Diogo Rendeiro Figueira

Licenciado em Bioquímica

Valorization of agro-industrial waste through chemical and microbiological approaches

Dissertação para obtenção do Grau de Mestre em

Biotecnologia

Orientadora: Professora Doutora Susana Filipe Barreiros, FCT-UNL & LAQV

Coorientadora: Professora Doutora Madalena Salema Oom, ISCSEM & UCIBIO

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro de 2017

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Resumo

O principal objetivo desta tese foi a utilização do bagaço de uva (GP) como fonte de hidratos de carbono para o crescimento de leveduras e posterior produção de lípidos, e de carotenóides, conseguindo assim a valorização de um resíduo agroindustrial muito importante. Para esse efeito, a tecnologia de água subcrítica (SBW) foi utilizada como um tratamento para superar a resiliência da matriz lignocelulósica do GP e despolimerizá-la em estruturas mais elementares. Foram realizadas algumas experiências de crescimento de estirpes de levedura no extrato de GP resultante do licor rico em hidratos de carbono gerado durante o tratamento com SBW, em condições propícias à acumulação de lípidos e produção de carotenóides.

As experiências de extração / hidrólise com SBW foram realizadas a uma pressão constante de 70 bar, com um caudal de água de 10 mL / min e temperaturas máximas entre 190 ° C e 240 ° C. Foram utilizados dois programas de temperatura, nomeadamente um em que a temperatura foi aumentada continuamente para a temperatura alvo, e outro consistindo numa série de passos, cada passo consistindo numa rampa seguida de um patamar, visando o fracionamento de GP em extratáveis (patamar a 130 ºC), constituintes da hemicelulose (patamar a 190 ºC) e glucose ou gluco-oligossacarídeos provenientes da celulose (patamar a 240 ºC).

Os melhores resultados foram obtidos num ensaio por patamares atingindo 240 ºC. Nessas condições, a eficiência de extração / hidrólise do tratamento SBW de GP foi de 71% em peso, levando a um rendimento de extrato de GP de 31% em peso e um rendimento de hidratos de carbono de 27% em peso. O último valor corresponde a uma recuperação de aproximadamente 84% da quantidade total de hidratos de carbono (solúveis e estruturais) de GP, o que indica que a hidrólise da celulose ocorreu até certo ponto. Da quantidade total de hidratos de carbono medida no extrato de GP, aproximadamente 80% estavam na forma de oligossacarídeos. Os monossacarídeos mais abundantes no extrato de GP foram glucose e frutose, que existem no GP como hidratos de carbono solúveis, seguidos de arabinose, xilose e galactose, da hemicelulose.

Visando a hidrólise dos oligossacarídeos nos licores produzidos pelo tratamento SBW, o Viscozyme, um complexo enzimático que exibe atividade de celulase e hemicelulase, foi imobilizado em micropartículas de quitosano ativadas com glutaraldeído. O rendimento da imobilização enzimática foi de aproximadamente 79% e a enzima manteve cerca de 75%dae sua atividade específica, conforme determinado na hidrólise de um substrato modelo (arabinogalactano). Verificou-se que a hidrólise enzimática do GP e do resíduo de GP que fica no reator após o tratamento com SBW era muito lenta. A hidrólise enzimática do extrato de GP foi também efetuada, mas a análise por HPLC não foi conclusiva.

Os extratos de GP foram utilizados como fonte de carbono para o crescimento de duas leveduras oleaginosas: *Rhodotorula babjevae* e *Lipomyces starkeyi*. Uma análise comparativa revelou que a produção de biomassa e de lípidos era semelhante em meios de cultura com pH=4 e com pH=6. A levedura *Lipomyces starkeyi* apresentou valores mais elevados de biomassa e

de lípidos, quer no meio de controlo (9.6 g/L de biomassa, dos quais 5.3 g/L eram lípidos) contendo glucose como fonte de carbono, quer no meio com extrato de GP (0.9 g/L de biomassa, dos quais 0.2 g/L eram lípidos) contendo hidratos de carbono numa quantidade igual à da glucose no meio de controlo. A levedura *R. babjevae* atingiu o teor mais elevado de carotenóides (6.3 mg carotenóides / g peso seco) no meio de controlo com glucose.

Termos-chave: carotenóides, hidratos de carbono, hidrólise com água subcrítica, hidrólise enzimática, leveduras oleaginosas, lípidos, massas vínicas.

Abstract

The main objective of this thesis was to use grape pomace (GP) as a source of carbohydrates for yeast growth and subsequent production of lipids, or carotenoids, thereby achieving the valorization of a very important agro-industrial residue. To that effect, subcritical water (SBW) technology was used as a treatment to overcome the resilience of the GP lignocellulosic matrix, and depolymerize it into more elementary structures. Experiments were conducted on the growth of yeast strains on the GP extract resulting from the carbohydrate-rich liquor generated during SBW treatment, under conditions conducive to lipid accumulation and carotenoid production.

SBW extraction / hydrolysis experiments were carried out at a constant pressure of 70 bar, a water flow rate of 10 mL/min, and maximum temperatures between 190°C and 240 °C. Two temperature programs were used, namely one in which temperature was increased continuously to the target temperature, and another consisting of a series of steps, each step consisting of a ramp followed by a plateau, aiming at the fractionation of GP into extractives (plateau at 130 ºC), hemicellulose constituents (plateau at 190 ºC), and glucose or gluco-oligosaccharides from cellulose (plateau at 240 ºC).

The best results were obtained in a plateau-type assay reaching 240 ºC. At these conditions, the extraction / hydrolysis efficiency of the SBW treatment of GP was 71 wt.%, leading to a yield of GP extract of 31 wt.%, and a yield of carbohydrates of 27 wt.%. The latter value corresponds to a recovery of approximately 84% of the total amount of carbohydrates (both soluble and structural) of GP, which indicates that the hydrolysis of cellulose occurred to some extent. Of the total amount of carbohydrates measured in the GP extract, approximately 80% were in the form of oligosaccharides. The most abundant monosaccharides in GP extract were glucose and fructose, which exist in GP as soluble carbohydrates, followed by arabinose, xylose and galactose, from hemicellulose.

With a view to breaking down the oligosaccharides in the liquors produced in the SBW treatment, Viscozyme, an enzyme complex exhibiting cellulase and hemicellulase activity, was immobilized on glutaraldehyde-activated chitosan microparticles. The yield of enzyme immobilization was approximately 79%, and the enzyme kept about 75% of its specific activity, as determined in the hydrolysis of a model substrate (arabinogalactan). The enzymatic hydrolysis of GP and GP residue left in the reactor after SBW treatment was found to be very slow. That of GP extract was carried out but HPLC analysis was inconclusive.

GP extract was used as carbon source for the growth of two oleaginous yeasts: *Rhodotorula babjevae*, and *Lipomyces starkeyi*. A comparative analysis of biomass and lipid production was made, in media with different pH and composition. Similar results were found with media at pH 4 and pH 6. *Lipomyces starkeyi* exhibited both highest cell dry weight and lipids dry weight in both the control medium (9.6 g/L cells, of which 5.3 g/L were lipids) containing glucose as carbon source, and medium with GP extract (0.9 g/L cells, of which 0.2 g/L are lipids)

containing carbohydrates in an amount identical to that of glucose in the control medium. *R. babjevae* produced the highest carotenoid content, reaching 6.3 mg carotenoids / g cell dry weight in control assays with glucose.

Key-words: grape pomace, subcritical water hydrolysis, carbohydrates, oleaginous yeasts, lipids, carotenoids, enzymatic hydrolysis.

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List of Abbreviations

DW – dry weight

GC – gas chromatography

GP – grape pomace

GP-SBW – grape pomace hydrolyzed by hot compressed water

GP-SBWR – grape pomace remaining in the reactor after hydrolysis with hot compressed water

HCW – hot compressed water

HPLC – high performance liquid chromatography

HTW – high temperature water

LCM – lignocellulosic material

NR – Nile Red

OD – optical density

SBW – subcritical water

SCO – single cell oil

SPS – scintillation per second

TAG - triacylglycerol

1 STATE OF THE ART

1.1. BIOREFINERY & BIOTECHNOLOGY

A biorefinery is an integral unit that can accept various biological nonfood feedstocks and convert them into a range of useful products, including chemicals, energy, and materials (Fig.1.1).

