foods to enrich them. Carotenoids are also precursors for many important chemical compounds, responsible for the aroma of some foods, flowers fragrance, coloring and specific photoprotection (Sanchez, 2002).

Production of carotenoids by microorganisms competes primarily with the production by synthetic procedures. Currently, the carotenoids are industrially or chemically produced or obtained by extraction from plants and/ or algae. The chemical synthesis generates hazardous wastes that can affect the environment and in the case of compounds from plants origin there are some problems regarding seasonal and geographic variability that cannot be controlled. The biotechnological production of carotenoids shows great interest and has been increasing due to factors such as the possibility of using low cost raw material; designation of natural substances; small space for production, independence of environmental and climate conditions, season or soil

composition(Mata-Gomez *et al.*, 2014). Thus, microbial synthesis offers a promising alternative for carotenoids production.

1.5.2.1 Carotenoids synthesis by microorganisms

Carotenoids can be biosynthesized by photosynthetic microorganisms, for example, algae and cyanobacteria (blue and green) and by bacteria, fungi and yeast (Mata-Gomez *et al*., 2014).

Yeast are particularly interesting due to their ability to growth on media with high sugar concentration. Species such as *Xanthophyllomyces dendrorhous*, *Rhodotorula glutinis , Rhodotorula mucilaginosa, Sporobolomyces spp.*, *Phaffia rhodozyma*, *a*re being studied in order to maximization and/or optimization of carotenoids production, with a view to industrial use(Mata-Gomez *et al*., 2014).The production of carotenoids varies between species, and is affected by medium constituents and environmental conditions. Table 4 lists yeast with potential to be used in the production of the major carotenoids.

Table 4. Carotenoids produced by pigmented yeast

Yeast	Carotenoids produced
Phaffia rhodozyma	Astaxanthin and β-carotene (Liu, *et al*., 2006)
Rhodosporidium babjevae	Astaxanthin and β-carotene(Johnson *et al*., 1995)
Rhodotorula glutinis	Torularhodin, β-carotene and Torulene (Buzzini, *et al*., 1999)
Rhodotorula graminis	Torulene (Buzzini, *et al*., 1999)
Rhodotorula mucilaginosa	Torularhodin, β-carotene and Torulene (Buzzini, *et al*., 1999)
Rhodotorula rubra	β-carotene (Shih, *et al*. 1996)
Sporobolomyces roseus	Torularhodin, β-carotene and Torulene (Davoli, *et al*., 2004)
Sporobolomyces ruberrimus	Torularhodin , β–carotene(Razavi *et al*., 2006)
Xanthophyllomyces dendrorhous	Astaxanthin (Hu, *et al*., 2006)

METHODS

2. Methods

2.1Apple pomace

Apple pomace was provided by University of Birmingham in collaboration with Supercritical Group and stored at -20°C. For use in all experiments, frozen apple pomace was lyophilized and milled in a grinder to making the mixture morphologically more homogeneous.

2.1.1 Chemical characterization of Apple pomace

In order to estimate maximum potential and a reference based on the monosaccharides, phenolic compounds, ash and proteins present in the AP residue, carried to the following methods:

2.1.1.1 Monosacharides, 5-HMF and Furfural

For chemical characterization of biomass was used acidic hydrolysis with sulfuric acid. After lyophilization and grinding, 0.5g of AP were added to 5mL 72% H_2SO_4 and maintained at 30°C for 1 hour. Distilled water was added to the mixture to dilute the 72% H_2SO_4 to 4% H_2SO_4. The mixture was incubated at 121°Cfor 1 hour. After 1 hour de mixture was cooled to room temperature. The solid fraction was separated from the liquid fraction by filtration with porous crucible. The solution was neutralized with sodium hydroxide (NaOH) to pH 4-6and filtered with NY Filters (0.20 µm GVS SpA).

The monosaccharides, furfural and 5-HMF analysis was performed with Chromatographo HPLC Thermo Scientific, Finnigan Surveyor Autosampler Plus, Finnigan Surveyor LC Pump Plus, Finnigan Surveyor RI Plus Detector as explained in Chapter 2.3.3 and 2.3.4 respectively.

2.1.1.2 Ash determination

For determination of total ash was weighed approximately 0.5 g of lyophilized AP in tared porcelain crucible. The sample was introduced into the furnace, where it remained for 24 hours at 300°C. By the difference in mass, ash present in the original waste was determined.

2.1.1.3 Proteins Content

A sample of lyophilized AP residue was subjected to nitrogen analysis by the elemental analysis method CHNS performed by the Analytical Laboratory REQUIMTE - Chemistry and Technology Network Department of Chemistry, Faculty of Science and Technology, New University of Lisbon. The protein content was derived from the N content found on dry biomass, using the conversion factor of 6.25.

2.1.2 Phenolic compounds

For determination of phenolics compounds in AP three methods was tested:

Hydro-alcoholic extraction: 1g of AP residue was added to 20 mL of water:ethanol (Scharlau 99.5%) mixture (75:25 v/v) and stored for 18h at 50°C with constant magnetic agitation (150 rpm). Samples were filtered with NY Filters (0.20 µm GVS SpA).

Extraction with acetone:water: 1g of AP residue was extracted twice for 15 min with 10mL of water:acetone (SIGMA Aldrich 99.5%) mixture (20:80 v/v) containing HCl (Scharlau 38%) (0,1:10 v/v) to prevent oxidation of the polyphenols in an ultrasonic bath at room temperature and then stirred for 30 min on a magnetic agitation. After centrifugation (3000 rpm for 10 min) the supernatants from both extractions were combined and made up to final volume of 25 mL with Mili-Q water and filtered with with NY Filters (0.20 µm GVS SpA).

Extraction with citric acid 1g of AP residue was added to 10 mL of aqueous solution of citric acid (Scharlau 99.8%) (3g/L) and stored for 30 min at 40°C with constant magnetic agitation (150 rpm). Samples were filtered with NY Filters (0.20 µm GVS SpA).

2.2 Hydrolysis reactions Apparatus

For the hydrolysis reactions with HCW of AP residues, the installation represented in the schematic diagram on Figure 10 was used. The Figure 11 is photography of the installation used in this work. The main sections of this semi-continuous reactor are the heat exchange section and the reaction section. The samples are recovered after the BPR valve, which control the pressure of the system, at the end of the installation.

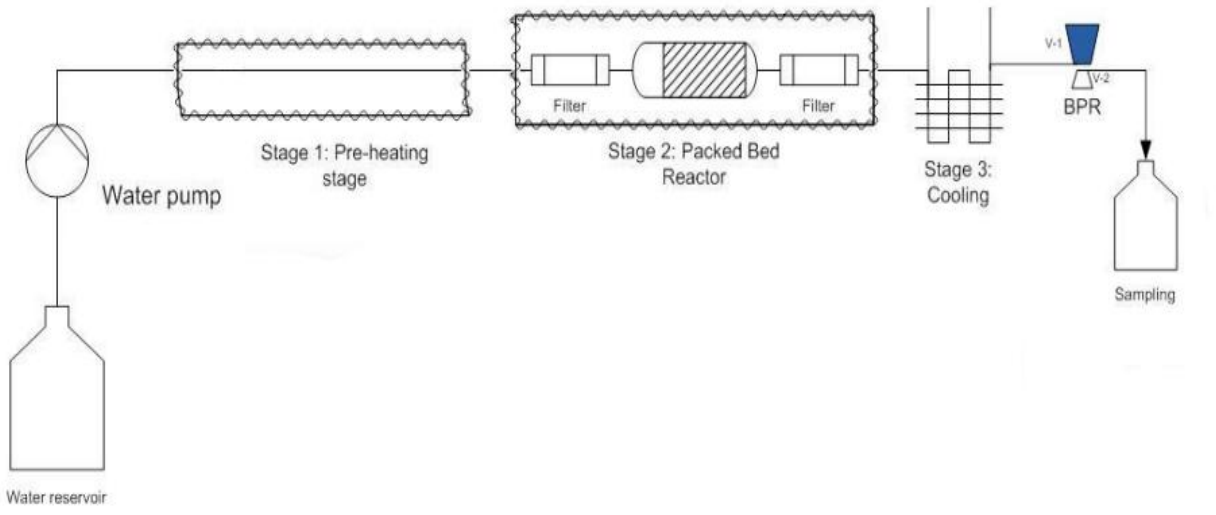
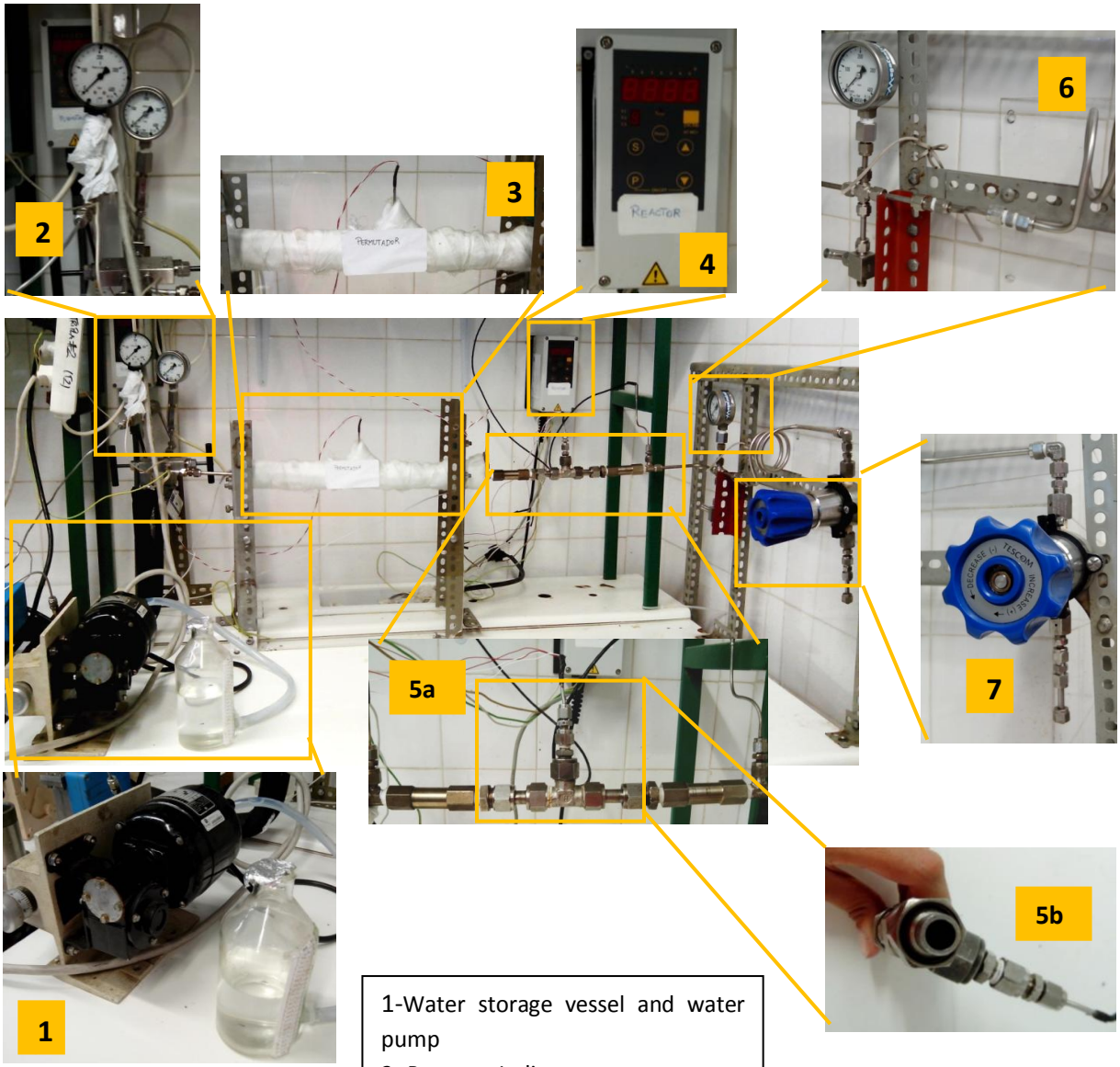


Figure 10. Schematic diagram of experimental set-up



1-Water storage vessel and water pump
2- Pressure Indicator
3- Pre-heating system
4- Temperature controller
5a- Reactor section
5b- Open reactor
6-Pressure indicator
7- BPR (Pressure Controller) and hydrolysate exit

Figure 11. Hot compressed water installation

This apparatus (Figure 10 and 11) is composed by a water storage vessel followed by a liquid pump (Minipump®, model LDC Analitical, max. flow 10 mL/min) (Figure 11.1) that pumped the water to the pre-heating section(Figure 11.3) before passing through the reactor section (Figure 11.5a and 11.5b)already filed with AP residue. The pre-heating section consists in a tubular stainless steel vessel, with an inner diameter of 10.5 mm and total length of 140.20 mm, with a PT100 sensor, in order to measure and control the temperature. The reactor section (Figure 11.5a and 11.5b) is consisted in a tubular stainless steel vessel, 10.5 mm of the inner diameter and 127.62 mm of length, with a PT100 sensor inside in order to measure and control the temperature. Before and after the entire set of tubes forming the reactor full with the AP residue, there are two filters (Swagelok®, 1/8 in., Female NTP, 0.5 micro pore sizes). The heat exchanger and the reactor are heated and controlled by two temperature and process controllers (HORST, model HTMC1) (Figure 11.4) which are pre-programmed with a progressive sequence of temperatures until the temperature of the experiment is reached. The reaction pressure is controlled with a steel stainless back pressure regulator valve (BPR) (Tescom Europe®, 26-1000) (Figure 11.7) and it is measured by an analogical pressure meter (WIKA, 640.059). When the fluid exists the reactor loses heat and after passing through the BPR pressure is the atmospheric. The hydrolysate obtained during the reaction is collected after the exit of the BPR in a Falcon tube (14 mL).

2.2.1 Hydrolysis reactions conditions

For each assay were introduced about 2g of residue inside the reactor. Extractions were carried out at a pressure of 100 bar and a constant flow rate of 2 mL/min. To perform the study of temperature effect on the hydrolysis yields ten different temperatures were chosen: 50°C, 70°C, 90°C, 130°C, 150°C, 170°C, 190°C, 210°C, 230°C and 250°c. Samples (hydrolysates) being collected for a period of 20 min after reach the desired temperature. All the samples were collected at falcon tubes of 14mL, filtered with paper filter (FILTER-LAB). Part of these solutions was used directly to perform colorimetric test. The hydrolysates colected were lyophilised in the Lyophilizer CHRIST ALPHA 1-4, B. Braun Biotech. International, during 48h to calculate the total amount of water soluble compounds obtained by assay.

2.3 Sample Analysis

2.3.1 Folin – Ciocalteu test (Waterhouse, 2011)

This colorimetric test has the purpose to quantify Total Phenolic Content (TPC) of AP hydrolysates into gallic acid equivalent. Calibration standard curve was performed with five different concentrations of Gallic Acid monohydrate (Sigma 98%) (50, 100, 150, 250 and 500 mg/L) and for blank solution was used Mili-Q water. Aqueous solutions of gallic acid and blank prepared as aqueous samples of AP hydrolysates described below without protein precipitation step. Aqueous samples of AP hydrolysates from each assay were used to precipitate proteins that could interfere with the method. At an eppendorf tube to 800µl of the sample were added 120µl of Trichloroacetic Acid reagent (Scharlau 99.5%). These eppendorfs were stored at -20°C for 5min, then at 4°C for 15min and centrifuge (Heraeus sepatech, Biofuge 13 Centrifuge) for 15 min at 12 000rpm. To 20µl of resulted supernatant were added 1.58mL of Mili-Q water, 100µl of Folin-Cicalteu reagent (MERK) and the resulted solution were stored at room temperature for 8min. After that were added 300µl Sodium Carbonate (Sigma) solution (200g/L) and incubated in water bath at 40°C for 30min. The absorbance were measured at 750nm (it should be 765nm but due to spectrophotometer limitation it was measured at 750nm) with DU®800 Spectrophotometer from Beckman Coulter, Brea, USA.

2.3.2 Colorimetric carbohydrate analysis (modified) (Masuko, 2005)

This colorimetric test has the purpose to verify the potential of AP hydrolysates in reduced sugars and derived compounds. Calibration standard curve was performed with eight aqueous solutions of 5-HMF monohydrate (SIGMA Aldrich) (0.005; 0.025; 0.05; 0.1; 0.15; 0.2; 0.25; 0.3 g/L) and for blank solution was used Mili-Q water. Aqueous solutions of 5-HMF and blank prepared as aqueous samples of AP hydrolysates described below. To 500 µl of aqueous samples of AP hydrolysates were added 1.5mL of H_2SO_4 acid (Panreac 96%) and 300 µl of 5% of phenol (SIGMA Aldrich 99-100%) in water. After incubating for 5 min at 90°C into AccuBlockTM Digital Dry Bath samples were cooled to room temperature into water bath and absorbance measured at 490 nm with DU®800 Spectrophotometer from Beckman Coulter, Brea, USA.