The first stage of an integrated biorefinery is the extraction of valuable primary and/or secondary metabolites. Primary metabolites include carbohydrates, proteins, and lipids. Secondary metabolites, which unlike primary metabolites are not required for normal growth and development of the species that produce them, include alkaloids, steroids and phenolic compounds.

Biorefining can provide a sustainable approach to valuable products while improving biomass processing economics, as well as environmental footprint.

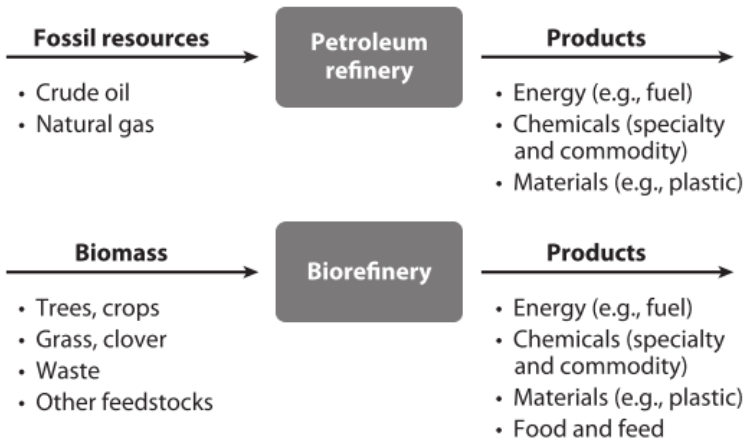

Figure 1.1 *The biorefinery concept: from biomass to valuable products (adapted from reference* 1*).*

The transition to a knowledge-based bio-economy is the prevalent vision among European countries. Supporting the ambition of achieving bio-based economic development is the increased utilization of industrial biotechnology. [2]

Industrial biotechnology, or white biotechnology, refers to the use of living cells or enzymes to create industrial products that are easily degradable, require less energy to manufacture, create less waste during production, and sometimes perform better than products created using conventional methods.

Green biotechnology is defined as the application of biological techniques to plants with the aim of improving the nutritional quality, quantity and production economics, and producing more environmentally friendly solutions than traditional agriculture.

Combined green biotechnology and white biotechnology will play a crucial role in this century to produce feedstock chemicals and fuels derived from biomass. This requires efficient breakdown and conversion of lignocellulosic material, which remains as one of the biggest handicaps currently holding back the development of successful biomass-based biorefineries that can dethrone traditional petroleum refineries.[1]

The biorefinery approach can lead to the production of green and sustainable chemical products with less negative impact on the environment,[3] as well as foster regional development and a fairer distribution of economic benefits than the actual model.

1.2. FROM AGRO-INDUSTRIAL WASTE TO ADDED-VALUE RESIDUES

Food production is wasteful. From crop residues, through processing (where substantial losses occur), to sale and consumption, approximately one-third of the food produced is thrown away.[1]

Waste substances can be viewed as secondary resources in the wrong place.[4] Agro-industrial wastes are generated during the industrial processing of agricultural products. These wastes are produced in great amounts throughout the year, and are the most abundant renewable resources on the planet.[5] Agro-industrial wastes such as grape pomace, apple pomace, sugarcane bagasse, wheat bran, rice bran, corn cob and wheat straw, are cheap and abundantly available natural carbon sources.

Such wastes can be used as low-cost substrates to produce added-value compounds. Moreover, most of the agro-industrial wastes contain molecules that may cause damage to the environment when discharged without prior treatment.[5] Thus it is crucial to reutilize these wastes and mine them for added-value, and to prevent their disposal to the environment and consequences therefrom.

The annual growth of plant-derived biomass is estimated to be 118×10^9 tons per year on a dry matter basis. The lignocellulosic biomass materials are abundant, cheap and renewable.

1.2.1. LIGNOCELLULOSIC BIOMASS

Lignocellulose (Fig.1.2) is a renewable organic material and is the major structural component of all plants.[5] Lignocellulose consists of three major components: cellulose, hemicellulose and lignin.

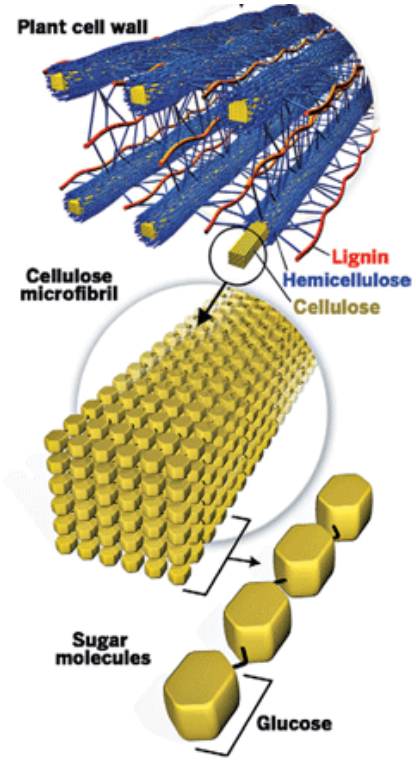

Figure 1.2 *Lignocellulose and its constituents (adapted from reference* 6*).*

Cellulose (Fig. 1.2) is the major constituent of all plants and the most abundant organic molecule on earth. It is a linear polymer of D-glucose units, connected by β-1,4-glycosidic bonds.[7] It is thus a homopolysaccharide.

Hemicellulose (Fig. 1.2) is a heteropolysaccharide formed by pentoses (mainly xylose and arabinose), hexoses (mainly mannose, less of glucose and galactose), and sugar acids.[7]

Cellulose and hemicellulose are bound together by lignin.[4] Lignin (Fig. 1.2 - 1.3), which is the second most abundant organic carbon source on the planet, is a heterogeneous polymer generally containing three aromatic alcohols, namely coniferyl, sinapyl and p-coumaryl alcohol.

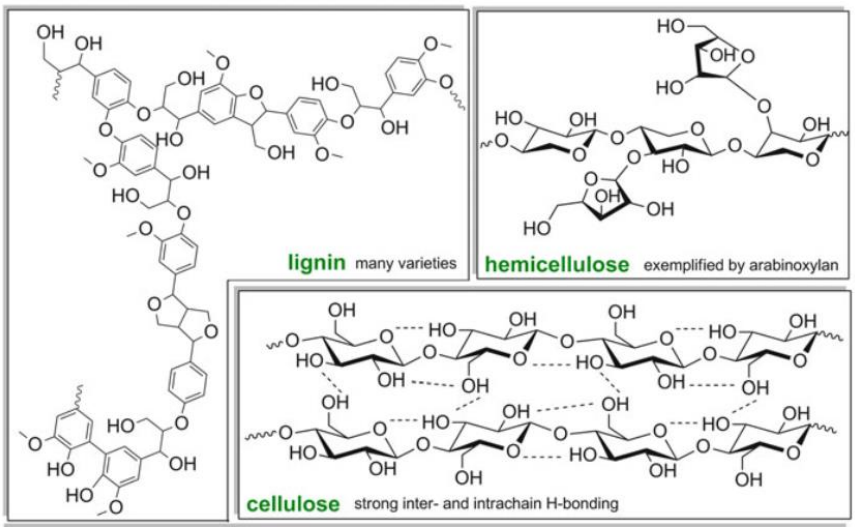

Figure 1.3 *The general chemical structure of the major biomass components.*

The valorization of lignocellulosic materials is highly desirable because their constituents originate compounds that have high potential and commercial value. Such is the case of glucose, a preferred carbon source for microorganism growth, xylitol, furfural or xylo-oligosaccharides from hemicellulose that can be used as a low glycemic sweetener, a platform chemical, or prebiotics, respectively, and adhesives, or high-quality carbon fibers, obtained from lignin.[8]

Other important biomass constituents, although appearing in comparatively minor amounts, are phenolic compounds with antioxidant and antimicrobial activity, with applications in the pharmaceutical and food industries.[9] Other added-value compounds appear further down the value chain of biomass constituents. Examples include biofuels, such as bioethanol, lipids and carotenoids, which can be produced by microorganisms grown on biomass-derived carbohydrates.

1.3. GRAPES AND GRAPE POMACE

1.3.1. GRAPES

According with FAO (Food and Agriculture Organization of the United Nations), grape crops are one of the main extensive agro-economic activities in the world. An average of 67.5 million tons of grapes were produced annually between 2000 and 2014 (Fig. 1.4).

In 2014, the most important contribution to the annual world grape production came from Europe (42.5%), followed by Asia (28.5%), and the Americas (20.2%). In Portugal, grapes represent the third major crop cultivated annually, reaching 818 thousand tons in 2014, making grape by-products a promising renewable biomass source for biorefinery integration processes.

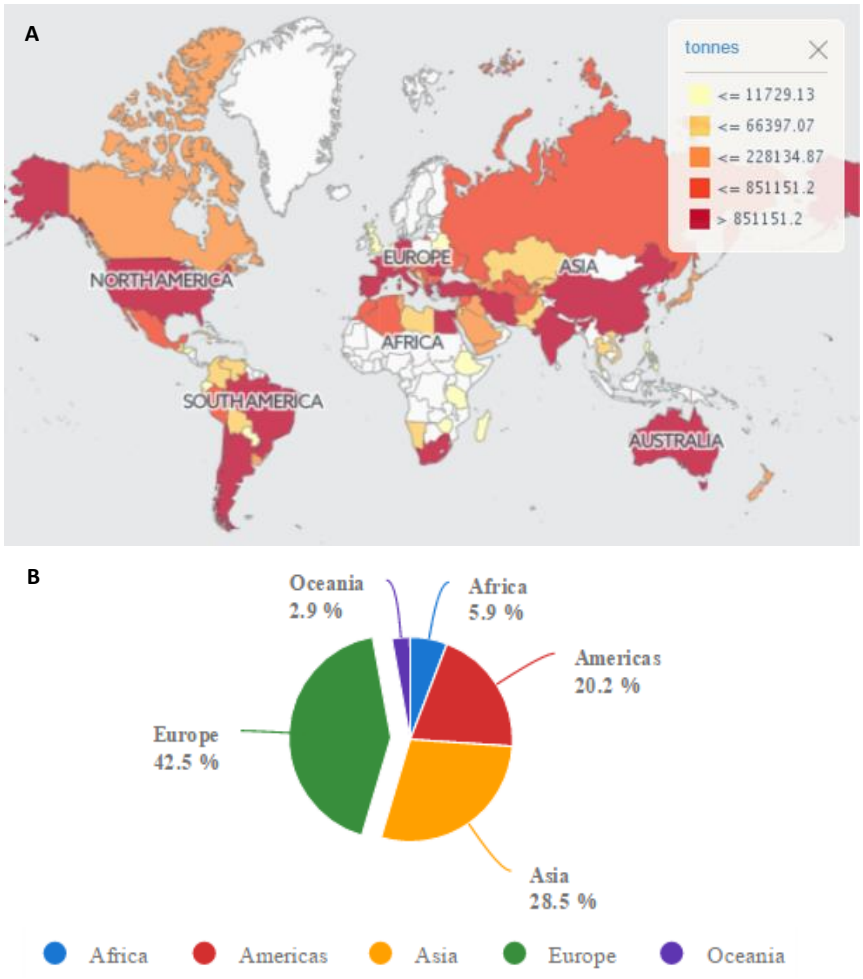

Figure 1.4 *Worldwide production of grapes (A). Europe is the main world grape producer followed by Asia and America (B) (content taken from reference* 10*).*

1.3.2. GRAPE POMACE

Grape by-products are produced in massive quantities, especially by the winemaking industry. During wine production, approximately 25% of the grape weight ends up as the by-product/waste designated as 'pomace' (Fig. 1.5).[11] This pomace comprises stalks (30%), seeds (30%) and skin and pulp (40%).[12] Basically, grape pomace is the residue that remains after pressing grapes for obtaining juice.

Figure 1.5 *Red grape pomace.*

Grape pomace has a water content between 60% - 80%.[13] Its composition varies widely, especially depending on the type of wine produced. For example, white wine grape pomace has a high content of soluble, immediately available glucose and fructose, because it does not suffer fermentation. These monosaccharides are abundant in grapes at the time of harvest. On the other hand, fermented, red wine grape pomace, has a low amount of soluble carbohydrates, and a high amount of structural ones. The composition of red wine grape pomace varies according to the type of grape, indicative values being approximately 5% soluble carbohydrates, 30% cellulose, 15% hemicellulose, 30% lignin, 10% protein and 10% lipids.[14] The disposal of grape pomace is a problem for the wine industry, because it represents thousands of tons, and if not treated, grape pomace can create various environmental threats, from surface and ground water pollution to fetid odors that attract insects (due to composting), pests, and can easily spread diseases.[15]

Composting is a natural process whereby organic material suffers aerobic microbial decomposition, originating a rich soil known as compost. Organic compounds are broken down into natural elements, such as carbon and nitrogen. The high C:N ratio provides nutrients for the microbes to survive and continue degradation.[11] When the purpose is the valorization of grape pomace, composting must thus be avoided, and this is usually accomplished by freezing to prevent microbial proliferation.

1.4. BIOMASS HYDROLYSIS

Lignocellulose has become a major target for biofuel and chemical feedstock production. Nonetheless, the lignocellulosic matrix is a very resilient material. To access its structural constituents, its complex, polymeric structure, must be disrupted.

Steam explosion and acid hydrolysis are common methods to hydrolyze lignocellulosic biomass and break it into its main constituents. Steam explosion combines thermal and

mechanical action to break down hemicellulose and transform lignin, increasing the accessibility of cellulose. Water is a cheap, non-toxic, non-flammable, non-explosive, sustainable solvent. But vaporizing water into steam requires a high amount of energy.[7]

Through acid hydrolysis, cellulose and hemicellulose are hydrolyzed to sugar monomers. Although acid hydrolysis is a common method to characterize lignocellulosic biomass, it generates a high environmental load, and causes corrosion problems.

Development of a lignocellulose pretreatment featuring moderate reaction conditions is highly desired. One alternative to the methods outlined above is hydrothermal hydrolysis, also referred to as hot compressed water hydrolysis, or subcritical water hydrolysis. As in steam explosion, only water is used, but in this case water is not vaporized and hence the energy involved is much lower. Also the process can be carried out without additives.[16]

1.4.1. SUBCRITICAL WATER

Supercritical fluids are substances at pressures and temperatures above their critical values (Figure 1.6). When the fluid reaches the supercritical region, it exhibits simultaneously a set of gas-like and liquid-like properties.

A low viscosity, high diffusivity and almost no surface tension[17] are properties that supercritical fluids share with common gases, which facilitate the permeation of solid matrices and improve mass transfer rates. But a main characteristic of supercritical fluids, which is at the root of their utility as solvents, is a density/solvent strength similar to that of liquids. Also, this parameter is easily adjustable close to the critical point, which allows the fractionation of mixtures of different solutes.

The critical point of water is attained at 374 ºC and 218 bar. Supercritical water is used for biomass gasification (Table 1.1) into a mixture of essentially methane, hydrogen and carbon dioxide. But when biomass degradation is to be avoided and the emphasis is biomass hydrolysis to obtain carbohydrates – hydrothermal liquefaction – subcritical water can be used. Subcritical water (SBW) is water below its critical point but above its vapor pressure (Fig. 1.6).

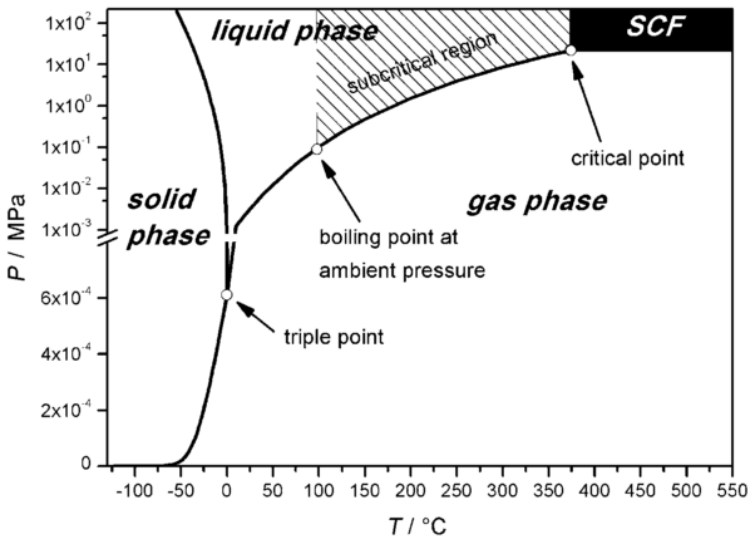

Figure 1.6 *Water phase diagram, indicating the subcritical region below the critical point and above the vaporization curve (content taken from reference* 18*).*

Generally, solid, liquid, and gaseous products are formed at all temperatures when using water. However, the extent of the respective main product formation shifts from solid (at low temperature), to liquid (medium temperature), to gaseous (at high temperature) products.[18]

Table 1.1 *Prevailing hydrothermal biomass conversion processes (content taken from reference* 18*).*

Process	Temperature window (°C)
Hydrothermal carbonization	100-200
Hydrothermal liquefaction	200-350 (5 – 28 MPa)
Hydrothermal gasification	350-750

Subcritical water (SBW) has a set of very interesting properties for biomass pretreatment. It features different behavior compared to water at ambient conditions due to the dramatic changes in physical properties, namely dielectric constant and ionic product.