2.3.3 Monosaccharides analysis by HPLC

The HPLC analysis has purpose to identify and quantify monosaccharides presented in AP hydrolysates. The analysis was performed with Chromatographo HPLC Thermo Scientific, Finnigan Surveyor Autosampler Plus, Finnigan Surveyor LC Pump Plus, Finnigan Surveyor RI Plus Detector. Software used to data treatment was ChromQuest 5.0. Column used was HyperRez XP Carbohydraten Ca++ with dimensions 300x7.7 mm at constant temperature 85°C. As mobile phase was used Mili-Q water at flow rate of 0.6 mL/min.

There were expected low concentrations of monosaccharides in AP residue so the Method of Standard Addition Calibration was used. Standard curves were performed to glucose, fructose,

arabinose, xylose and galactose (99% SIGMA Aldrich) with 100 ppm as a standard concentration (25; 50; 75; 100; 150; 250 and 500 ppm).

There were prepared two solutions of standards due the fact that fructose and galactose retention times are too closed to arabinose and xylose respectively. The solution A contained 500 ppm of glucose, xylose and arabinose. The solution B contained 500 ppm fructose and galactose.

Samples of AP hydrolysates HPLC analysis were prepared with the powder resulted from lyophilisation in Mili-Q water and filtered with NY Filters (0.20 µm GVS SpA). It was used 400 µl of aqueous AP sample and 100µl of standard solution so that the final concentration of standard was 100 ppm and concentration of AP hydrolysate 4000 ppm.

2.3.4 5-HMF anf Furfural analysis by HPLC

The HPLC analysis has purpose to identify and quantify 5-HMF and furfural in the AP hydrolysates. The analysis was performed with Chromatographo HPLC Thermo Scientific, Finnigan Surveyor Autosampler Plus, Finnigan Surveyor LC Pump Plus. Software used to data treatment was ChromQuest 5.0. The method used has an injection volume of 10 µl and flow rate of 1 mL/min. The absorbance is measured at two different wavelengths: 280nm with Accela UV/Vis Detector. The column (BDS HYPERSIL C18; length 250mm; I.D. 4mm.) was at a constant temperature of 40°C.

The mobile phase is a mixture of two eluents, eluent A is Mili-Q water with 2% (v/v) of acetic acid glacial (Scharlau 99.8%) and eluent B is a mixture of water and acetonitrile (CALO ERBA 99.9%) (50:50) with 0.5% (v/v) of acetic acid. The gradient program was as follows: 10% B to 55% B (50 min), 55% B to 100% B (10 min), 100% B to 10% B (5 min).

Standard curves were performed to 5-HMF (25; 50; 100; 150; 200; 300; 500 and 1000 ppm), Furfural (25; 50; 75; 100; 150; 200; 250; 300; 500 and 1000 ppm) in Mili-Q water. All the standards used were from SIGMA Algrich 98-99%. Samples of AP hydrolysates were prepared with lyophilized powder in Mili-Q water with concentration 5000ppm.

2.4 Oleaginous yeast growth conditions

All the yeast used in this work were supplied by the Portuguese Yeast Culture Collection (PYCC), Caparica, Portugal (Table 5).

The yeast were maintained in Petri dishes containing YMA previously sterilized by autoclaving and stored at 4°C in the cold chamber. For yeast inoculation, the petri dishes were previously transferred from the cold chamber to the chamber at 25°C.

Table 5. Yeast strains used in the pre-selection path of the work

Species names	Origin
Rhodosporidium babjevae	Leaves
Rhodosporidium toruloides	Wood
Rhodotorula glutinis	Sea water
Rhodotorula mucilaginosa	Water
Yarrowia lipolytica	Olives

2.4.1 Pre-inoculum

For each strain of yeast was prepared a pre-inoculum with final volume of 5 mL in a glass test tube, and YNB minimal medium containing 2% glucose as carbon source. Minimal media (YNB) (DifcoTM Yeast Nitrogen Base) were prepared ten times concentrated with 6.7g of YNB in 100 mL of sterile water. Carbon source were prepared ten times concentrated with 20g of glucose in 100 mL of sterile water, this solution was sterilezed by filtration.

This medium was inoculated with the strain of the corresponding yeast and placed under constant agitation at 150 rpm at 25°C for 24h.

2.4.2 Inocula

For each species of yeast two assays was performed: an assay control, with glucose as a carbon source and an assay where the carbon source was obtained from the AP hydrolysate. The monosaccharides concentration was calculated (through the results obtained by HPLC analysis) to obtain a culture medium with monosaccharides concentration equal to the test control. This assay was developed as shown in the Table 6.

Table 6. Inocula medium composition

Control	Hydrolysate
12 g/L glucose	12 g/L monosaccharides from AP hydrolysate
YNB medium	YNB medium
Yeast (A_{640}=0.2) provenients of pre-inoculum	Yeast (A_{640}=0.2) provenient of pre-inoculum
Volume = 1mL	Volume=1mL

This growth experiments were performed in 10mL test-tubes. The maximum liquid volume used was 1 mL to provide headspace necessary to cells respiration attending to the fact that all stains used are aerobic microorganisms.

Culture growths were performed at 25°C with constant agitation. Experimental data were collected every three hours at 640 nm with OD detector (Stat Fax – 2100, AWARENESS TECNOLOGY INC.) for about 50h. As a blank solution was used YNB with glucose, in the case of AP hydrolysates used as carbon sources there were performed blank solution (YNB with AP hydrolysate) with the AP hydrolysate.

2.4.3 Potencial of AP hydrolysate as carbon source and Lipid accumulation by yeast

For the study of the potencial of AP hydrolysates as carbon source for oleaginous yeast growth and lipid accumulation, was outlined four different assays. First, was conducted an experiment with all five yeast, using the total AP hydrolysate (mixture of all hydrolysates obtained through the temperatures from 50°C to 250°C) as the carbon source to determine the potential of AP hydrolyzate as carbon source (Chapter 3.4.1). In the second assay (Chapter 3.4.2)was evaluated the ability of the five yeast to accumulate lipids using the total AP hydrolysate (mixture of all hydrolysates obtained through the temperatures from 50°C to 250°C) as carbon source, using a specific medium to lipid accumulation described below. The most efficient yeast was selected.

In the three assay (Chapter 3.4.3) the yeast selected was grown in four different AP hydrolysates obtained by hydrolysis in four different temperatures (130°C, 150°C, 170°C and 190°C).

In all assays described before the inoculum with each yeast culture was prepared in 10 mL test tubes with a culture volume of 1mL, 200 rpm and 25°C. The culture are initiated with a specific amount of the pre-inoculum culture in order to initiated the shaking flask culture with initial OD_{640}=0.2.

Finally the fourth assay was outlined to scale up. The inoculum of yeast culture was prepared in Erlenmeyer flasks of 50 mL with a culture volume of 10 mL, 200 rpm and 25°C. The culture are initiated with a specific amount of the pre-inoculum culture in order to initiated the shaking flask culture with initial OD_{640}=0.2. Liquid medium was prepared as descript below, using as carbon source, AP hydrolysate obtained by the best temperature of hydrolysis.

Medium composition for lipid accumulation were described by Zhao *et al.*, 2008: 70 g/L carbon source (provenient of AP hydrolysate) dissolved in 125 mL of sterilized water and filtrated with 0,5 g/L Yeast extract, 2g/L $(NH_4)_2SO_4$, 7g/L KH_2PO_4, Na_2HPO_4, 0,5 g/L $MgSO_4.7H_2O$ dissolved in 100 mL of water and sterilized by autoclaving and 0,1g/L $CaCl_2.2H_2O$ dissolved in 100 mL of water and sterilized by autoclaving. At the end the 3 solution are mixed.

2.4.4 Fluorescence microscopy (Beopoulos, 2008)

For visualization of lipid droplets, Nile red (1mg/mL solution in acetone) was added to the cell suspension (1/10, vol/vol) and incubated for 1 h at room temperature. Cells were harvested, washed twice with distilled water, and resuspended in 50 mM sodium phosphate buffer (pH=6.8) to an A_{640} of 5. Microscopy was performed with a fluorescence microscope with a 100x oil immersion objective and the images was recorded.

2.4.5 Lipid quantification and analysis

Lipid droplets analysis was performed by microscopic observation of cell culture as described above. Ten representative cells were selected from fluorescence microscopy images to determine lipid droplets number for each assay.

2.4.6 Carotenoids extraction and analysis

Biomass was collected from 10 mL samples of yeast culture (centrifugation at 10000 rpm, 5 min, 25°C)frozen with liquid nitrogen and lyophilized. To fragillized the cells, primary dry biomass was submitted to extraction with small portions of acetone (5 mL) and glass balls (2mL) in a vortex agitation (15 min). Then extracted under agitation for 16h with acetone and the organic solvent was evaporated with gaz nitrogen.

To determine total carotenoids content in the extract, UV Spectrophotometry (DU®800 Spectrophotometer, Beckman Coulter) analysis was used. Spectra was run at λ=452 nm, according to the specific optical coefficient of β-carotene.

For a qualitative and quantitative analyses of carotenoids extracts were analyzed with Thermo Scientific (Finning Surveyor AutoSamples Plus) HPLC apparatus equipped with a reverse-phase analytical 5-µ particle diameter, polymeric C_{18} column and a UV diode array detector (Accela Uv/Vis Detector). The mobile phase consisted of (methanol and 0.2% H_2O)/ acetonitrile (75:25 v/v). Total run time was 30 min, with a injection volume of 10 µl. Quantification was performed with HPLC-DAD (λ = 450 nm) at a flow rate of 1mL/min, using a calibration curve (Annex 4) of trans-β-carotene-95% type I, Sigma as external standard.

RESULTS AND DISCUSSION

3 Results and discussion

3.1 Apple pomace

3.1.1 Chemical characterization

For evaluating the efficiency of different alternative processes to reuse agro-industrial waste is fundamentally a precise characterization of the biomass chemical composition. For chemical characterization of AP, was used an acidic hydrolysis with sulfuric acid. Thus, there is a depolymerization of the polysaccharide to form oligomers and its constituent sugars, i.e. their repeating units.

3.1.1.1 Monosacharides, furfural and 5-HMF contents in AP residue

By acid hydrolysis of AP was possible to determine the monosaccharides content present in the residue, as shown in Figure 12 it was concluded that the main monosaccharides present in the lignocellulosic structure of AP are fructose and arabinose. These values are the reference in order to determine the effectiveness of the hydrolysis method used in this work, the hydrolysis with HCW.

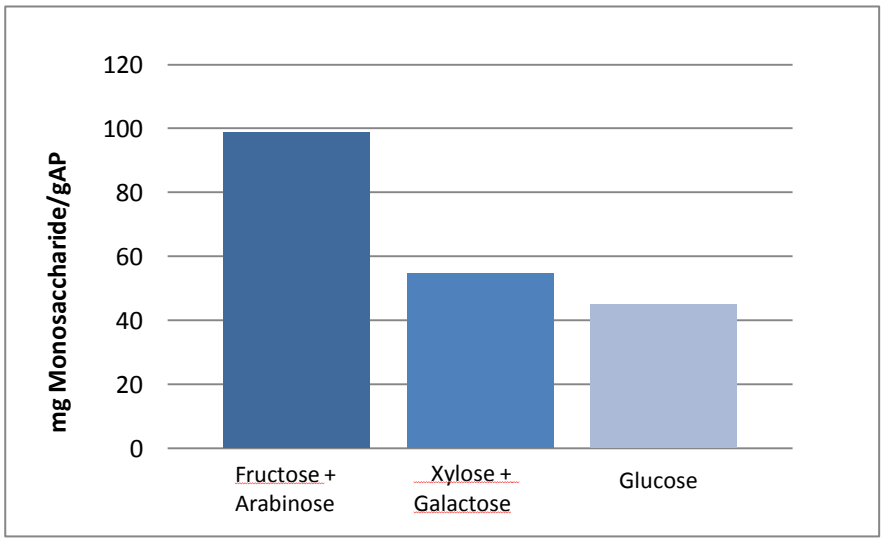


Figure 12. Monosaccharides content in AP after acid hydrolysis

As mentioned before furfural and 5-HMF results from the degradation of pentoses and hexoses respectively. These compounds can negatively affect the synthesis of lipids and carotenoids by yeast, since inhibit the specific growth rate of microorganisms, thus reducing biomass. Having into account identification of this compound in AP was extremely important. The Figure 13 shows the 5-HMF and furfural contents in AP after acid hydrolysis and provides an overview of the contents of these elements in the AP residue. However it must be noted that the degradation reactions will not

stop with the formation of 5-HMF and furfural and may be able to continue producing formic acid and levulinic acid.

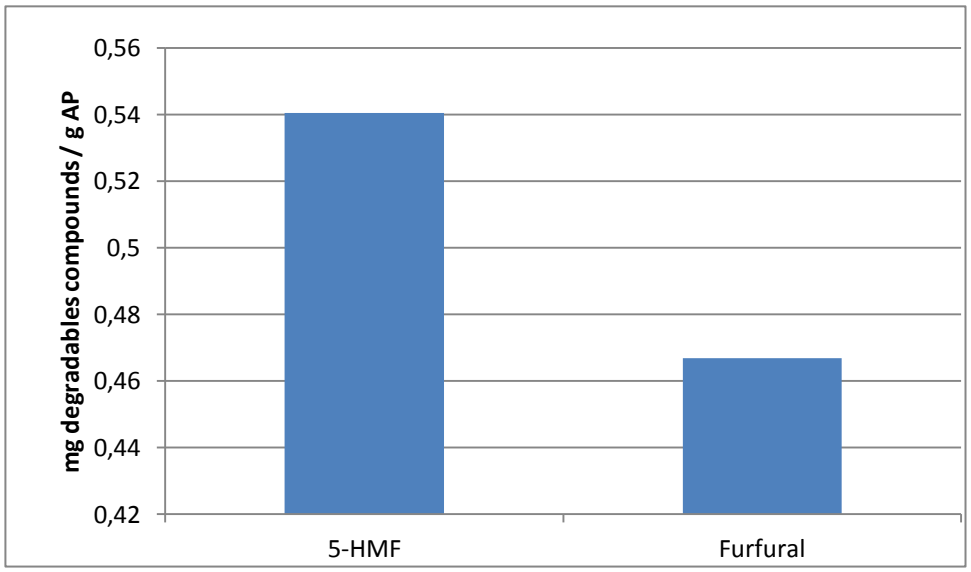


Figure 13. 5-HMF and furfural content in AP after acid hydrolysis

3.1.1.2 Ash

To determine the ashes three replicas were made. Ash content of apple pomace residue varied between 1.15% and 1.40% (w/w). These values are compatible with those found in the literature, such as 1.8% (w/w) by Sato, 2010 and 1.6% by Marcon *et al*. 2005.

3.1.1.3 Proteins content

The protein content obtained was 5.25% and was derived from the nitrogen content in AP obtained by the elemental analysis method, using the conversion factor of 6.25.This result is compatible with those found in the literature (2.73%- 36.7% (Renaud *et al*. 1999)(Sato, *et al.,* 1999). However, the protein content varies from apple species and within species, depending on culture conditions, temperature, photoperiod, light intensity, among others.

3.1.2 Polyphenols composition

In addition to the sugars was also evaluated the content of phenolic compounds in AP since they are also value added compounds being widely used in the pharmaceutical industry since it has many health benefits such as antimutagenic and anticarcinogenic activity, antioxidant and anti-inflammatory activities, prevention and delay of cardiovascular diseases.

To evaluate the extraction of phenolic compounds with HCW, other established methods of extraction of phenolic compounds were experimented, such as, extraction with water:ethanol

(75:25), water:acetone (20:80) and citric acid (3g/L). The TPC concentration extract by each solvent was evaluated by colorimetric analysis that gives an estimative of the total phenolic content (TPC) in the AP residue. The concentration of phenolic compounds extracted by each method is shown in the Table 7.

The color intensity (blue) is directly proportional to the concentration on polyphenols as it could be seen in the Figure 14 below there is a direct correlation between the color intensity of the samples and the results present on the Table 7.