The dielectric constant of SBW (water polarity) decreases abruptly with temperature, due to hydrogen bond dissociation. Hence, the solubility of ionic molecules decreases, while the solubility of hydrophobic molecules increases. This enables the extraction of compounds that are normally extracted with organic solvents.

Ionic strength is another water property that changes pronouncedly. The ionic product of SBW at 250 ºC is nearly three orders of magnitude higher than for ambient liquid water. [19] This leads to an increase in H^+ and OH^- concentrations, making water a more reactive medium. Water behaves as a reagent as well as a solvent.[18] This property makes subcritical water an ideal reaction medium for the hydrolysis of complex, lignocellulose polymers.[20]

Also using SBW, large-chain carbohydrates can be broken down into simpler structures, such as oligomers or even di- and mono-saccharides. The rate of hydrolysis varies between different carbohydrates, hemicelluloses and starch being hydrolyzed much faster than cellulose due to the crystallinity of the latter.[21]

SBW is being extensively researched and developed as a technology for converting lignocellulosic matrices into fuels and added-value products, as well as for converting protein-rich wastes into amino acids.[4],[22] A large amount of waste biomass has water contents of over 95%, whose elimination is extremely energy-intensive and thus costly. Hence there is increased interest in SBW as an alternative to other biomass pretreatment techniques.

SBW has lower viscosity and surface tension than water at ambient temperature, which increases mass transfer rates from solid matrices.[9]

SBW hydrolysis has been applied to a few agricultural and food industry residues, relevant examples including corn stalks and stover, sugarcane bagasse, rice bran, grape pomace, pressed palm fiber and coconut husk.[23] SBW hydrolysis is a clean technology and a fast hydrolysis method applicable to lignocellulosic biomass as a pretreatment step. As mentioned earlier, the simple sugars that can be obtained from the cellulose and hemicellulose fractions of lignocellulosic materials can be used as substrate to produce bioethanol or other precursors of bio-products.[24] Another possible product could be lipids, produced by yeasts grown on these simple sugars, as it will be developed further in chapter 1.6.

1.5. ENZYMATIC HYDROLYSIS

One of the most important roles of enzymes as natural biocatalysts is their ability to increase the rate of almost all chemical reactions within a cell, without themselves being permanently altered or consumed in the reactions. Enzymes work under mild reaction conditions, and are selective, avoiding the generation of by-products.

To use lignocellulosic residues as substrates, for example, for yeast growth and the production of industrially important products, it is necessary that the polysaccharides cellulose and hemicellulose be converted into simple sugars that can be assimilated by the microorganisms, through hydrolysis and consequent breaking down of glycosidic bonds.

1.5.1. CELLULASE AND HEMICELLULASE

Cellulase is a mixture of three types of enzymes, namely endoglucanases, which break β-1,4-glycosidic linkages randomly; exoglucanases, which preferentially release glucose monomer units from the end of the cellulose chain; and β-glucosidases, which release D-glucose from cellobiose dimers.

The conversion of cellulose to glucose units is most commonly made with cellulases, which do not generate degradation products from glucose, unlike what happens when using alternative hydrolysis treatments.

Much effort has been made to develop cellulosic bioethanol, directed towards making hydrolysis faster, at a more favorable and less costly enzyme:substrate ratio.

Hemicellulase is a term used to define a variety of enzymes that hydrolyze hemicellulose structures, such as xylan, arabinan, arabinogalactan. A common example of a hemicellulase is xylanase that hydrolyses xylan.

Together, cellulases and hemicellulases can hydrolyze the polysaccharides of lignocellulose, originating monosaccharides that can be assimilated by microorganisms to produce compounds of interest.

However, the lignocellulosic matrix is too complex to be hydrolyzed directly, and efficiently, with enzymes. A pretreatment is required, to increase enzyme accessibility and allow enzyme action.

1.5.2. ENZYME IMMOBILIZATION

To make enzyme application in biotechnological processes more favorable, different methods for cost reduction have been put in practice, such as immobilization. Enzyme immobilization consists in physically confining enzymes in a certain region of space, usually resorting to a material acting as support, with retention of catalytic activity and enhanced stability, allowing repeated, continuous use, and easy recovery.

Basically, immobilization methods can be divided into two general classes: physical and chemical methods. Physical methods are characterized by noncovalent interactions, such as hydrogen bonds, hydrophobic interactions, van der Waals forces, ionic interaction of the enzyme within the support material, or mechanical constraint of enzyme within the support. In chemical methods, formation of covalent bonds achieved through ether, thioether, amide or carbamate bonds between the enzyme and support material are involved.[25]

There are four main techniques for immobilization of enzymes, namely adsorption, entrapment, covalent bonding, and cross-linking (Fig. 1.7).

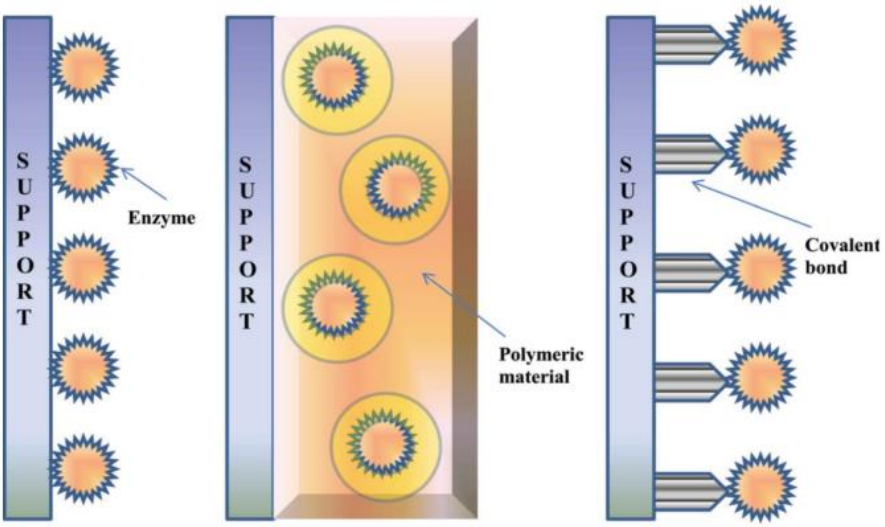

Figure 1.7 *Schematics of the three most common enzyme immobilization techniques: (A) physical adsorption, (B) entrapment and (C) covalent attachment / cross-linking (content taken from reference* 25*).*

Adsorption consists on enzyme immobilization either at the external surface of a support, or within pores in the support. The support or carrier is commonly mineral, such as a zeolite, or modified sepharose, or an ion exchange resin. There is no permanent bond formation between carrier and the enzyme in this method. Only weak bonds stabilize enzyme-support interactions, which may lead to enzyme leaching. When the enzyme is adsorbed within pores of the support, internal diffusion limitations may exist.

Covalent bonding involves the formation of strong, directional bonds, between the chemical groups on the enzyme and the chemical groups on the support. Hydroxyl and amino groups of support or enzyme form covalent bonds more easily. Carriers commonly used in this method are carbohydrates, such as cellulose, or Eupegit® (copolymer of methacrylamide). One advantage of covalent bonding is the strong linkage of enzyme to the support, but one disadvantage is the chemical modification of the enzyme that can lead to the loss of its functional conformation.

Through entrapment the enzyme is physically restrained inside a porous matrix. Bonds involved in stabilizing the enzyme in the matrix may be covalent or noncovalent. The pore size of the matrix is adjusted to prevent the loss of the enzyme, through manipulation of experimental parameters. Commonly used matrixes are polyacrylamide gels, cellulose triacetate, agar, gelatin, carrageenan, alginate, and silica, as in sol-gel. Again, leaching of the enzyme may occur, as well as diffusion limitations. Enzymes can also be entrapped within micelles, formed by using a surfactant.

Cross-linking is an immobilization method that does not involve a support matrix. Enzymes can be cross-linked through covalent bonds established between enzyme molecules and polyfunctional reagents, such as glutaraldehyde or diazonium salt. One disadvantage of this

method is that the reagents used in cross-linking may denature or structurally modify the enzyme, leading to the loss of catalytic activity.

1.6. Oleaginous Yeasts & Lipid Biosynthesis

1.6.1. Oleaginous Yeasts

The growth of oleaginous yeast on lignocellulosic biomass is being exploited as an effective option for lipid accumulation and generation of microbial oil.[26] Oleaginous yeasts are usually non-pathogenic, and known to accumulate lipids up to 40% of their total dry weight. Under starvation conditions, oil production can exceed 70% of cell dry weight.[27]

The ability to grow on a broad spectrum of carbon sources, such as grape pomace agricultural waste, makes oleaginous yeasts economically interesting. They can be used as microbial factories to produce various compounds with industrial utility.

Approximately 1500 species of yeast belonging to over 100 genera have been described so far.[28] Among the huge number of species that have been described, only about 30 are able to accumulate more than 25% of their dry weight as lipids.[29] These include *Lipomyces starkeyi*, *Yarrowia lypolytica*, *Rhodoturula glutinis*, *Rhodotorula toruloides*, *Cryptococcus curvatus*, *Trichosporon fermentans*, *Rhodotorula mucilaginosa*, and *Rhodotorula babjevae*.

Rhodotorula babjevae, Rhodotorula mucilaginosa and *Lipomyces starkeyi were* used for lipid production in this thesis (Fig. 1.8).

Figure 1.8 *Oleaginous yeast species used in the study. From the left to the right: L. starkeyi, R. babjevae and R. mucilaginosa.*

Oleaginous yeast can accumulate various types of lipids. These include triacylglycerols (TAGs), diacylglycerols (DAG), and monoacylglycerols, sterols, fatty acids, sterol esters, free sterols, carotenoids, and others.

Lipids are found mainly in the form of neutral lipids, glycolipids, phospholipids and free fatty acids. The fractions of neutral lipids, in general, accounts for more than 90% of total lipids.[30] The classes of lipids present, as well as the ratio of membrane lipid to storage lipid, vary among species and strains, culture conditions, such as pH and carbon-source concentration, and culture growth phase.[31]

A screening of lipid production by over sixty yeast strains, with analysis of fatty acids proportion and profile, showed that the major fatty acids represented were oleic (18:1ω9), palmitic (16:0), stearic (18:0), and linoleic (18:2ω6) acids, with minor contributions from lignoceric acid (24:0), palmitoleic (16:1ω9), behenic acid (22:0), myristic acid (14:0), α-linolenic (18:3ω3), and arachidic acids (20:0). Other fatty acids were observed in trace amounts.[31]

1.6.2. LIPID BIOSYNTHESIS PATHWAY

In oleaginous yeasts, the initiation of TAG accumulation (Fig. 1.9) often occurs under nitrogen-depletion conditions. Upon nutrient limitation, the carbon-source is diverted from energy production, via the tricarboxylic acid cycle (TCA), to TAG synthesis.

Although TAG is generally the desired end-product, carbon may also be redirected to other metabolites including citric acid, polysaccharides, and other secondary metabolites. The change in carbon flux is initiated upon activation of nitrogen-scavenging enzymes, such as adenosine monophosphate deaminase (AM). This enzyme deaminates AMP releasing ammonia, which can be utilized by the cell as a nitrogen source. The reduced cellular AMP content results in inhibition of isocitrate dehydrogenase (ICDH), the enzyme responsible for the conversion of isocitrate to oxoglutarate in the tricarboxylic acid (TCA) cycle. This inhibition causes citrate accumulation and transportation into the cytoplasm, where ATP citrate lyase (ACL) catalyzes the conversion of citrate into acetyl-CoA and oxaloacetate. ACL is an enzyme required for oleagenesis, and its absence limits the flux of carbon to fatty acid (FA) synthesis.[32]

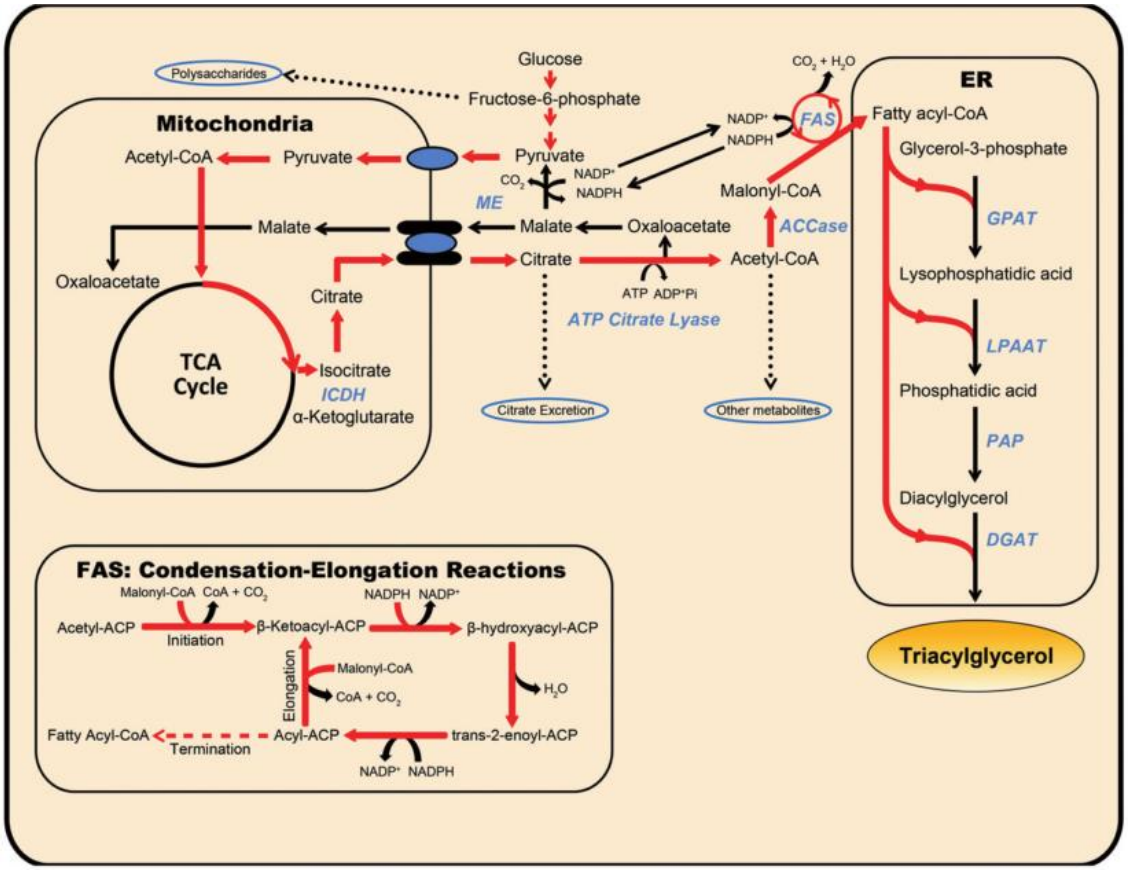

Figure 1.9 *Biochemistry of triacylglycerol (TAG) accumulation in oleaginous yeast (Probst et al., 2015). Abbreviations: endoplasmic reticulum (ER), tricarboxylic acid (TCA), isocitrate dehydrogenase (ICDH), malic enzyme (ME), fatty acid synthase (FAZ), acetyl-CoA carboxylase (ACCase), glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), phosphatidate phosphatase (PAP), diacylglycerol acyltransferase (DGAT) (content taken from reference* 32*).*

The general mechanism of lipid accumulation in oleaginous yeast has not yet been fully clarified. Examination of enzymatic activity[33] and comparative genomics[34] suggest that ATP:citrate lyase (ACL) may play a role in directing excess carbon to be stored as lipids, rather than carbohydrates, in oleaginous yeasts.