Table 7. TPC concentration in AP using three different solvents to extract

Solvent	TPC concentration (mg/mL)
Citric acid	6.81
Water:Acetone	18.55
Water:Ethanol	32.61

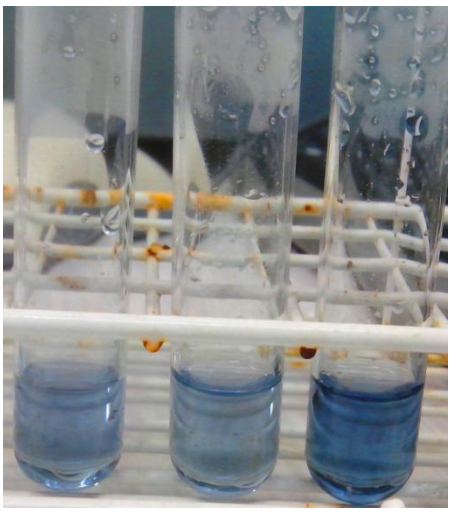


Figure 14. Different color intensity obtained by extraction with Citric acid (1); Water:Acetone (2) and Water:Ethanol (3).

Solubility of phenolics compounds is governed by their chemical nature in the plant residue, which may vary from simple to very highly polymerize. There is a possibility of interaction of phenolics with other plant components such as carbohydrates and proteins that may lead to the formation of complexes that may be quite insoluble. Likewise, the solubility of phenolics is affected by the polarity of solvent(s) used. Therefore, it is very difficult to develop an extraction procedure suitable for the extraction of all phenolics compounds in AP residue.

The results obtained by colorimetric methods show that among the extraction methods tested the most efficient is using ethanol as a solvent, allowing extract 32.61 mg/mL of TPC.

The table 8 shows the final chemical characterization of AP.

Table 8. Chemical characterization of AP

Compound	Content
Fructose + Arabinose	98.76 mg/gAP
Glucose	45 mg/gAP
Xylose + Galactose	54.78 mg/gAP
5-HMF	0.542 mg/gAP
Furfural	0.471 mg/gAP
Total phenolics compounds (Water:Ethanol)	32.61 mg/ml
Ash	1.15%-1.40%
Proteins	5.25%

3.2 Study of hydrolysis efficiency with HCW

3.2.1 Preliminary assay

To evaluate the temperature influence on the hydrolysis, first about 2g of AP residue was subject to hydrolysis at 11different temperatures. The samples of the liquid phases (aqueous solution of extracted and hydrolysed compounds) were collected in 14 mL falcon tubes (Figure 15) during 20 min after reaching the desired temperature of hydrolysis. In this assay was noted color difference that means that chemical composition of samples varies along time and that there is fractionation of the extracted/hydrolysed compounds.

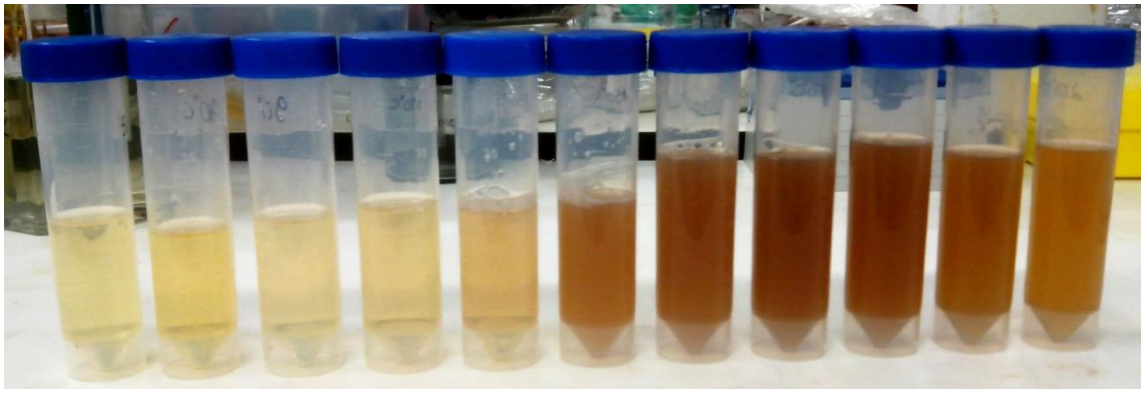


Figure 15. Example of samples collected during hydrolysis assay.

After lyophilization of hydrolysates there was estimated the mass of resulted powder to calculate the water soluble compounds obtained by assay. The mass of the water soluble compounds increases with the temperature as show in the Figure 16, however after reaching 130°C the amount of soluble mass decreases considerably reaching a minimum value at 250°C. With increasing temperature glycosidic bonds, between the polysaccharides of the lignocellulosic structure of the residue are broken, resulting in chemically simpler structures which are soluble in water. After the 190°Cthere is a drastic decrease in the recovered soluble compounds. Higher temperatures lead to severe degradation processes, and these results could be explained by degradation of the residue. The thermal degradation could result in ashes (that could precipitate and been removed afterwards from the samples during filtration), proteins (that could be precipitated) and furfural (product of thermal degradation of polysaccharides that sublimates during the lyophilisation process).

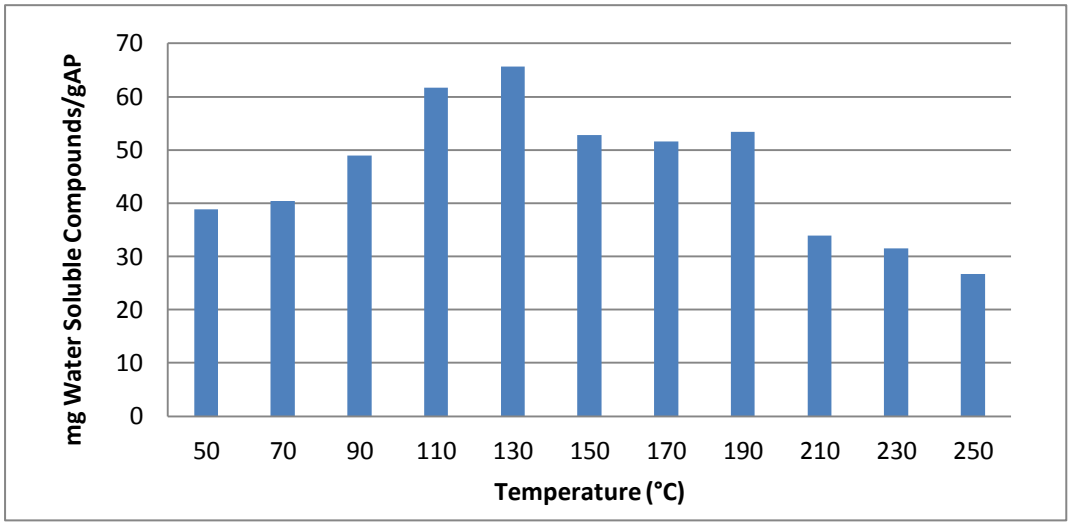


Figure 16. Amount of water soluble compounds at different temperatures

3.2.1.1 Polysaccharide content in AP hydrolysates

The total sugar content present in the AP hydrolisates samples was evaluated by colorimetric assays performed at its liquid phase, this method measures the soluble compounds present in the hydrolyzate (polysaccharides, trisaccharides, disaccharides, monosaccharides, 5-HMF and furfural). The method was performed only with the aim to estimate the potential of hydrolysates into carbohydrate composition. In Figure 17, is shown the amount of total sugars obtained at each temperature hydrolysis.

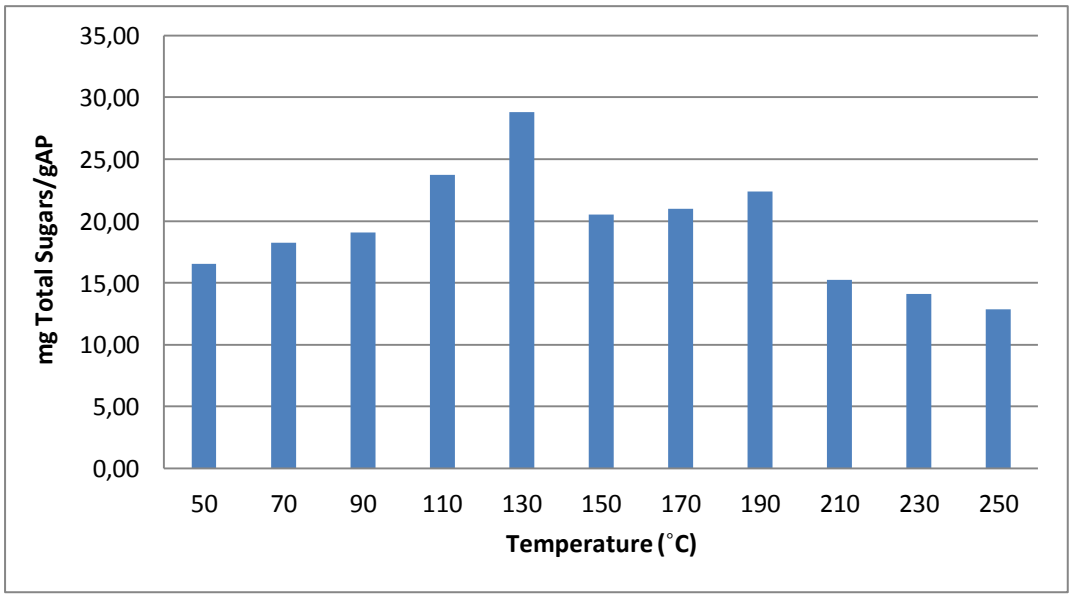


Figure 17. Amount of total sugars obtained at each temperature at different temperatures

As it can be seen on the Figure 17 polysaccharides and its derivates content shows the same behavior of the water soluble compounds mass variation (Figure 16). The amount of total sugars is

directly proportional to amount of soluble compounds and increases with the temperature showing higher rates for the 130°C hydrolysis assay and then decreases.

3.2.1.2 Monosaccharide content in AP hydrolysates

To get more precise data about hydrolysates composition, HPLC analysis were performed (Figure 18). Experimental data confirms variation of monomer composition in hydrolysates that resulted from assays performed at different temperatures. It means that fractionation of monosaccharides is possible.

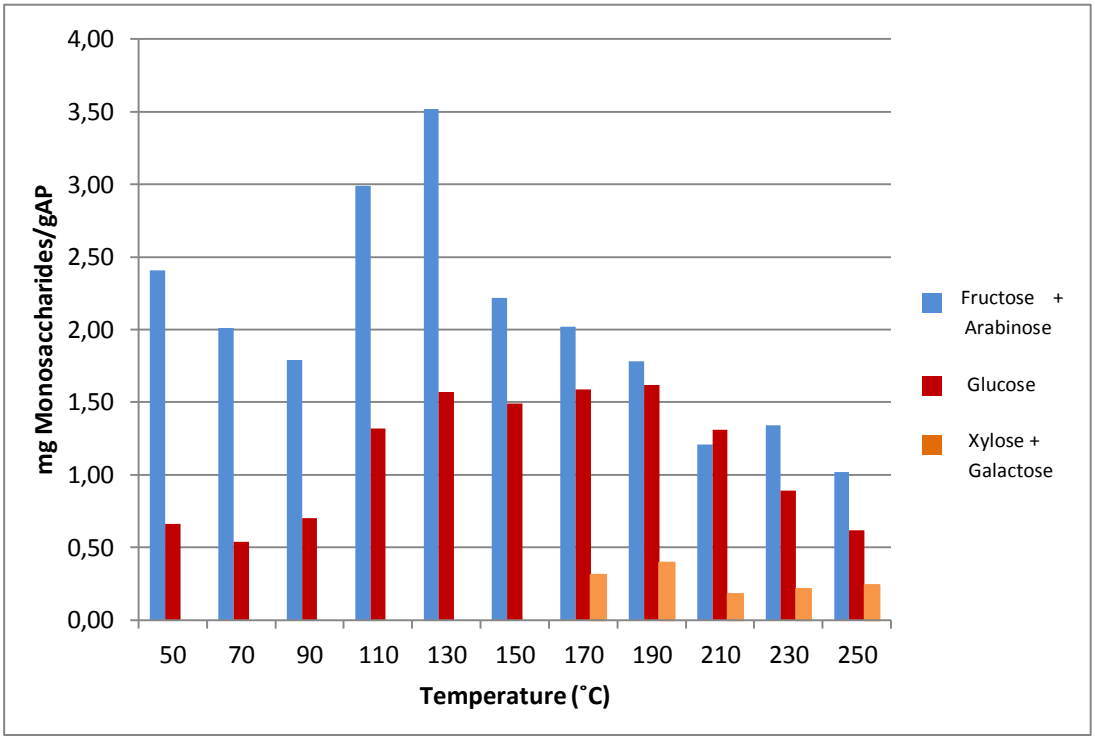


Figure 18. HPLC analysis of chemical composition of AP hydrolysates obtained at different temperatures

There were identified five sugars mentioned in the literature as the main monomers present in the apple pomace (fructose, glucose, xylose, arabinose and galactose). Glucose and arabinose could exist as residual sugars and as building blocks of cellulose (glucose) and hemicellulose (glucose and arabinose). Hemicellulose also contains xylose and galactose. Therefore, two main groups of monomeric structures should result from the hydrolysis process: the extracted saccharides (residual sugars like fructose) and monomers resulted from hydrolysis of polysaccharides (cellulose and hemicellulose). As it could be seen in the Figure 18 the behavior of monosaccharides composition at lower temperatures 50-90°C suggest that it is part of residue sugars and that it was extracted from the residue and didn't result from hydrolysis.

The gradual increase of glucose along the temperature (110°C-190°C) suggests that at these and higher temperatures there were hydrolysed sufficient amount of hemicellulose to achieve cellulose structures from which glucose comes from.

3.2.1.3 Degradable compounds in AP hydrolysates

As mentioned before, from degradation of biomass, namely monosaccharide species, during subcritical water processing are formed furfural and 5-HMF (degradable compounds) which have been shown in previous studies to be potential inhibitors of the growth of yeast. One of the main objectives of this work is to use AP hydrolysates as carbon source to oleaginous yeast growth. Having into account the identification of this compound was extremely important.

There were performed HPLC analyses to identify the presence and amounts of 5-HMF and furfural in AP hydrolysates. The Figure 19 shows the variation of 5-HMF and furfural through the different temperatures.

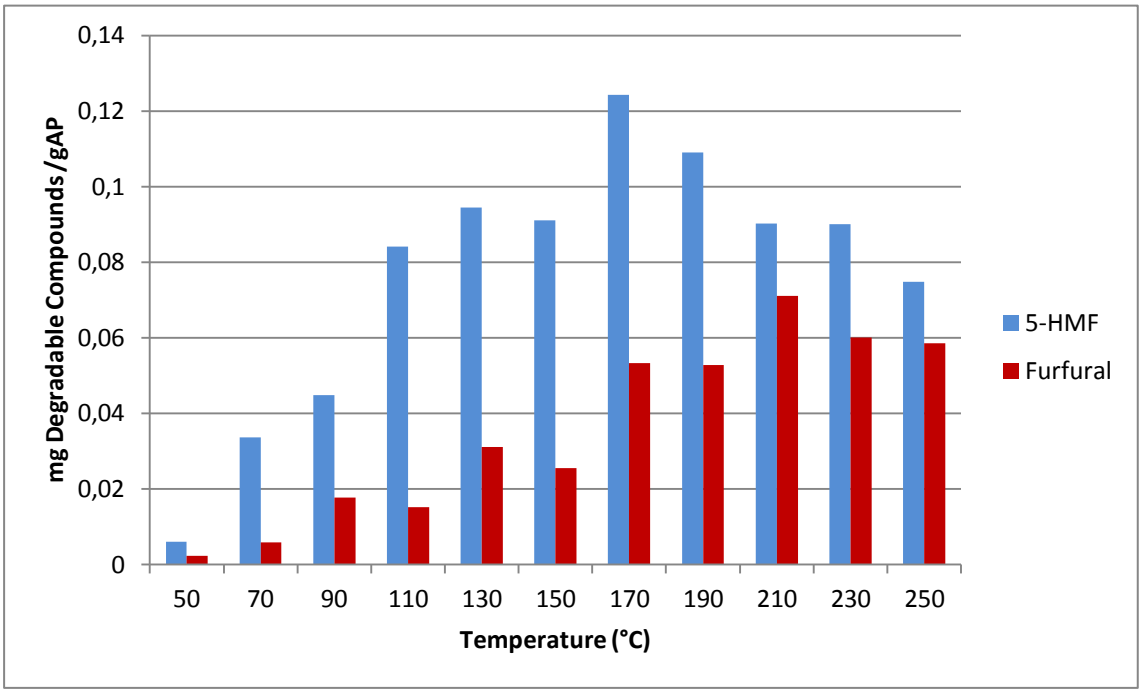


Figure 19. Variation of 5-HMF and furfural with increasing temperature

The results shows that recovered amount of those compounds is dependent on temperature. The higher amount of 5-HMF is achieved when biomass was subjected to subcritical water at 170°C decreasing slightly in the hydrolysate from 190°C. 5-HMF is also described as easily decomposable, yielding degradation products such as levulinic acid, formic acid. During reactions with higher temperatures (190°C-250°C) 5-HMF could be partially degraded into compounds mentioned above (it wasn't perform any test to identify its presence) shifting the maximum yield peak to 190°C.