Lipid accumulation is prompted by a nutrient limitation combined with an excess of carbon. Lipids can be accumulated within the cell in two different ways[35], namely *de novo* synthesis and *ex novo* synthesis. In the former, lipid accumulation is induced by depletion of an essential nutrient in the culture medium, such as nitrogen. In this case, lipid accumulation reaches a peak in the early stationary phase, when the growth medium is nitrogen-depleted, but there is still excess carbon source in the medium.[36] In *ex novo* synthesis, lipid accumulation occurs simultaneously with cell growth, being completely independent of the nitrogen deprivation in the growth medium.

Usually nitrogen limitation is used to trigger lipid accumulation, but other nutrients, such as phosphorus and sulphur, have been shown to have the same effect .[27]

1.6.3. SINGLE CELL OIL

Single cell oil (SCO) is a designation that applies to neutral lipids accumulated by oleaginous microorganisms (yeast, fungi, microalgae) [32]

Microorganisms can use organic carbon to produce SCO more efficiently, with higher productivity, than the best producing oil crops. SCO from oleaginous yeast may serve as a renewable source of edible oil, as well as an intermediate "building block" for oleochemicals, such as fuels (for example, biodiesel[38]), soaps, plastics, paints, detergents, textiles, rubber, surfactants, lubricants, additives for the food and cosmetic industry, and many other chemicals.[30]

The cost of yeast SCO has been estimated as 2.9 €/kg, excluding feedstock costs, or 4.7 €/kg, including the cost of glucose as a feedstock.[13] Use of low cost waste and by-product streams, such as those from an integrated biorefinery, will be needed for yeast SCO to be competitive with other commodity type oils. In this respect, grape pomace is a good candidate to be used as a carbon-source material to produce SCO.

1.6.4. CAROTENOIDS

Carotenoids are pigments whose biological effects in humans are related to their antioxidant activity. The most common carotenoids include carotenes, such as α- and β-carotene, lutein and astaxanthin, which are thought to have beneficial effects on eye and vision, and lycopene, a known food coloring found in tomatoes.

The production of carotenoids by oleaginous yeast varies between species, and is affected by medium constituents and environmental conditions. The yeasts studied in this thesis have the potential to be used in the industrial production of the fatty acids and carotenoids indicated in Table 1.2. Torularhodin, β-carotene and torulene have provitamin-A activity.[37]

Table 1.2 *Dominant carotenoids and fatty acids produced by the oleaginous yeasts used in this study.*

Oleaginous Yeast	Carotenoids Produced	Fatty Acids Produced
Rhodotorula babjevae	Astaxanthin and β-carotene (Johnson et al., 1995)	Oleic (18:1ω9), Palmitic (16:0) and Stearic (18:0) acids (Sitepu et al., 2013)
Rhodotorula mucilaginosa	Torularhodin, β-carotene and Torulene (Buzzini, et al., 1999)	Oleic (v18:1ω9), Stearic (18:0) and Palmitic (16:0) acids (Sitepu et al., 2013)
Lipomyces starkeyi	None	Palmitic (16:0), Oleic (18:1ω9) and Palmitoleic (16: 1ω9) acids (Bonturi et al., 2015)

1.6.5. BIOMASS HYDROLYSATES AS CARBON SOURCE FOR YEAST GROWTH

Valuable chemicals, including oligosaccharides, monosaccharides, and secondary decomposition products such as 5-hydroxymethyl-furfural (5-HMF) and 2-furaldehyde, have been produced by hydrothermal degradation of various natural polysaccharides such as cellulose, hemicellulose, starch, guar gum, and polygalacturonic acid.[16] These natural polysaccharides can be obtained from corn stalks and stover, sugarcane bagasse, rice bran, grape pomace, pressed palm fiber and coconut husk, among others.[23]

Using hydrolysates for microbial oil production could reduce the cost of SCO production, which is critical to the success of industrial scale-up.[38-40] In the natural environment, yeast species have a broad set of carbon sources (e.g., polyols, alcohols, organic acids and amino acids) that can support their growth, but preferentially they metabolize sugars. The metabolism of different carbon sources, such as the sugar monomers glucose, fructose, galactose or mannose, and disaccharides, such as maltose or sucrose, as well as ethanol or acetate, is widely studyed.[39]

Examples of oleaginous yeasts that can grow on a wide variety of monosaccharides and some disaccharides include *R. babjevae* and *R. mucilaginosa,* shown to be able to grow in medium supplemented with glucose, xylose, galactose, arabinose, fructose, sucrose, maltose or cellobiose as carbon-sources.[40] The oleaginous yeast *Lipomyces starkeyi* also utilizes diverse carbon sources including glucose, xylose, glycerol.[26]

An important aspect is also the sensitivity of yeast species to the presence of inhibitors in the hydrolysates used for the conversion of carbohydrates to lipids. These inhibitors can not only inhibit the growth of the oleaginous species, but also lead to a decrease in cellular lipid content.[41]

1.7. THESIS OUTLINE

The main goal of this thesis is to use grape pomace as a source of carbohydrates for yeast growth and the production of lipids, and carotenoids.

Towards that end, grape pomace was submitted to SBW hydrolysis as a pretreatment to depolymerize the lignocellulosic structure and obtain a liquor (liquor A), consisting mainly of oligosaccharides. Experiments were conducted on the growth of yeast strains on the GP extract resulting from this liquor, under conditions conducive to lipid accumulation, and formation of carotenoids (Fig. 1.10).

The enzymatic hydrolysis of liquor A, using an enzyme complex containing cellulases and hemicellulases, was also studied, with a view to breaking down the oligosaccharides of liquor A into monosaccharides, resulting in a liquor B, to be also tested as growth medium for oleaginous yeast species in future work.

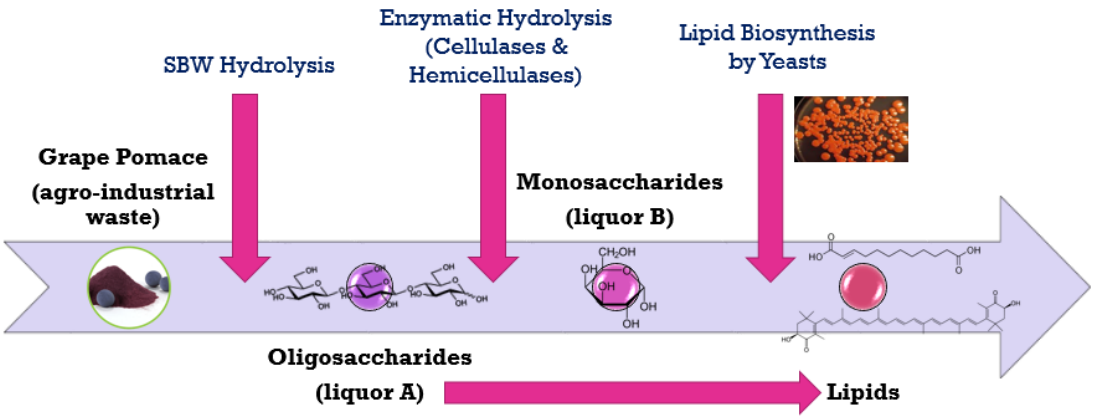

Figure 1.10 *Outline – From grape pomace to single cell oil production using oleaginous yeast species, after subcritical water pretreatment with or without subsequent enzymatic hydrolysis.*

2 MATERIALS AND METHODS

2.1. CHEMICAL CHARACTERIZATION OF GRAPE POMACE AND DERIVED MATERIAL

2.1.1. GRAPE POMACE AND GP POWDER

Red wine grape pomace was supplied by a Portuguese wine producer. Grapes were destemmed before pressing. Grape pomace (GP) from grapes pressed on a given day was delivered the following day. It was immediately put in plastic bags and placed in the freezer, where it was kept refrigerated at -20 ºC.