The amount of furfural revealed to be substantially lower than amount of 5-HMFand increase with temperature, which means increasing amounts of pentoses are degradeted to form furfural.

3.2.1.4 Polyphenolic content of AP hydrolysates

This study focused on the valorization of AP residue through obtaining value-added products. As mentioned before, the sugars presents in the AP were used as carbon source to oleaginous and pigmented yeast, through the assimilation of these substrates will produce carotenoids and lipids.

Another pathway studied in this work for AP valorization was the extraction of phenolic compounds, which, as mentioned above has benefits for health and is widely used in pharmaceutical industry. So in this chapter was evaluated the potential of phenolic compounds in AP.

To evaluate the potential of AP hydrolysates in polyphenols, colorimetric analysis were performed on the same samples that were used in the previous study.

From the experimental data of Folin-Ciocalteu colorimetric test higher amounts of TPC were between 170°C and 250°C without significant differences (Figure 20).

The results show that the total phenolics compounds extraction is more efficient from the 170°C to 250°C, however, the Folin-Ciocalteu method does not allow the identification of individual compounds and tend to overestimate TPC due to interference of reducing substances such as sugars, aromatic amines, sulfur dioxide ascorbic acid, organic acids, as well as nonphenolic organic substances that react with the Folin-Ciocalteu reagent Its principal aim is to evaluate the potential of the material to perform later, if necessary, more rigorous analysis such as HPLC.

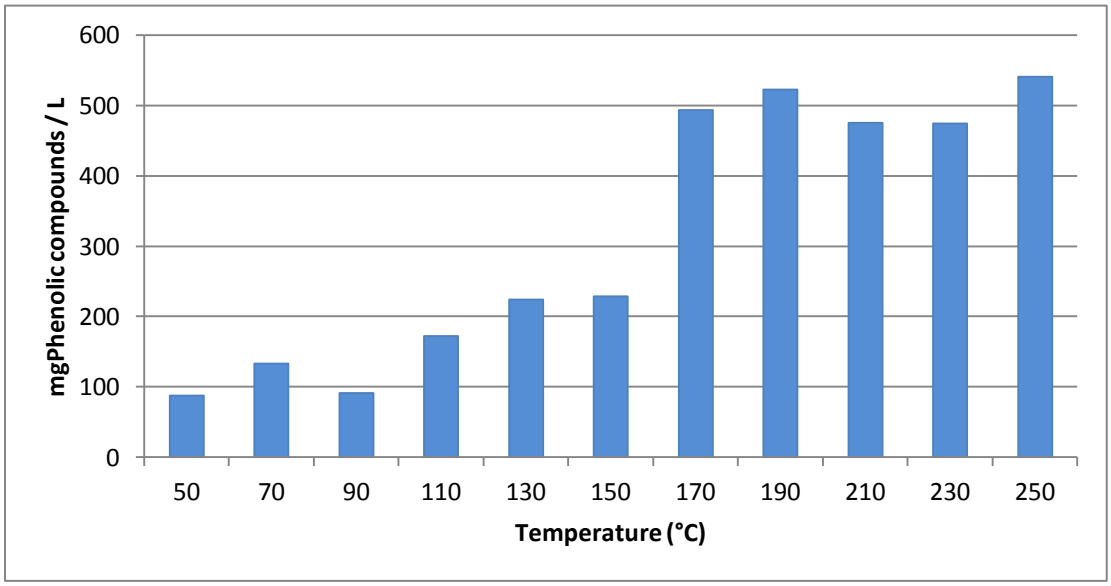


Figure 20. Amount of total phenolics compounds at different temperatures

3.3 Second assay

Unlike previous assay wherein the temperature gradually increased from 50°C to 250°C, in this second assay was evaluated each individual temperature, ie for each tested temperature, a new sample of AP residue was used and the assay was finished 20 minutes after reaching the desired temperature. Was only selected the temperatures that showed the best concentration of sugars (110°C-190°C)once the hydrolysate from these temperatures will be used as a substrate for growing oleaginous and pigmented yeast.

As might be expected, the mass of the water soluble compounds varies in the same way as in the previous assay, increase with the temperature up to 130°C (Figure 21). Regarding the concentration of total sugars, analyzed by a colorimetric method, it was found that the maximum value is reached at 130°C. Among the 150°C-190°C there is no major changes in the total sugars amount (Figure 22).

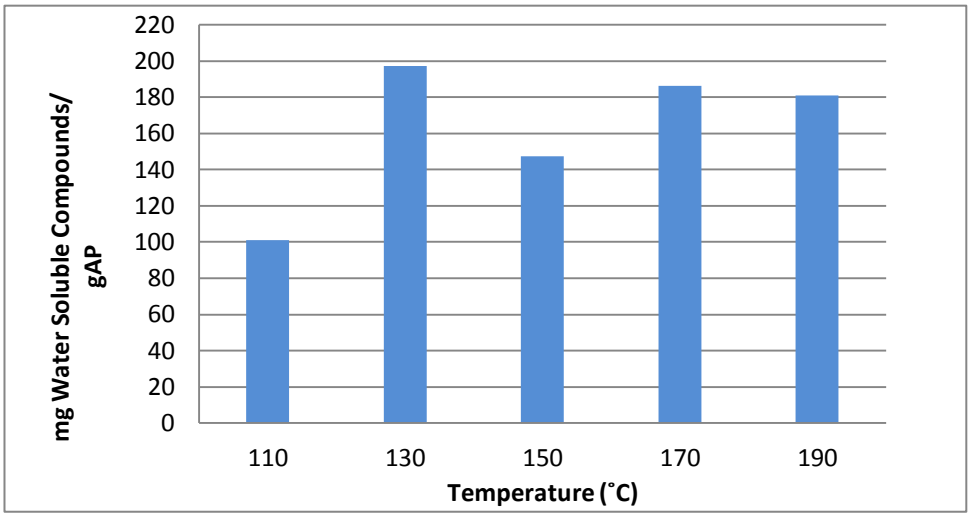


Figure 21. Amount of water soluble compounds at different temperatures

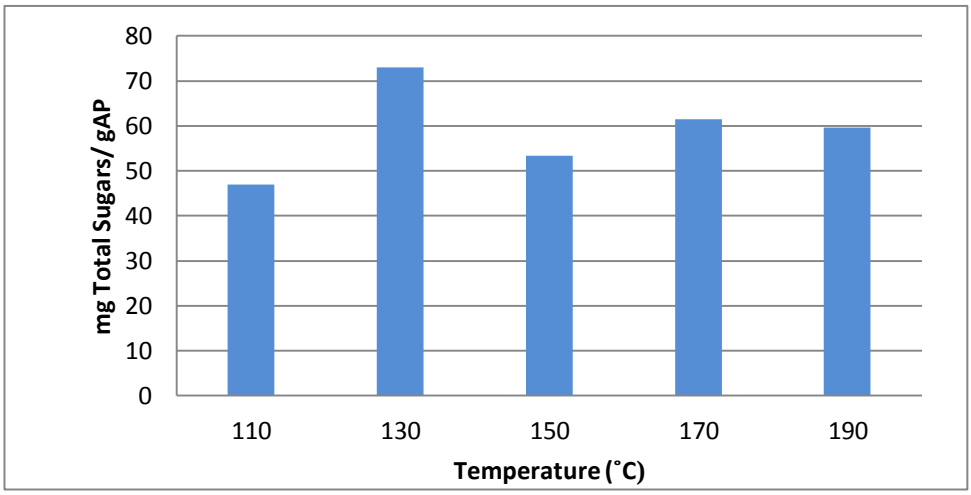


Figure 22. Amount of total sugars in different temperatures

HPLC analysis was performed in the lyophilized hydrolysates samples, to more accurate results. As it could be seen in the Figure 23 the composition of hydrolysis assays performed at 110°C it only occur fructose + arabinose and glucose. There was no xylose and galactose identified at 110°C. However with increasing temperature the amount of xylose + galactose also increases which means that higher amounts of hemicellulose are hydrolysed.

The amount of glucose varies differently along temperatures. Between 110°C and 130°C the amount of glucose increase, however at 150°C there is a decrease and subsequent increase from 170°C. At 150 °C the results suggests that this temperature is not sufficient to hydrolyze significant amount of hemicellulose. At higher temperatures glucose concentration increase suggests that there were hydrolysed sufficient amount of hemicellulose to achieve cellulose structures.

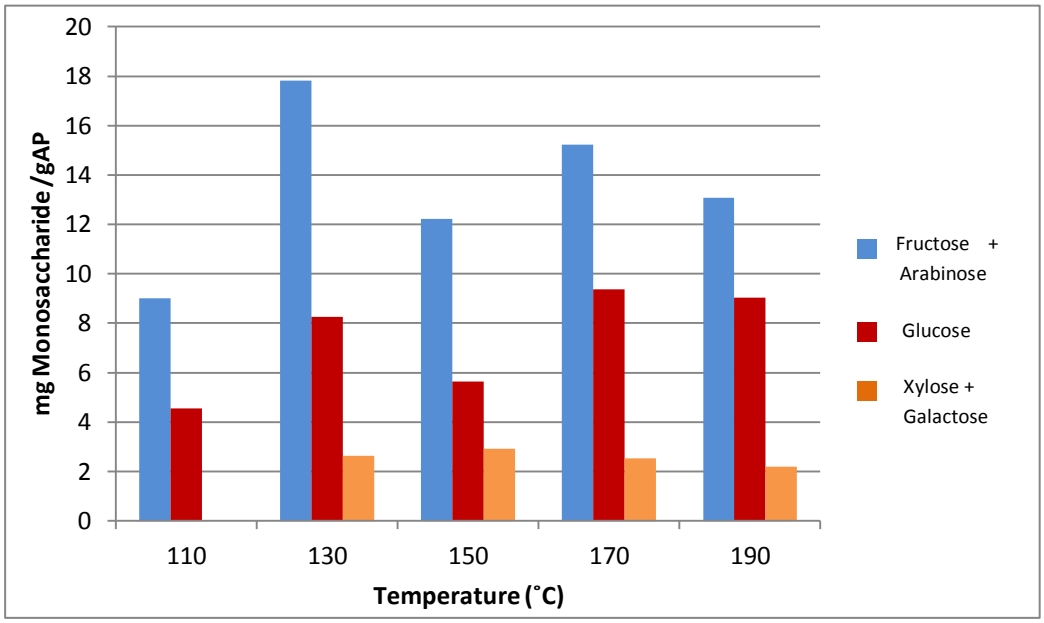


Figure 23. HPLC analysis of chemical composition of AP hydrolysates obtained at different temperatures

3.3.1 Degradable compounds in AP hydrolysates (second assay)

Since these compounds are potential inhibitors of the yeast growth it is important to identify these compounds in AP hydrolysate. There were performed HPLC analyses to identify the presence and amounts of 5-HMF and furfural in AP hydrolysates. The Figure 24 shows the variation of 5-HMF and furfural through the different temperatures.

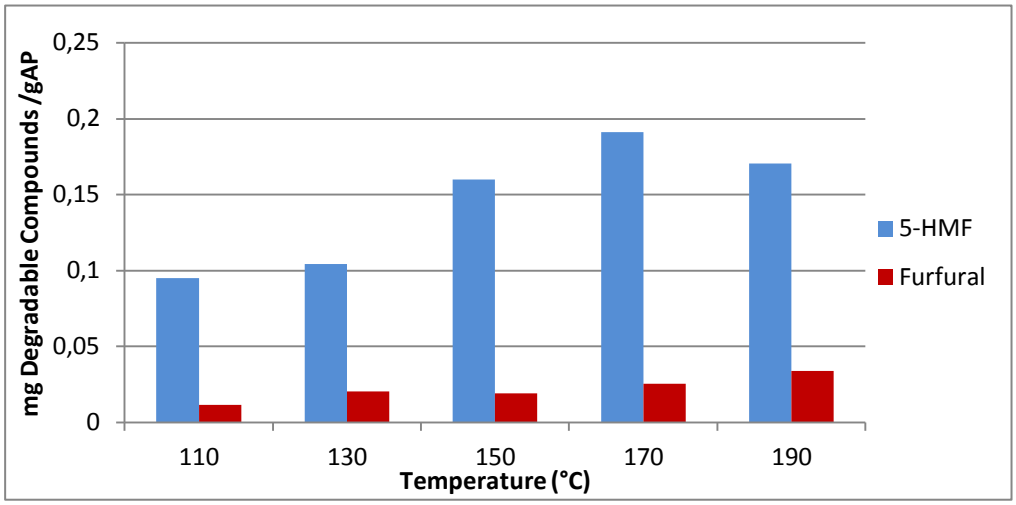


Figure 24. Amount of degradable compounds at different temperatures

The results of this analysis confirm the results obtained previously in the first assay. 5- HMF and furfural amounts are low and have the same behavior discussed in the previous chapter along the temperatures. As it was mentioned before, furfural concentration in hydrolysates increases with the temperature and the amount of 5-HMF showed to be higher into samples from the assays performed at 170°C-190°C.

Comparing these results with the variation of soluble compounds (Figure 21) and total sugars (Figure 22) over the temperatures it is possible to realize that at the same time as the concentration of degradable compounds increases the composition into water soluble compounds and total sugars decreases. This result can be explained since there is a balance between hydrolysis and decomposition of the AP residue.

3.3.2 Polyphenolic content of AP hydrolysates

The results obtained in this assay validate the results above, showing that higher temperatures are more effective in the extraction of phenolic compounds from AP (Figure 25).

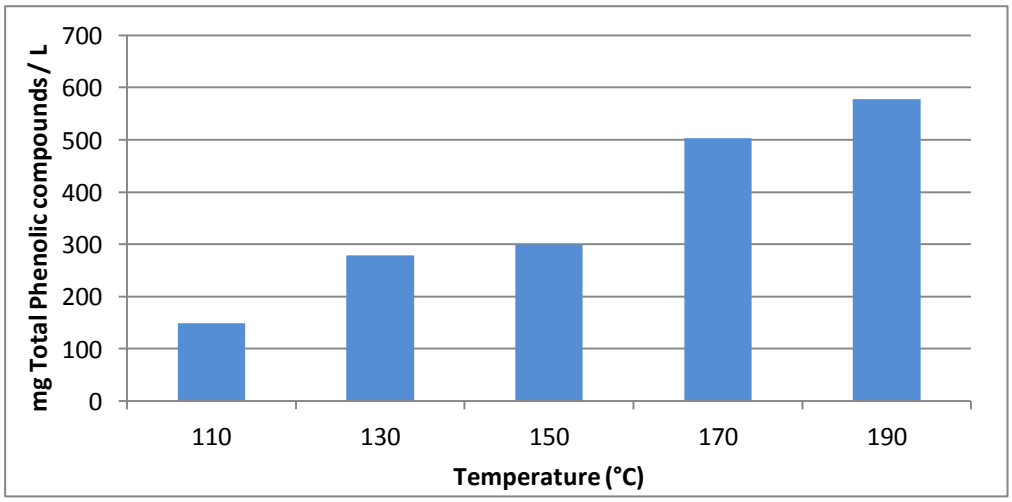


Figure 25. Amount of TPC at different temperatures

Overall, the extracts obtained by HCW were significantly higher than solvent extraction as shown in Table 9. Temperature is expected to have a significant effect on extraction efficiency. The TPC extraction by HCW presents advantage over traditional methods, such as high product quality due to the absence of solvents in the solute phase.

Table 9. Experimental results for TPC extraction with different methods

TPC extration method	TPC concentration (mg/mL)
Citric acid	6.8
Water:Acetone	18.6
Water:Ethanol	32.6
HCW (90°C-190°C)	14.9-58.4

3.4 Oleaginous yeast

The aim of this second part of the present work was to:
- Confirm the possibility of using AP hydrolysates as the unique carbon source to be assimilated by the pre-selected oleaginous yeast;
- Identify the best conditions yielding suitable AP hydrolysates to be used as a carbon source to be assimilated by the pre-selected species;
- Identify the yeast able to accumulate lipids and carotenoids;
- Quantify the carotenoids produced by yeast grown in the AP hydrolyzate.

For this study, yeast capable of accumulating lipids and produce carotenoids were chosen (Table 10). The selection of yeast was based on type of substrate from where they were isolated and carbon assimilation pattern already known.

Table 10. Yeast strains used in the pre-selection path of the work

Species names	Origin
Rhodosporidium babjevae	Leaves
Rhodosporidium toruloides	Wood
Rhodotorula glutinis	Sea water
Rhodotorula mucilaginosa	Water
Yarrowia lipolytica	Olives

Among these five species only *Yarrowia lipolytica* is non-pigmented and was selected because it is one of the most intensively studied oleaginous yeast and one that accumulate more lipids. The table 11 shows the ability of yeast to assimilate the monosaccharides and some disaccharides present in the AP hydrolyzate.

Due to limitations of the experimental set-up used to hydrolize AP, only small amount of each hydrolysate sample were obtained. Thus, growth experiments were carried out in small-scale trials.

Table 11. Assimilation of differents sugars by yeast (w-weak; d- delayed growth)

	Glucose	Galactose	Xylose	Arabinose	Fructose	Sucrose	Maltose	Cellobiose
R. mucilaginosa	+	+	+	+	+	+	+	+
R. toruloides	+	+	+	d	+	+	+	-
R. glutinis	+	+	+	+	+	+	+	d
R. babjevae	+	w	+	+	+	+	+	+
Y. lipolytica	+	d	+	+	+	-	-	-

3.4.1 Study of the potential of Apple Pomace hydrolysates as a carbon source for oleaginous yeast growth

Furfural and HMF is described in the literature as the two most important inhibitors in biomass hydrolysates that, depending on its concentration in the culture medium, can delay and reduce cells growth rate. Thus, the first assay focused on the potential of apple pomace hydrolysates as a carbon source for yeast growth.

For this assay, AP hydrolysates from 11 different hydrolysis temperatures: 50°C, 70°C, 90°C, 110°C, 130°C, 150°C, 170°C, 190°C, 210°C, 230°C and 250°C, (Figure 26) were used after lyophilization, blend (Figure 27) and homogenization (Figure 28). The amount of monosaccharides present was determined and adjusted to achieve a concentration of 12 g/L. To this end, the concentration of six most common monomers present in the AP (glucose, fructose, mannose, galactose, xylose and arabinose) was obtained the by HPLC. Concentration of 12 g/L corresponds to the sum of the six different monomers mentioned above. In parallel, a control assay was carried out containing 12 g/L glucose as carbon source. The main differences between both media are: proportion between monomeric carbohydrates, presence of di- trisaccharides or higher molecular weight sugars and possibly some 5-HMF and furfural or even other inhibitory compounds.

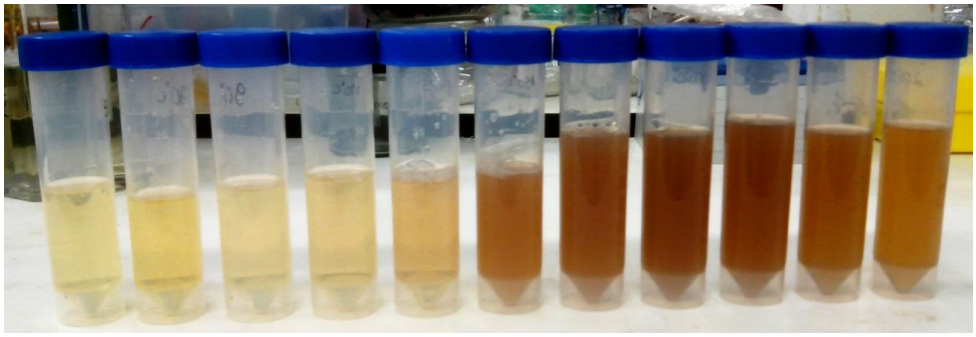


Figure 26. AP hydrolysate obtained at 11 different temperatures

Figure 27. Mixture of AP hydrolysates (after lyophilization) obtained from 11 different hydrolysis temperatures

Figure 28. Mixture of AP hydrolysates homogenized

As it could be seen by growth curves in the Figures 29, 30, 31, 32, 33 and 34 all five strains were able to growth on hydrolysates but *Rhodotorula mucilaginosa* and *Rhodotorula toruloides* achieved higher cell biomass.

The comparision of the growth on AP hydrolysate and on the same amount of glucose indicates that, without exception, yeast grew worst on AP hydrolysates. Since that yeast strains are able to assimilate all the five monosaccharides, this difference may be related, to the amounts of 5-HMF and furfural or other inhibitor produced during hydrolysis. The presence of other complex sugars can be other reason to the obtained results. In a heterogeneous and complex residue as apple pomace is, the hydrolysis reaction would never be complete and the final product would be composed by simple and complex carbohydrates and compounds resulted from its degradation. So, higher the variety of carbon sources which yeast are able to assimilate, the higher would be its probability to assimilate AP hydrolysates. Thus, another necessary study for better conclusions of these results is the influence of the presence of polysaccharides in the yeast's growth.

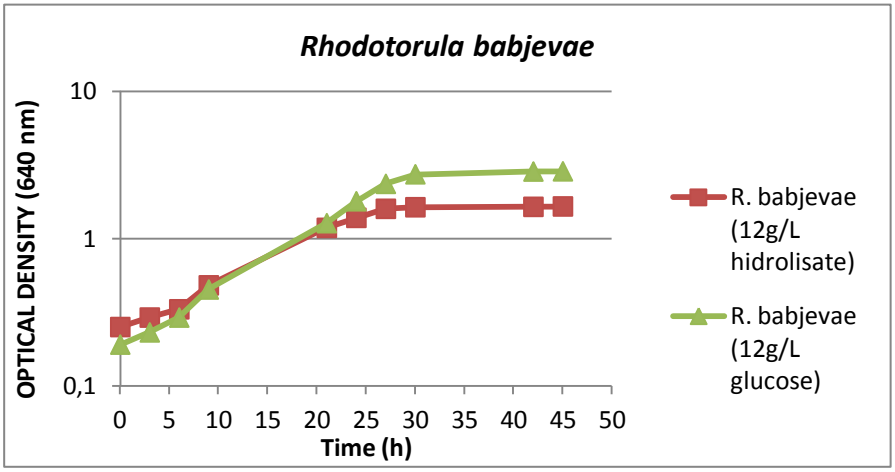


Figure 29. Growth curves of *Rhodotorula babjevae* in the in the presence of AP hydrolysates and glucose

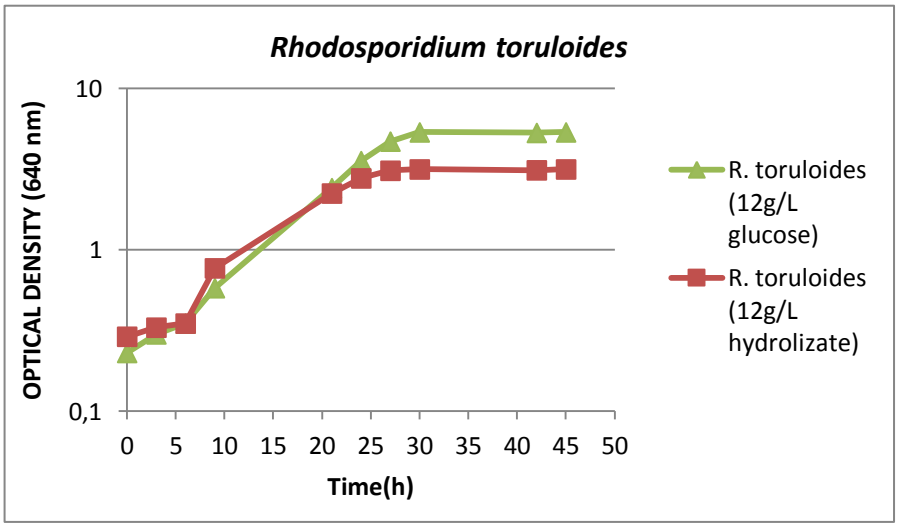


Figure 30. Growth curves of *Rhodosporidium toruloides* in the in the presence of AP hydrolysates and glucose

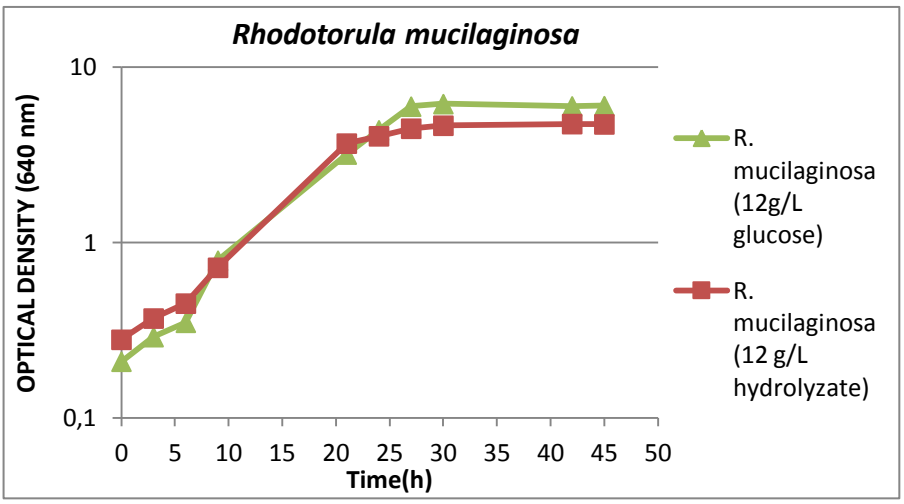


Figure 31. Growth curves of *Rhodotorula mucilaginosa* in the in the presence of AP hydrolysates and glucose

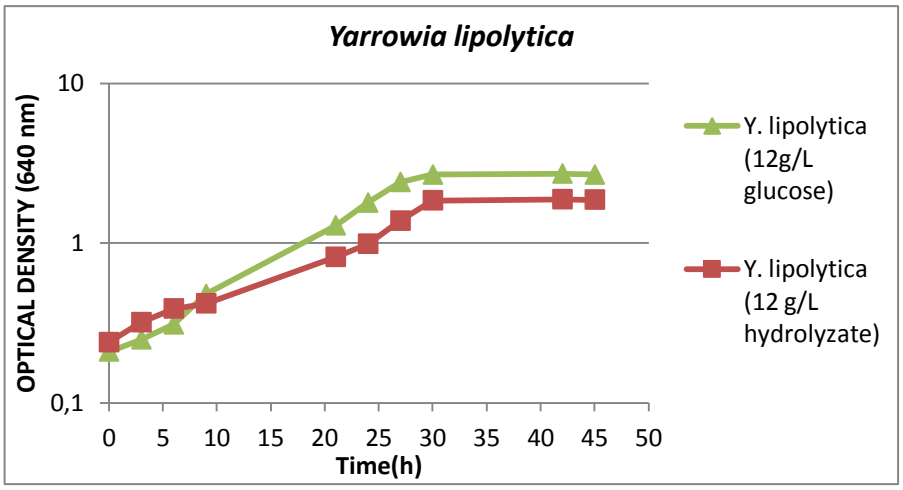


Figure 32. Growth curves of *Yarrowia lipolytica* in the in the presence of AP hydrolysates and glucose.

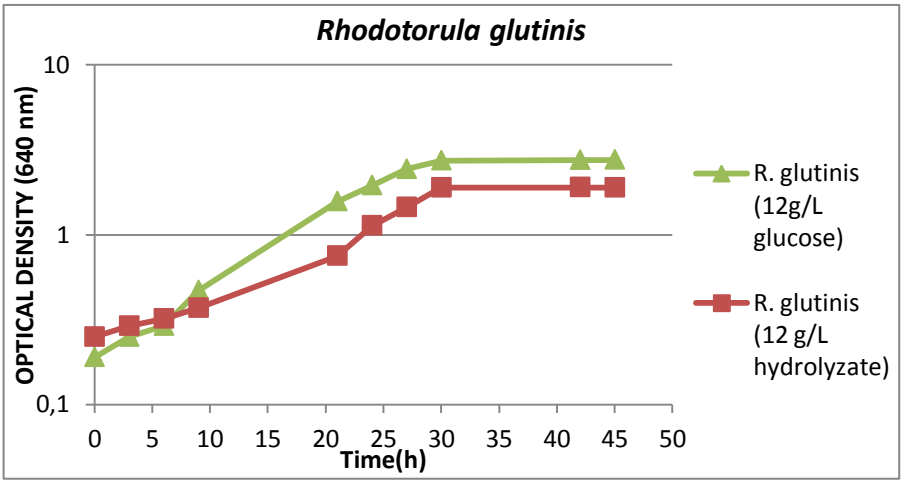


Figure 33. Growth curves of *Rhodotorula glutinis* in the in the presence of AP hydrolysates and glucose

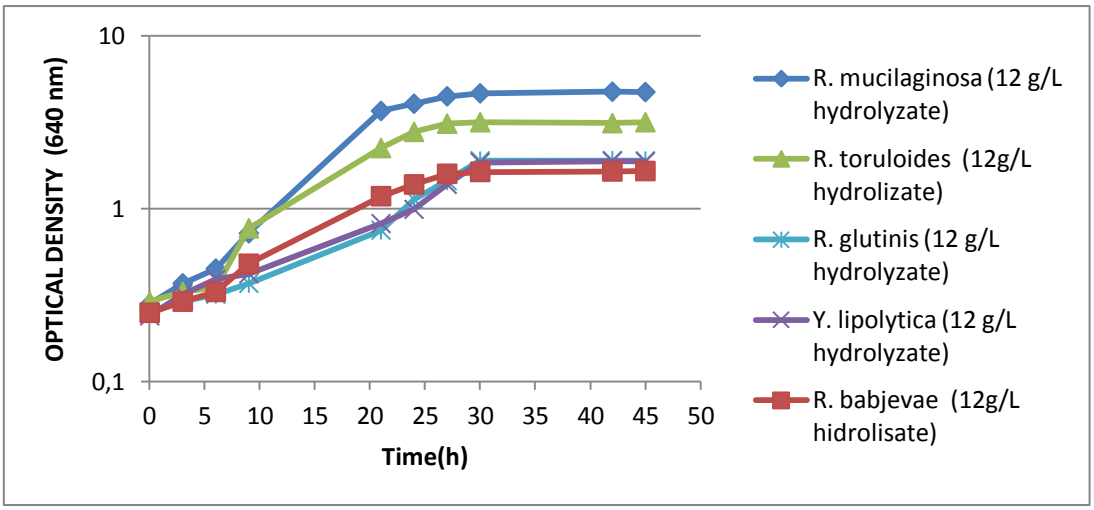


Figure 34. Growth curves of five yeast in the in the presence of AP hydrolysates

3.4.2 Study of lipid accumulation in yeast

The main goal of this work, as mentioned before, was the lipid production by yeast. Thus, this experiment was designed in three parts: determine the best fitted yeast for lipid accumulation on this type of hydrolysates, select the most adequate temperature to yield apple pomace, hydrolysates with higher sugars concentration, and finally, perform a scale-up trial with the selected yeast on the selected AP hydrolysate.

3.4.2.1Selecting the most efficient yeast to lipid accumulation

Thus a primary goal was studied the potential for lipid accumulation by the 5 yeast strains described above, using the AP hydrolysate prepared as described previously (Chapter 3.4.1). The medium was designed to promote lipid accumulation containing 70 g/L AP hydrolysate as the only carbon source. In parallel, a medium with 70 g/L glucose as carbon source was used as control.

The growth curves of each strain yeast are presented in the following Figures (35, 36, 37, 38, 39, 40).