A comparative analysis was made on the method of drying GP, namely lyophilization (freeze drying), or oven drying. In the first assay, GP was taken from the freezer, immersed in liquid nitrogen and lyophilized under vacuum for 48 h in a lyophilizer (CHRIST ALPHA 1-4, Braun Biotec International). In the second assay, GP was heated at 60 ºC in an oven, for 48 h. In both cases, dried GP was allowed to reach room temperature, milled to ca. 2 mm, and the water content of each residue was determined gravimetrically, by measuring its mass before and after drying overnight in an oven at 105 ºC.

When not in use, GP powder was kept refrigerated at -20 ºC.

2.1.2. PROTEIN DETERMINATION

The nitrogen content of GP powder was determined by elementary analysis performed at Laboratório de Análises, REQUIMTE-LAQV. To determine protein content, a nitrogen-to-protein conversion factor of 6.25 was used.

2.1.3. ASH DETERMINATION

To determine ash content, 0.10 g of GP powder were weighed in a glass filter in a porcelain crucible, and placed in a muffle at 550 ºC. The crucible was removed after 6 h and placed in a desiccator, overnight, to cool down, after which ash content was determined by gravimetry.

2.1.4. CARBOHYDRATES AND LIPID CONTENT

Before carbohydrate analysis, 2.00 g of GP powder were defatted through Soxhlet extraction, for 4 hours, using 65 mL of *n*-hexane (Carlo Erba Reagents) as solvent. The resulting residue was dried overnight at 40 °C to evaporate the solvent, after which it was weighed. The solvent in the liquid solution was also removed through evaporation, aided by a gaseous nitrogen flux, and the remaining oil was weighed. The direct and indirect measurements of GP powder fat content – the dried deffated residue and the extracted oil measurements - were in good

agreement.

For soluble carbohydrate analysis, the defatted GP powder (0.80 g) was extracted with 40 mL of a (80:20, v/v) ethanol:water solution, in an ultrasonic bath, for 15 minutes, at room temperature. The extraction was finished by centrifugation (10000 rpm, for 10 min, at 4 °C). The process was repeated three times. The three supernatants were combined and ethanol was evaporated at 50 °C, under vacuum, in a rotary evaporator. The remaining solution was diluted with 80 mL of water and then analyzed by UV-Vis spectrophotometry – colorimetric method, described in section 2.1.5 – to quantify soluble carbohydrates. It was also analyzed by HPLC (high performance liquid chromatography). The remaining residue – defatted GP powder without soluble carbohydrates – was dried at 40 °C overnight.

To hydrolyze the insoluble structural carbohydrates, 3 mL of 72% (w/w) H_2SO_4 were added to 0.30 g of the remaining residue. The mixture was incubated in a water bath at 30 °C, under stirring, for 1 hour. After that, the mixture was diluted to 4% (w/w) by adding 84 mL of water, and incubated at 121 °C in a silicone bath, under stirring, for 1 hour. The mixture was then filtered in porcelain filters, and the supernatant analyzed by UV-Vis spectrophotometry – colorimetric method described in section 2.1.5 – to quantify the sugar monomers resulting from structural carbohydrates. It was also analyzed by HPLC.

The solid remaining after acid hydrolysis was washed with water, dried at 105 ºC overnight, and weighed. Its ash and nitrogen contents were determined as stated above. The amount of Klason lignin was obtained by subtracting resistant protein and acid insoluble ash from the weight of dry residue.

2.1.5. COLORIMETRIC METHOD FOR CARBOHYDRATE ANALYSIS

This method (phenol-sulphuric) is used to quantify the reducing sugar content of the sugar-rich liquors, using a calibration curve built with D(+)-glucose monohydrate (SIGMA Aldrich) solutions. These were prepared from a 1 g/L stock solution, with concentrations of 0.25, 0.1, 0.05, 0.025 and 0.005 g/L in milli-Q water. The control was milli-Q water.

To 500 µL of either a standard solution or sugar-rich liquor were added 1.5 mL of H_2SO_4 (Carlo Erba Reagents, 96%) and 300 µL of a 5% (w/v) aqueous solution of phenol (Sigma Aldrich, 99-100%). The resulting mixtures were well stirred. After incubation for 5 min at 90 ºC in an Accu BlockTM Digital Dry Bath, the mixtures are well stirred, and cooled to room temperature by immersion in a water bath. Absorbance was measured at 490 nm with a DU®800 Spectrophotometer from Beckman Coulter, Brea, USA. The results obtained are expressed in g/L glucose equivalent.

2.1.6. HPLC Method for Carbohydrate Analysis

The HPLC technique was used to identify and quantify sugars (monosaccharides, such as glucose, fructose, galactose, arabinose, mannose, rhamnose, and xylose) in GP-derived liquors and extracts. All the analyses were performed at Laboratório de Análises, LAQV-REQUIMTE, with a Dionex ICS-3000 system, with electrochemical detection, using a 4x50 mm Thermo BioLC Dionex AminoTrap pre-column and a 4x250 mm Thermo Dionex CarboPac SA10 column, at a constant temperature of 40 ºC. A 1 mM NaOH solution was used as mobile phase, at a constant flow rate of 1.2 mL/min. Calibration curves were built for the monosaccharides (25, 50, 75, 100, 150, and 250 ppm).

Alternatively, HPLC analyses were performed with a Dionex P580 system, using a differential refractometer LKB, model 2142, and an Aminex HPX-87P column (BIORAD), at a constant temperature of 65 ºC. Sterile water was used as mobile phase, at a constant flow rate of 0.6 mL/min. Calibration curves were built for the monosaccharides, covering the range of concentrations required, and peak integration and analysis were made automatically using the Chromeleon data acquisition software.

2.2. Hot Compressed Water Hydrolysis

2.2.1. Hot-Compressed Water Apparatus and Reaction Conditions

A high pressure, high temperature, semi-continuous reactor (Fig. 2.1) was used to extract / hydrolyze GP using SBW.

The body of the reactor is of stainless steel, and it is 51 cm long, having a 5 cm external diameter and a 2.6 cm internal diameter.

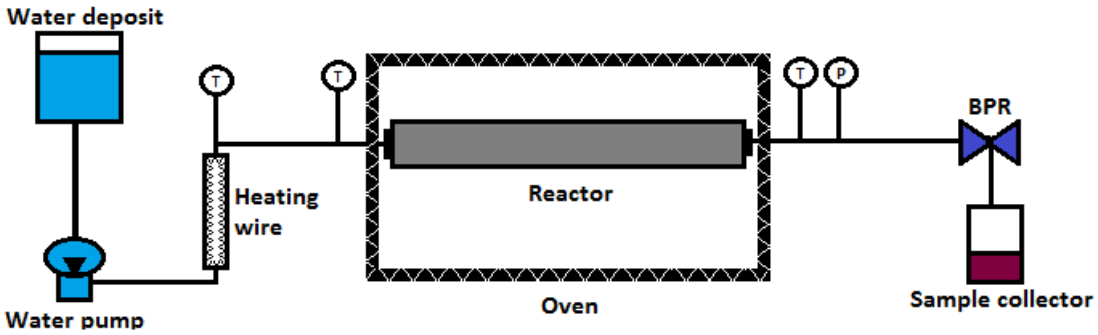

Figure 2.1 *Schematic representation of the subcritical water (SBW) semi-continuous experimental set-up. P – pressure regulator (manometer); T - temperature regulator (thermocouple); BPR - back pressure regulator.*

Distilled water is heated by a heating wire connected to a temperature regulator (T). Its pressure is measured with a pressure indicator (P). Water is pumped by a water pump (KNAUER

Preparative pump 1800) through a high-pressure tube connected to the reactor, filled with GP powder and glass spheres, to avoid compacting the GP bed. The reactor is placed inside an oven with temperature control.

After passing through the reactor, water goes through a filter, on to the sample collector for further analysis. Water temperature and pressure are monitored before and after the reactor. The pressure of the system is controlled by a Back-Pressure Regulator (BPR, Tescom Europe®, 26-1000).

In all the assays, pressure was kept constant at 70 bar, and four target temperatures were used: 190, 200, 220 and 240 ºC. The water flow rate was kept constant at 10 mL/min.

To initiate an experiment, the water pump is turned on with the selected flow rate, and the BPR is set for 70 bar. When pressure reaches this value, the water heating wire and the oven are turned on, and sample collection (in common Schott flasks) begins.