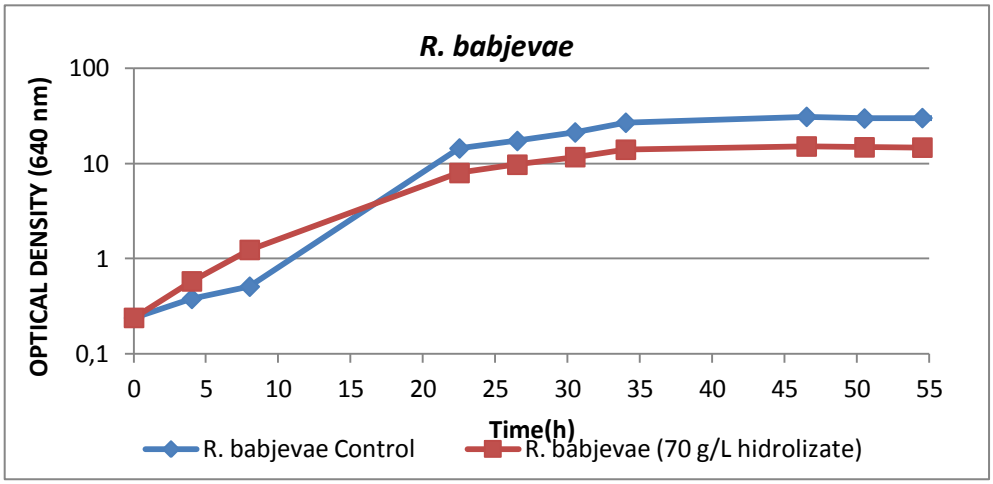


Figure 35. Growth curves of *R. babjevae* in the in the presence of AP hydrolysates and glucose

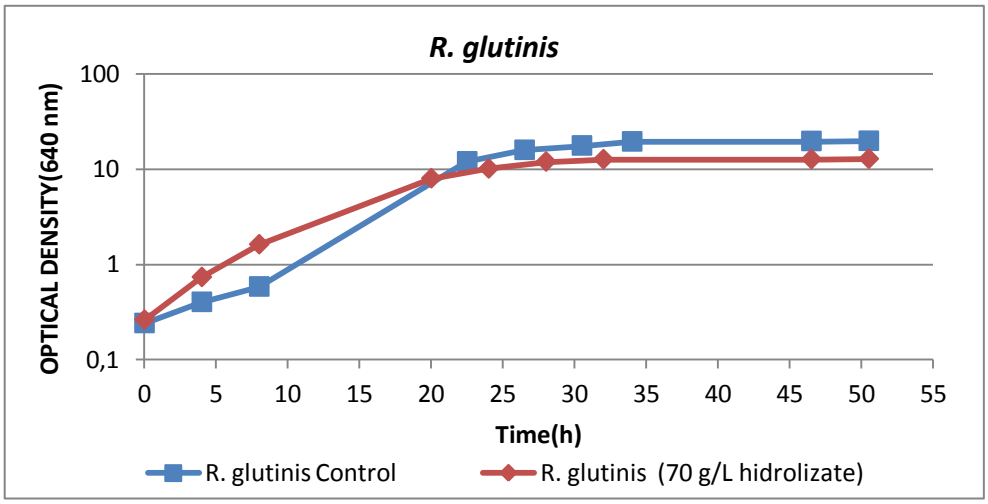


Figure 36. Growth curves of *R. gutinis* in the in the presence of AP hydrolysates and glucose

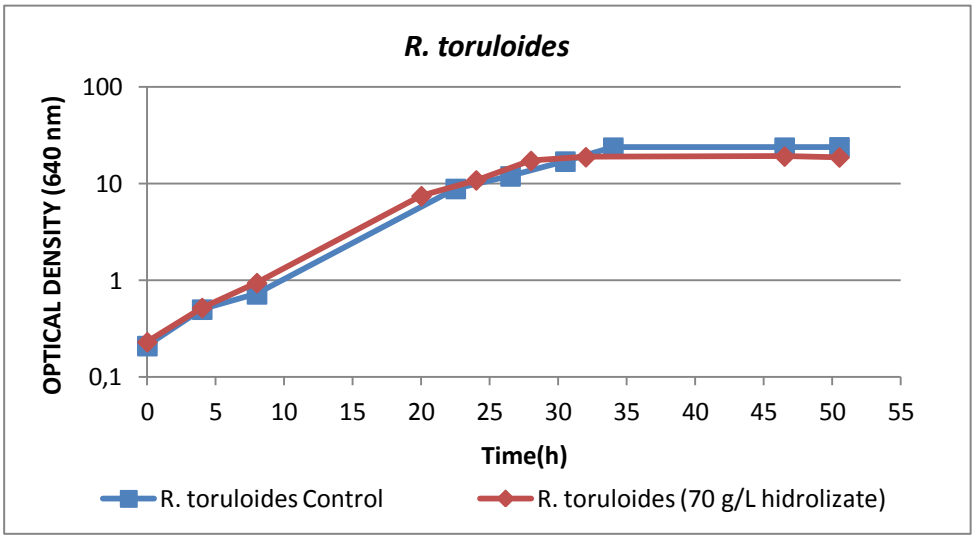


Figure 37. Growth curves of *R. toruloides* in the in the presence of AP hydrolysates and glucose

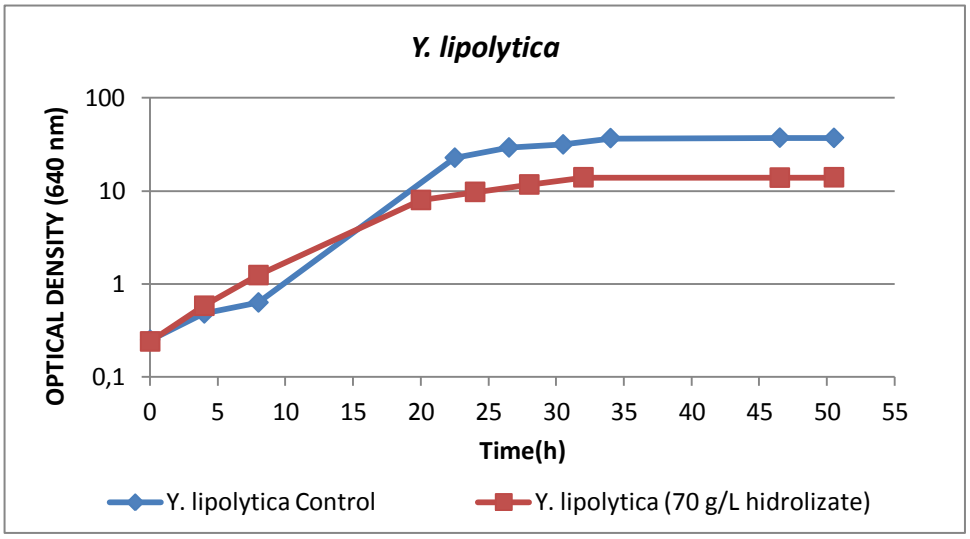


Figure 38. Growth curves of *Y. lipolytica* in the in the presence of AP hydrolysates and glucose

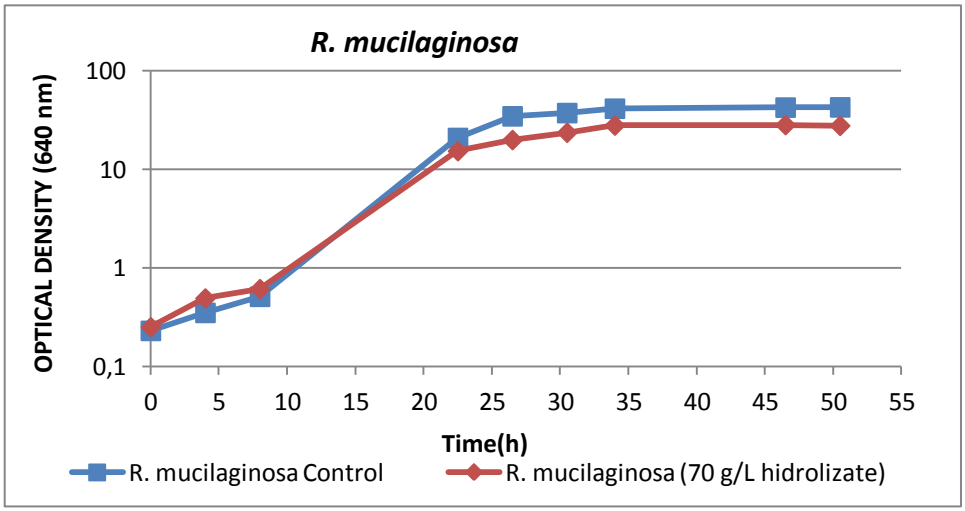


Figure 39. Growth curves of *R. mucilaginosa* in the in the presence of AP hydrolysates and glucose

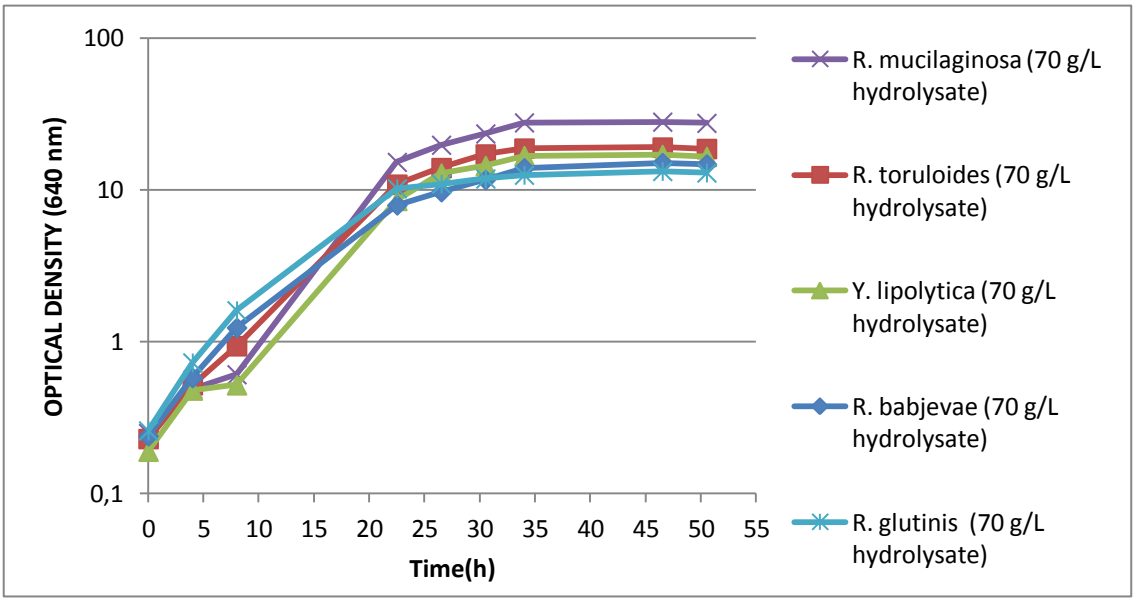


Figure 40. Growth curves of five yeast in the in the presence of AP hydrolysates

As it can be seen in the Figure 40 the strain that showed higher growth and cell biomass on the hydrolysate was *R. mucilaginosa*, followed by *R. toruloides* and *Y. lipolytica*. Although the presented growth curves on each substrate showed for the five yeast a better performance on glucose, the difference on both growth and total cell biomass are rather small for *R. mucilaginosa*, and *R. toruloides.* There are two main factors that can contribute to the observed diversity. The sensibility of the each yeast to the inhibitors eventually present and the capability of the each yeast to assimilate the higher molecular weight sugars not considered in calculation of added carbon.

For a better interpretation of these results, it would be necessary a further study to gather information about the assimilation of di- and trisaccharides and also more complex sugars by each yeast strain.

The lipid accumulation in the yeast cells was evaluated by fluorescence microscopy (Figures 41, 42, 43, 44 and 45). A rough estimation of lipid droplets present in each strain was attempted by counting the number of cells containing lipid droplets in a total 10 cells randomly selected from each of five captured images from integrated fluorescence microscope software. The following figures are representative images from lipid droplets (white dots within the cell) present in different strains, observed with 100x objective, and detailed cell expanded 70%.

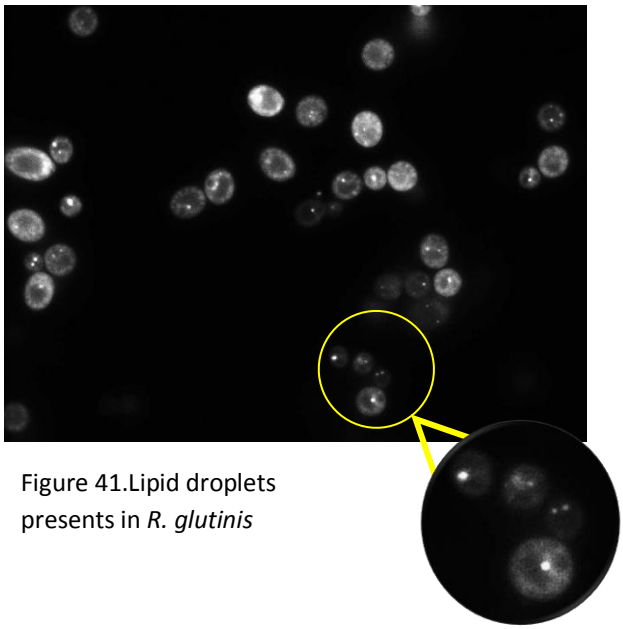


Figure 41.Lipid droplets presents in *R. glutinis*

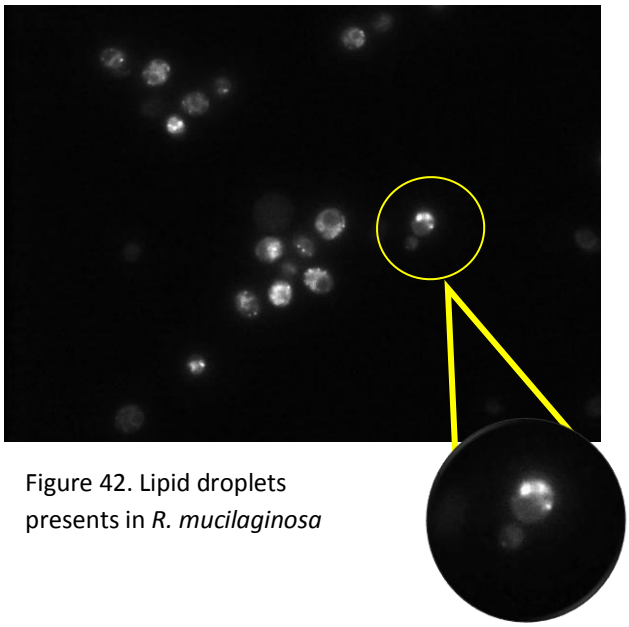


Figure 42. Lipid droplets presents in *R. mucilaginosa*

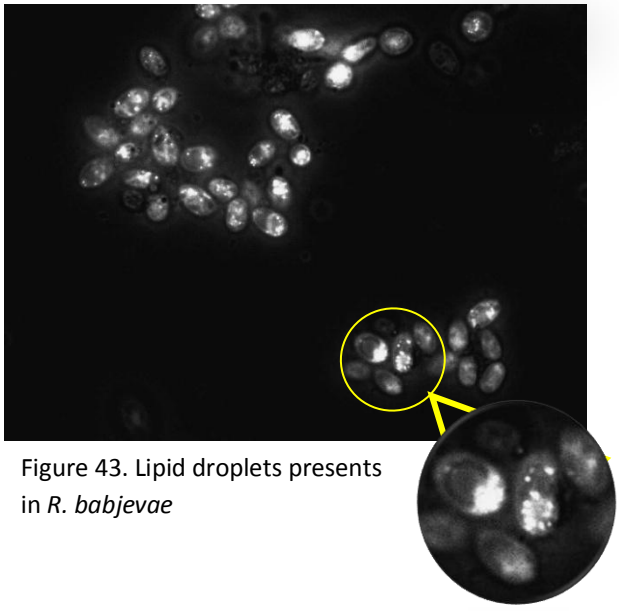


Figure 43. Lipid droplets presents in *R. babjevae*

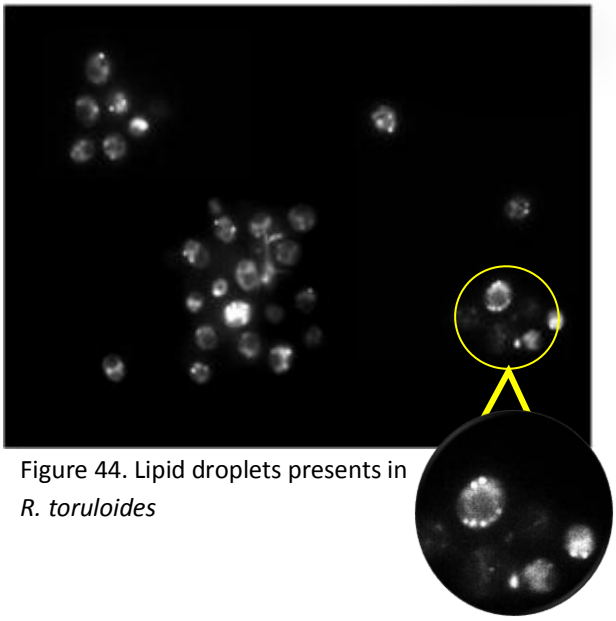


Figure 44. Lipid droplets presents in *R. toruloides*

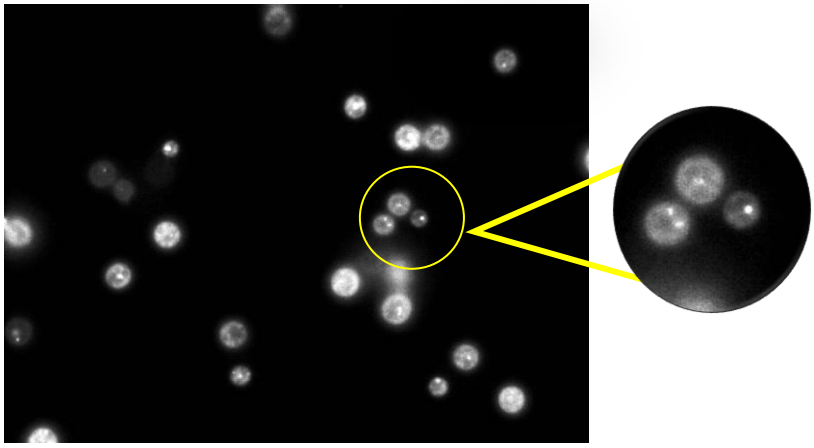


Figure 45. Lipid droplets presents in *Y. lipolytica*

Similar results were obtained for the five yeast species with approximately 90% of cells containing lipid droplets. Once the analysis was superficial, it will be necessary for a further analysis of this subject to extract, quantify and analyze the type of lipids. However, this method and this experimental small-scale allow the preliminary conclusion that, AP hydrolysate has the potential to be the carbon source used for oil production.

The results obtained by fluorescence microscopy not allowed a distinction between the performance of each strain in the accumulation of lipids, however, attending to the fact that the desired final products are essentially fatty acids accumulated in the cells, it is important to achieve high biomass yield that will consequently result in high fatty acids amount. Based on this premise, the strain that showed better performance when grown in AP hydrolysate, was *R. mucilaginosa* (Figure 40)and selected for the following experimental part.

3.4.3 Selecting the best temperature hydrolysis temperature

The second phase was to select the best hydrolysis temperature. AP hydrolysates obtained at 4 different temperatures, 130 °C, 150 °C, 170 °C and 190°C (Figures 46, 47, 48 and 49) were used as substrates to growth *R. mucilaginosa*. The medium was prepared as described above and the total amount of monosaccharides in each hydrolysate was determined and the growth medium were prepared with lyophilised powders in order to maintain the concentration of 70g/L (in carbon source) used in previous studies.

As it could be seen by growth curves in the Figure 50 the higher growth rates were reached in the presence of AP hydrolysate obtained at 130°C. It is also observed no significant difference in on yeast growth on the other temperatures. As observed in the chapter 3.3 at 130°C was obtained a higher amount of total sugars (Figure 22) and total monosaccharides (Figure 23).

The growth rates in the presence of hydrolysates are lower than in the presence of glucose (control) as previously seen. This result may be due to the different proportions of monosaccharides, the presence of other complex sugars and inhibitors presence in AP hydrolysate. For a better analysis of all the results, it would be necessary, as already mentioned in the previous chapter, to conduct a further study on the assimilation of monomers by *R. mucilaginosa* and more complex sugars and influence of 5-HMF and furfural.

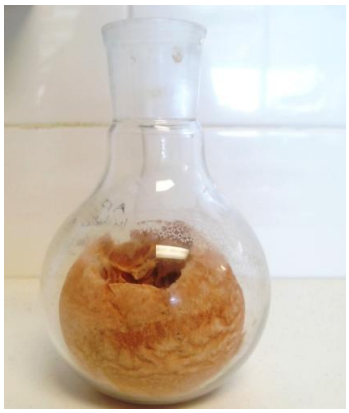


Figure 46. AP hydrolysate 130°C

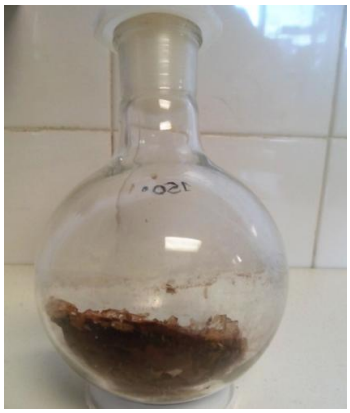


Figure 47. AP hydrolysate 150°C

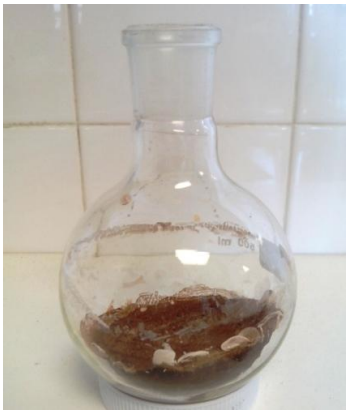


Figure 48. AP hydrolysate 170°C

Figure 49. AP hydrolysate190°C

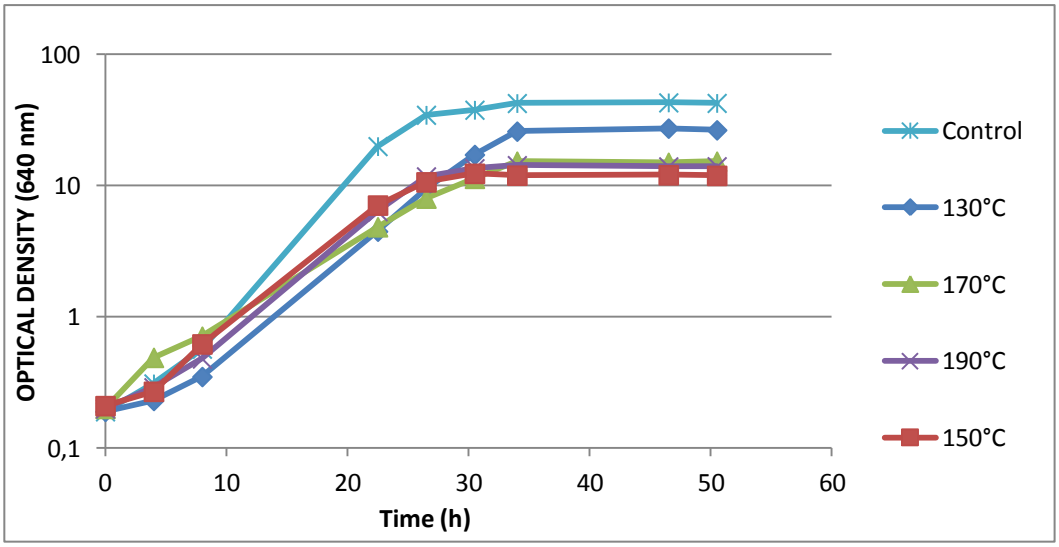


Figure 50. Growth curves of *R. mucilaginosa* in the in the presence of four AP hydrolysates an glucose

The lipid accumulation in the cells was also evaluated in this assay; the analysis was performed as in the previous chapter. As expected, all assays were positive for the lipids droplets (Figures51, 52, 53 and 54) and for all assays the percentage of cells presenting droplets was about 90%.

Comparing these results with the results of the previous chapter where *R.mucilaginosa* has grown in a mixture of AP hydrolysate from the hydrolysis reactions at 11 different temperatures it was noticed larger and higher numbers of droplets, for a more accurate analysis of these results would be necessary a detailed analysis of LD in each cell through an image editing software, for which this work could not be obtained.

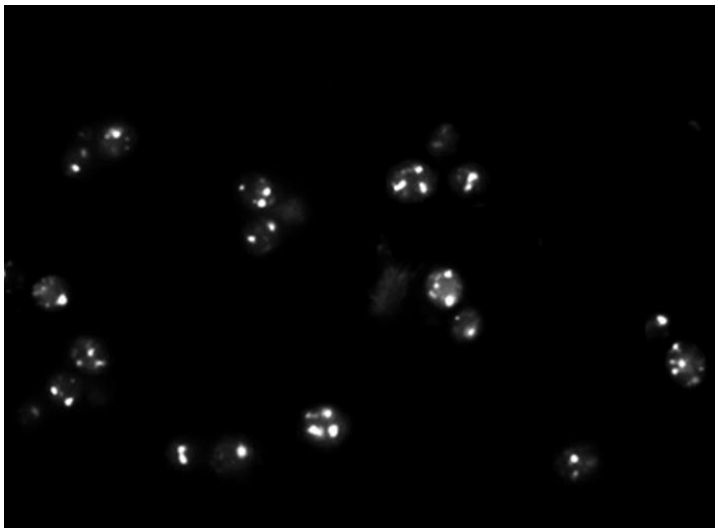


Figure 51.Lipid droplets in *R.mucilaginosa* growth in 130°C AP hydrolysate

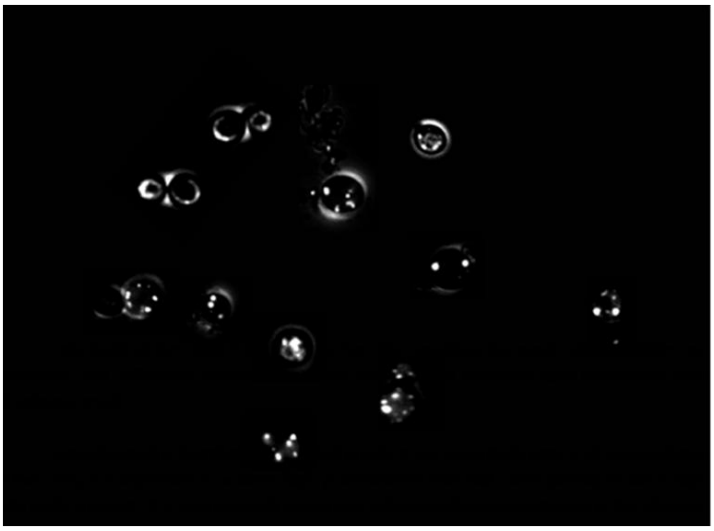


Figure 52.Lipid droplets in *R.mucilaginosa* growth in 150°C AP hydrolysate

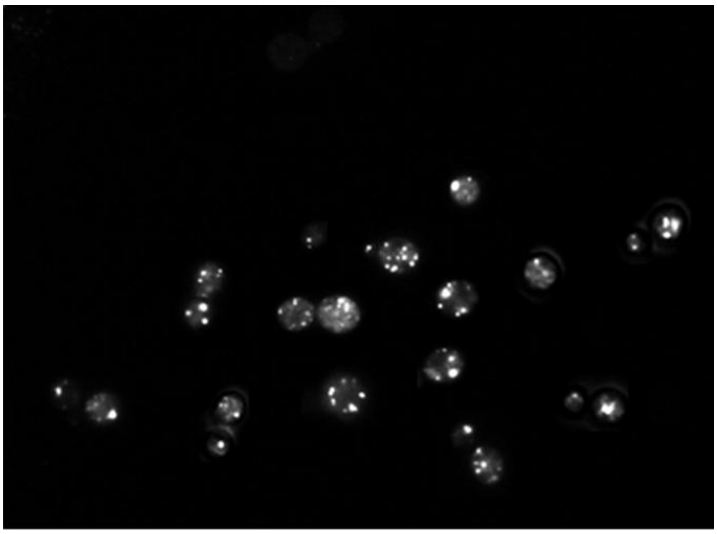


Figure 53.Lipid droplets in *R.mucilaginosa* growth in 170°C AP hydrolysate

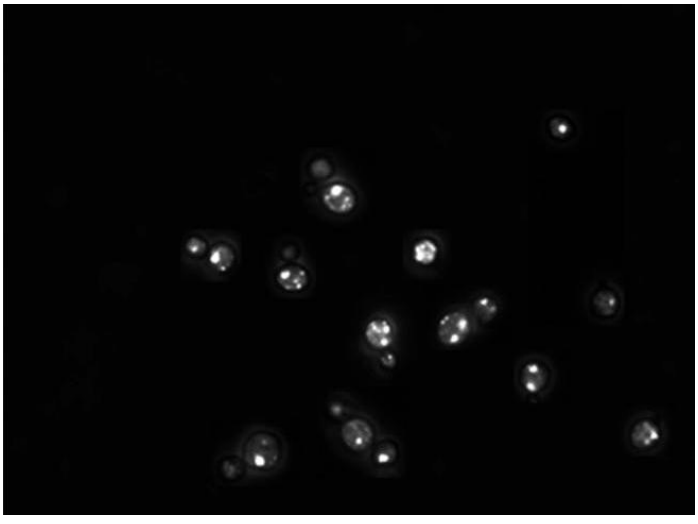


Figure 54. Lipid droplets in *R.mucilaginosa* growth in 190°C AP hydrolysate

The main goal of this assay was to analyze which AP hydrolysate provide the best growing conditions and lipid accumulation by *R.mucilaginosa*, to proceed to a scale up. Comparing the results from the R. *mucilaginosa* growth rate in different AP hydrolysates (130°C, 150°C, 170°C and 190°C) with lipid accumulation occurred in each assay, it was possible to select the best hydrolysis temperature that provides the best conditions for that purpose. It is known that the quantity of biomass directly influences the amount of accumulated lipids, then for further study, we selected the AP hydrolysate obtained by hydrolysis at 130°C.

Another important factor to consider when promoting a scale up trial is the economic viability of the process, and the higher the temperature supplied to the system, the higher energy costs and reaction time. Thus, AP hydrolysate obtained at 130°C is shown to be most advantageous at all levels.

3.4.4 Scale up

In this study, was used a volume 10 times higher than that used in the previous assays (1 mL), The conditions of culture media were identical to those already described. Again, the total amount of monosaccharides present were determined in order to maintain the concentration of 70 g/L. In parallel there was a control assay where the carbon source was glucose (70 g/L).

These results are satisfactory and show the great potential of AP as a substrate for growing oleaginous and pigmented yeast. The results observed in this assay (Figure 55) were quite similar to those obtained in previous assays, validating the results.

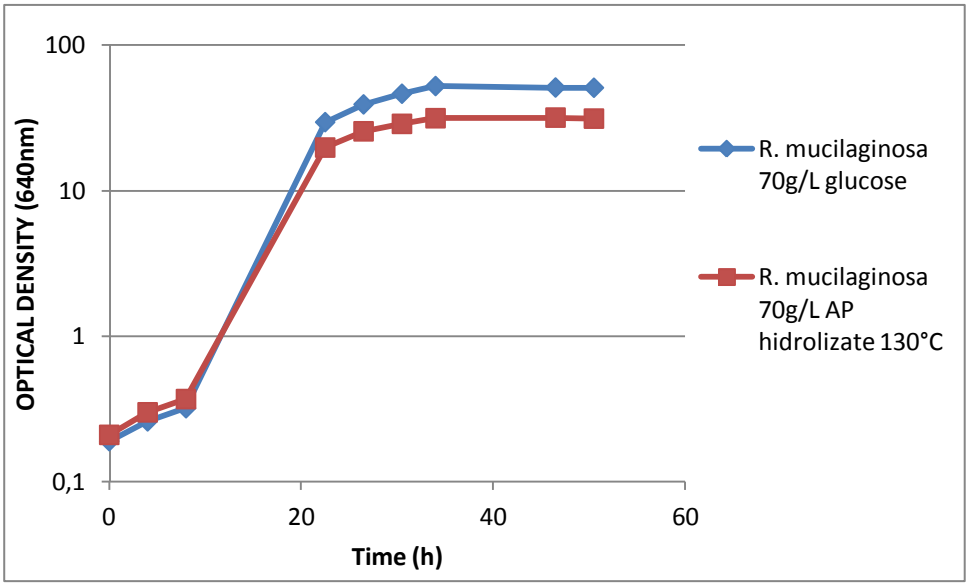


Figure 55. Growth curves of *R. mucilaginosa* in the in the presence of AP hydrolysate (130°C) and glucose

Regarding the lipid accumulation, a different accumulation pattern was observed. In the previous assay (3.4.2 and 3.4.3) the LD were small and in large quantity within a single cell, in this case, most cells exhibit only one droplet but apparently larger (Figure 56 and 57). This occurs for the control assay and for AP hydrolysate assay. Moreover, as occurred in all assays all cells had droplets. It is known that lipid contents of oleaginous yeast are generally influenced by the growth conditions, especially the carbon source, the nitrogen source, minerals, and aeration. Increasing the volume of culture medium and the use of an Erlenmeyer flask instead of a tube can have promoted a better aeration. In the future, exploration of other conditions might be an important step to maximize lipid production by oleaginous yeast. So far, the most commonly used carbon source for single cell oil production is glucose. Therefore, it is very important to use a low cost raw material instead of glucose in order to reduce the cost of single cell oil production, thus this method and this experimental scale allow the preliminary conclusion that AP hydrolysate has great potential for this purpose.

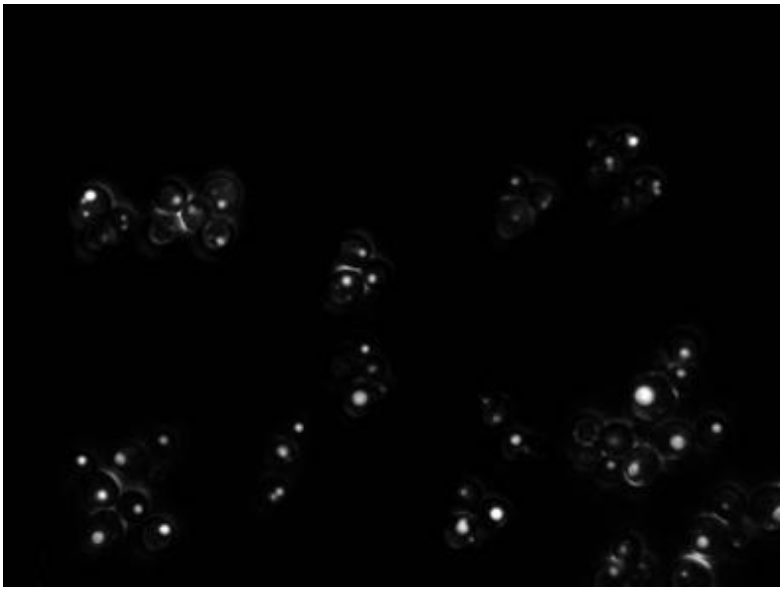


Figure 56. Lipid droplets presents in *R. mucilaginosa* (AP hydrolysate)

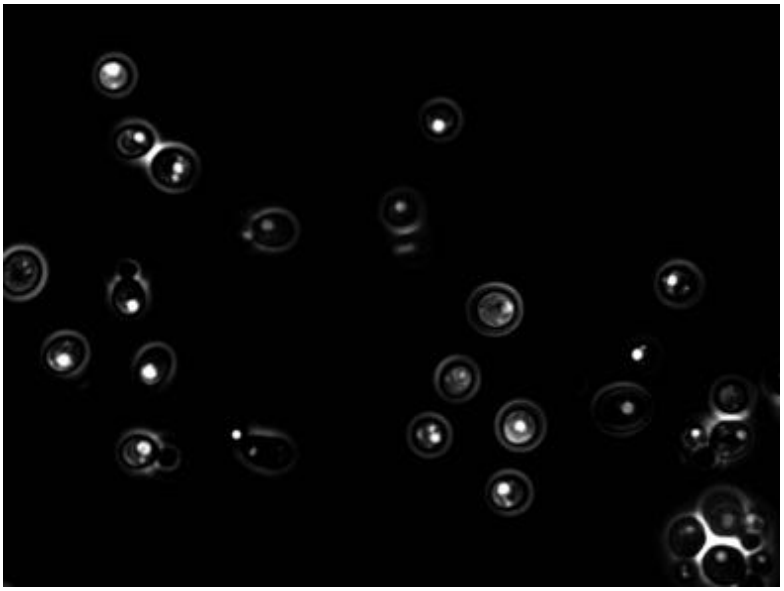


Figure 57. Lipid droplets presents in *R. mucilaginosa* (Control assay)

3.4.5 Quantification of lipid extract produced by *R. mucilaginosa*

3.4.5.1 Accumulated biomass

The lipid extraction was performed after obtaining the dry biomass (by cells lyophilization) accumulated in the previous assay (Figure 58). In control assay, was obtained 286 mg of cell dry weight and in the assay with AP hydrolysate as carbon source was obtained 197 mg of cell dry weight.

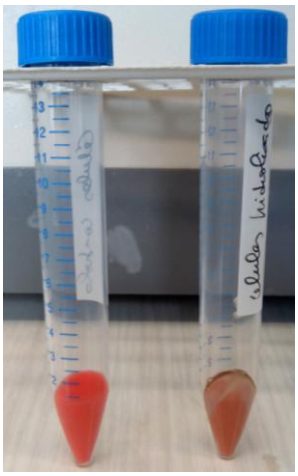


Figure 58. Lyophilized cells to carotenoids quantification (Control and AP hydrolysate assays)

The lipids were extracted using hexan as solvent Figure 59. In control assay, was obtained 69.27 mg of lipids extract and in the assay with AP hydrolysate as carbon source was obtained 22.15 mg of lipids extract, corresponding to a percentage equal to 24.22 % (cell dry weight) to control and 11.24 % (cell dry weight) for the assay using AP hydrolysate.

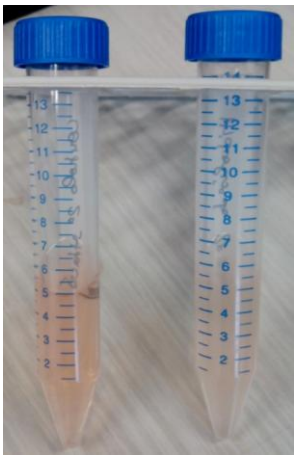


Figure 59. Lipids extration using hexan (Control and AP hydrolysate assays)

Quantification of total carotenoids was achieved by UV-vis spectrometry directly from the lipids extracted with hexan samples and analyzed. As expected, yeast grown in glucose has a higher content of carotenoids equal to 9.31µg carotenoids/g cell dry weight, while carotenoids extracted from yeast grown in presence of AP hydrolysate has a content equal to 7.02µg carotenoids/g cell dry

weight. These results were lower than those found in the literature (152 µg/g) (Maldonade, *et al*., 2012). Studies shows that the culture medium and conditions such as temperature, pH and oxygen have influence on the yeast biomass accumulation and carotenoids production (Naghavi, *et al*., 2014). Thus it is necessary to optimize the culture conditions so that yeast can accumulate carotenoids at much higher levels and have a greater commercial potential.

3.4.6 Quantification of β-carotene produced by *R. mucilaginosa*

HPLC analysis was carried out to determine the content of β-carotene in the extracts. The β -carotene is the most studied carotenoid and has an important role in the context of nutrition and medicine. This carotenoid, which confers many fruits and vegetables its yellow pigmentation, plays an important physiological role, being converted into vitamin A (retinol) and retinoic acid in human body.

In control assay, was obtained 5.11µg β-carotene/g cell dry weight and in the assay with AP hydrolysate as carbon source was obtained 2.31µg β-carotene/g cell dry weight, corresponding to a percentage equal to 54,8% of total carotenoids in control and 33% for the assay using AP hydrolysate. These results are lower than those found in the literature. Many studies shows that the conditions for the bioproduction of carotenoids, as cultivation, aeration, pH, inducing and inhibiting substances have important roles in the activity of formation and composition of carotenoids. However, this was a preliminary assay to determine the ability of yeast to produce carotenoids having as substrate the AP hydrolysate.

3.5 Future work

To obtain reduced sugars and explore all potential of AP as carbon source for oleaginous yeast grown it would be important promote the enzymatic hydrolysis after pre-treatment of AP residue with HCW technology.

It is also important: scale up the hydrolysis reaction apparatus to minimize the effect of morphological and chemical heterogeneity of the residue on the experimental data; perform HPLC analysis to identify the phenolic groups extracted by HCW and analyze the antioxidant potential of phenolic compounds extracted.

Regarding the assays with oleaginous yeast it would be important: optimizing growth conditions of the yeast, studies of toxicity of AP hydrolysates, essentially for furfural, 5-HMF; verify lipid accumulation by yeast and analyzing its composition in TAG's, for instance, by GC analysis.

CONCLUSIONS

4. Conclusions

The main goal of the present work was the valorization of an agro-industrial residue, the apple pomace (AP) using HCW technology. Two main pathways to AP valorization was studied, the extraction of value-added by-products such as antioxidants and the hydrolysis of complex polysaccharide structures. Antioxidants are extensively used in the food, cosmetic and pharmaceutical industry, whereas by hydrolysis with HCW results a sugar-reach medium for yeast growth and production of lipids and carotenoids that can be used in the food, cosmetic and pharmaceuticals industry. The main goal is to integrate these two pathways into one continuous "green" process, HCW that represents an environmentally friendly solvent and an attractive reaction medium for several applications.

The best extraction yield of total phenolic compounds (proved through Folin-Ciocalteu tests) was achieved at higher temperatures, from 170°C (100bar) and the identification of the phenolic compounds presents in AP residue represents one of the main topics of the future work.

Hydrolysis reaction performed at eleven different temperatures showed that the amount of water soluble compounds (sugars, 5- HMF and furfural) varies with temperature. Between 130°C and 190°C recovered water soluble compounds are higher then those recovered by other temperatures (50°C-110°C and 210°C-250°C). This fact was attributed to the tenuous balance that exists between hydrolysation and decomposition of biomass during reaction. At higher temperatures (210°C-250°C) was obtained a smaller amount of water soluble compounds, total sugars and monosaccharides, whereas the amount of 5-HMF and furfural were higher for these temperatures. This means that in terms of recovered mass yield there is no need to work at higher temperatures turning the process economically more advantageous.

On the other hand the main goal of hydrolysis of AP was obtain a substrate to been used as carbon source to the yeast growth. Final composition of hydrolysates in terms of monosaccharides obtained at different temperatures, show that between 110°C and 190°C was obtained higher amounts of the monosaccharides.

Between the five yeast tested to assimilate the AP hydrolysate as carbon source the best result was achieved with hydrolysates collected at 130°C by *Rhodotorula mucilaginosa.* Comparing the results obtained in control, where the carbon source was glucose and the results obtained when the carbon source was de AP hydrolysate it is possible to conclude that (despite the lower growth) yeast can assimilate more complex structures such as disaccharides, trisaccharides and even more complex compounds (presents in AP hydrolysate). It is necessary to perform more test of the chemical composition of AP hydrolysates, essentially to identify the presence of toxic compounds that could prevent yeast growth.

The final objective of this work was to obtain lipids and carotenoids produced by yeast. The microscopic analysis of the yeast after growth in medium to lipids accumulation showed that all five strains accumulated lipids after using AP hydrolysate as carbon source.

R. mucilaginosa produced carotenoids using AP hydrolysate as a carbon source, with satisfactory results (9.31µg carotenoids/g cell dry weight to control and 7.02µg carotenoids/g cell dry weight to AP hydrolysate) the quantification of β-carotene showed that 33% of total carotenoids produced by *R. mucilaginosa* grown in the AP hydolysate are β-carotene.

The process of AP valorization proposed in the present work was performed at laboratorial scale and several steps must be optimized. Experimental data proves the viability of this process, adding value to apple pomace, an underexploited byproduct.

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APPENDIX

Appendix I

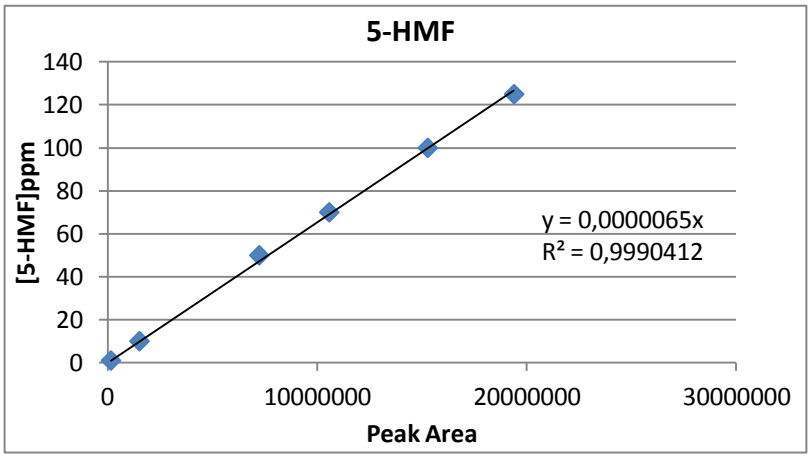

Figure 60. Calibration plot for 5-HMF

Appendix II

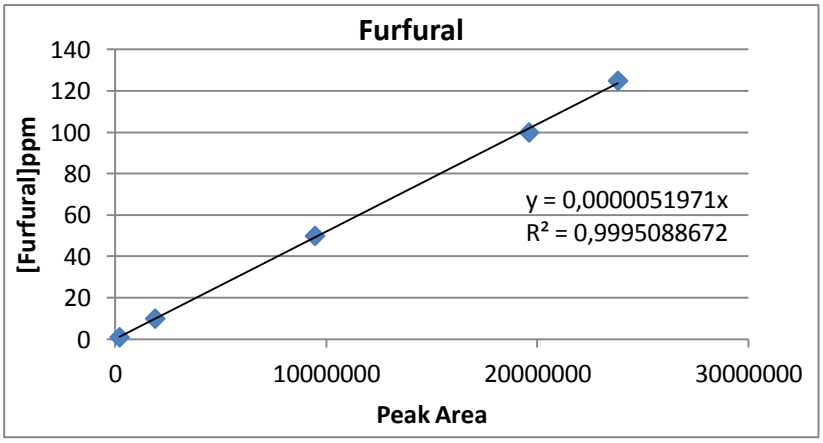

Figure 61. Calibration plot for Furfural

Appendix III

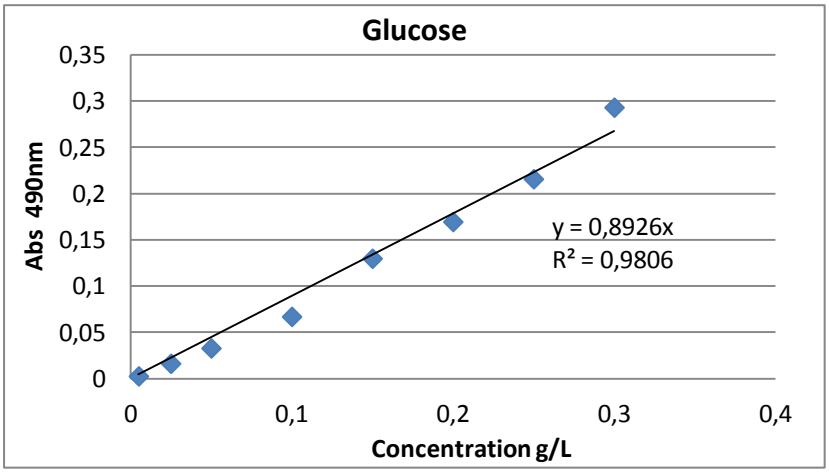

Figure 62. Calibration plot for glucose

APPENDIX IV

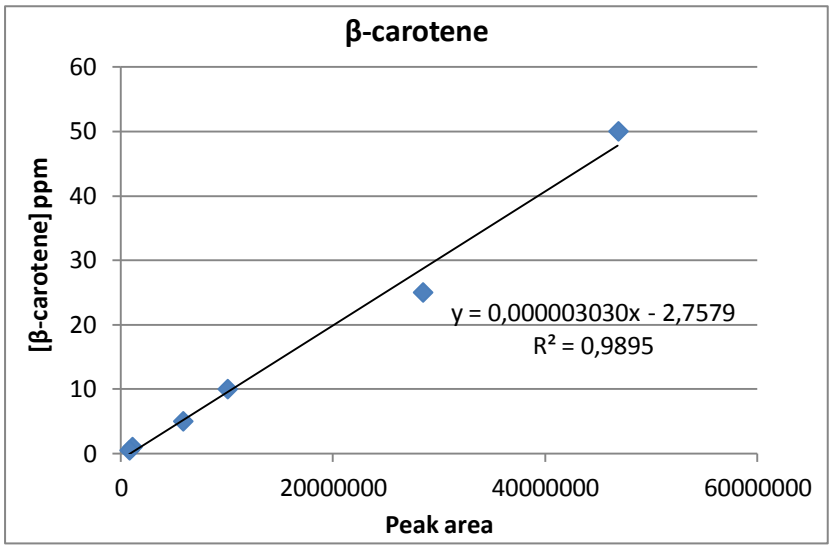

Figure 63. Calibration plot for β-carotene

APPENDIX V

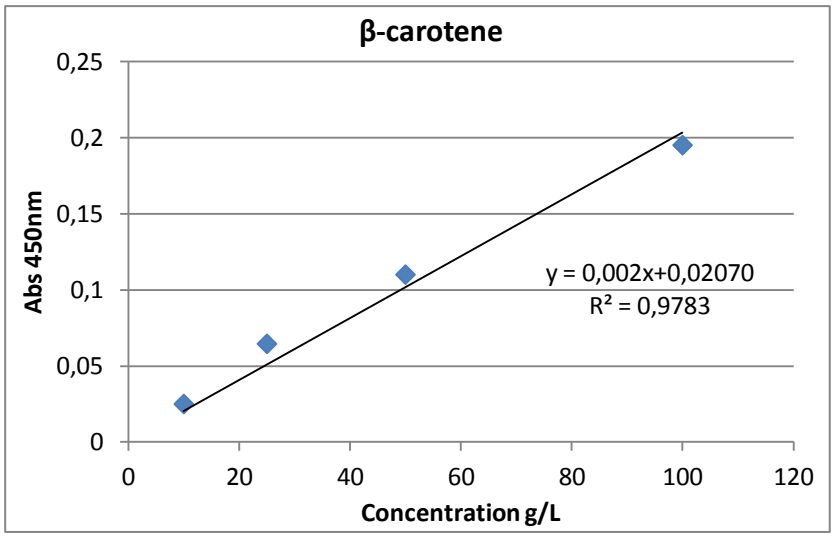

Figure 64. Calibration plot for β-carotene (carotenoids quantification)