Two different types of SBW extraction/hydrolysis assays were performed. In the first ones, temperature increased continuously to the target temperature. The initial sample was collected until the temperature of the water exiting the reactor reached 50 ºC, the second sample as the water outlet stream temperature varied from 50 to 130 ºC, and the third sample as the water temperature varied from 130 to the maximum temperature chosen for that assay. From then on, temperature was kept constant for 30 minutes and the final sample was collected.

In the second ones, referred to as plateau assays, the temperature program established a series of two or three steps, with each step consisting of a ramp followed by a plateau. For example, in the assay targeted at 240 ºC, six samples were collected. One sample was collected in each of the three temperature ramps, as the temperature of the water exiting the reactor varied from ambient to 130 ºC, from 130 to 190 ºC, and from 190 to 240 ºC. One sample was also collected in each of the three plateaus, as temperature was kept constant, for 30 minutes, at 130 ºC, at 190 ºC, and finally at 240 ºC.[42]

Samples – liquors – were stored at -20ºC.

2.2.2. Extraction/hydrolysis efficiency, Yield in GP extract, and Carbohydrate quantification

Each liquor sample was used for sugar quantification based on the colorimetric method. Also sugar monomer quantification was performed, using HPLC. For this purpose, a 5 mL sample from each liquor collected was lyophilized to obtain GP extract. The amount of GP extract generated in each assay allowed the calculation of the yield in GP extract.

The residue remaining in the reactor was dried in an oven at 105 ºC for two days and its weight determined and compared to the amount of GP mass loaded into the reactor, to calculate the

degree of SBW extraction/hydrolysis. The residue was also characterized with the methodology described in chapter 2.1, and analyzed for its content in lipids, carbohydrates, lignin and protein.

The GP extract was submitted to a one-step (instead of a two-step) acid hydrolysis, a softer hydrolysis.[43]

After analyzing each liquor sample separately, all the samples collected throughout the whole SBW assay were mixed together and lyophilized. The extract obtained was stored at -20 ºC. The liquor containing the most carbohydrates was used for yeast growth.

2.3. ENZYMATIC HYDROLYSIS

2.3.1. PREPARATION AND ACTIVATION OF CHITOSAN PARTICLES

A (2% w/v) chitosan solution was prepared by dissolving 500 mg of chitosan (SIGMA-ALDRICH, ref: 448869) in 0.35 M acetic acid (25 mL). The solution was stirred for 5 hours without heating and placed for 20 minutes in an ultrasound bath to remove bubbles. The chitosan solution was added dropwise, with a syringe, to 100 mL of an alkaline coagulation solution composed by 1 M sodium hydroxide and ethanol (26% v/v), under gentle stirring (60 rpm), to obtain particles with approximately 2 mm diameter. The chitosan particles were washed with distilled water until neutrality.

Chitosan activation was achieved by incubation of the chitosan particles in 100 mL of a glutaraldehyde solution (4% v/v) prepared in 0.1 M sodium phosphate buffer at pH 7.0. Incubation was performed at room temperature, during 3 hours, in an orbital shaker at 120 rpm. An erlenmeyer was used to obtain a greater contact area between the particles and the solution. The activated support was exhaustively washed with 50 mM sodium acetate buffer at pH 5.,5 to remove excess glutaraldehyde.[44, 45]

2.3.2. ENZYME IMMOBILIZATION

Chitosan particles were incubated with an enzyme solution prepared by dissolving 125 µL of enzymatic solution (SIGMA-ALDRICH Viscozyme® L) in 100 mL of a 50 mM sodium acetate buffer at pH 5.5, and incubating with the chitosan particles for 3 hours at room temperature, under gentle stirring. The immobilized enzyme was washed with 50 mM sodium acetate buffer, pH 5.5, and washed over again with a 1 M sodium chloride and ethylene glycol (30% v/v) solution, to remove unbound, ionically and hydrophobically bound enzyme molecules. The washing liquids were collected to quantify protein by the Lowry method.[44,45,46]

Viscozyme is sold as a multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase, with a reported activity of 100 FBG/g, in which 1 FBG is the amount of enzyme which, at standard conditions - Somogyi-Nelson method - releases glucose or reducing carbohydrate with a reduction capacity equivalent

to 1 µmol glucose/min. Viscozyme has approximately 7 wt.% enzyme and 23 wt.% sucrose as stabilizer. It has a density of 1.21 g/mL, yielding an activity of approximately 0.121 FBG/µL. [44,47]

2.3.3. ARABINOGALACTAN AS A MODEL SUBSTRATE FOR ENZYMATIC HYDROLYSIS

Enzyme immobilization was tested with a model substrate, arabinogalactan (SIGMA-ALDRICH, ref: 10830). Both free and immobilized Viscozyme were tested for arabinogalactan hydrolysis, following the method below.

In a 15 mL vial, to a 150 mg sample of arabinogalactan were added 5 mL of 0.1 M sodium citrate buffer, pH 4.8, 20 µL of sodium azide 10% (v/v), 5 mL distilled water, and 75 µL of Viscozyme. The enzyme solution was always added last since the reaction is initiated by the addition of enzyme. In the assay with the immobilized enzyme, 250 mg of the activated support, with a bound amount of enzyme calculated to be equivalent to the free enzyme present in 75 µL of Viscozyme solution was used.

The contents of each vial were brought to 50 ºC by warming in the orbital shaker under stirring at 160 rpm. The vials were closed tightly and placed in a scintillation vial rack suitable for the shaking incubator, until the release of soluble sugars from the sample(s) became negligible when measured. To detect that condition, 500 µL aliquots were removed at predetermined times. The samples were centrifuged at 12000 rpm for 5 min, and then filtered through a 0.22 µm filter, and the supernatant was analyzed for carbohydrates.[48]

Blank assays were done without enzyme, and with enzyme but without substrate.

2.3.4. LIGNOCELLULOSIC BIOMASS SACCHARIFICATION

In a 15 mL vial, to a 150 mg sample of GP were added 5 mL of 0.1 M sodium citrate buffer, pH 4.8, 20 µL of sodium azide 10% (v/v), 5 mL distilled water, and 250 µL of Viscozyme. In the assay with the immobilized enzyme, 250 mg of the activated support, with a bound amount of enzyme calculated to be equivalent to the free enzyme present in 75 µL of Viscozyme solution was used.

Blank assays were done without enzyme, and with enzyme but without substrate.

The reaction conditions were the same as in chapter 2.3.3 (Arabinogalactan as a model substrate for enzymatic hydrolysis).

2.3.5. LOWRY METHOD FOR PROTEIN QUANTIFICATION

A calibration curve was made with BSA (bovine serum albumin) solutions, prepared from a standard 200 µg/mL solution. This solution was used as such, and was also diluted with 0.1 M sodium phosphate buffer (pH 7.0), added to 20, 50, 100, or 150 µL of BSA solution, making up a

total volume of 200 μL.

Each sample to be analyzed (200 μL) required the addition of 1 mL Lowry reagent, prepared as follows: 2% (w/v) sodium and potassium tartrate solution (diluted 1:100) + 2% (w/v) copper sulphate penta-hydrated solution (diluted 1:100) + filling with 30 g/L sodium carbonate and 4 g/L sodium hydroxide solution until 25 mL final volume.

After addition of 1 mL Lowry reagent to 200 μL of each sample in an Eppendorf, its contents were vortexed for 5 s, and the solution was left to stand at room temperature for 10 minutes. 200 μL of Folin-Ciocalteau reagent (diluted 1:2) were added to each eppendorf, vortexed for 5 s, and left to stand at room temperature for 30 minutes. Then, the samples were analyzed in the spectrophotometer at 750 nm wavelength.

2.4. OUTLINE OF THE PROGRESSION OF BIOMASS FRACTIONATION

Grape pomace (GP) was, first of all, chemically characterized, to determine its content in lipids, soluble and structural sugars, proteins, ashes and lignin. The weight of GP loaded into the reactor, together with the weight of GP residue left in the reactor after hydrolysis with subcritical water (SBW), allowed the determination of the extraction / hydrolysis efficiency of the process. The output of the SBW extraction / hydrolysis was a liquor that was lyophilized to obtain a GP extract. The weight of GP extract together with the weight of GP loaded into the reactor allowed the determination of the yield of GP extract.

The GP residue left in the reactor was chemically characterized. The GP extract was characterized only in what concerns its sugar content and sugar profile.

Figure 2.2 summarizes all these steps.

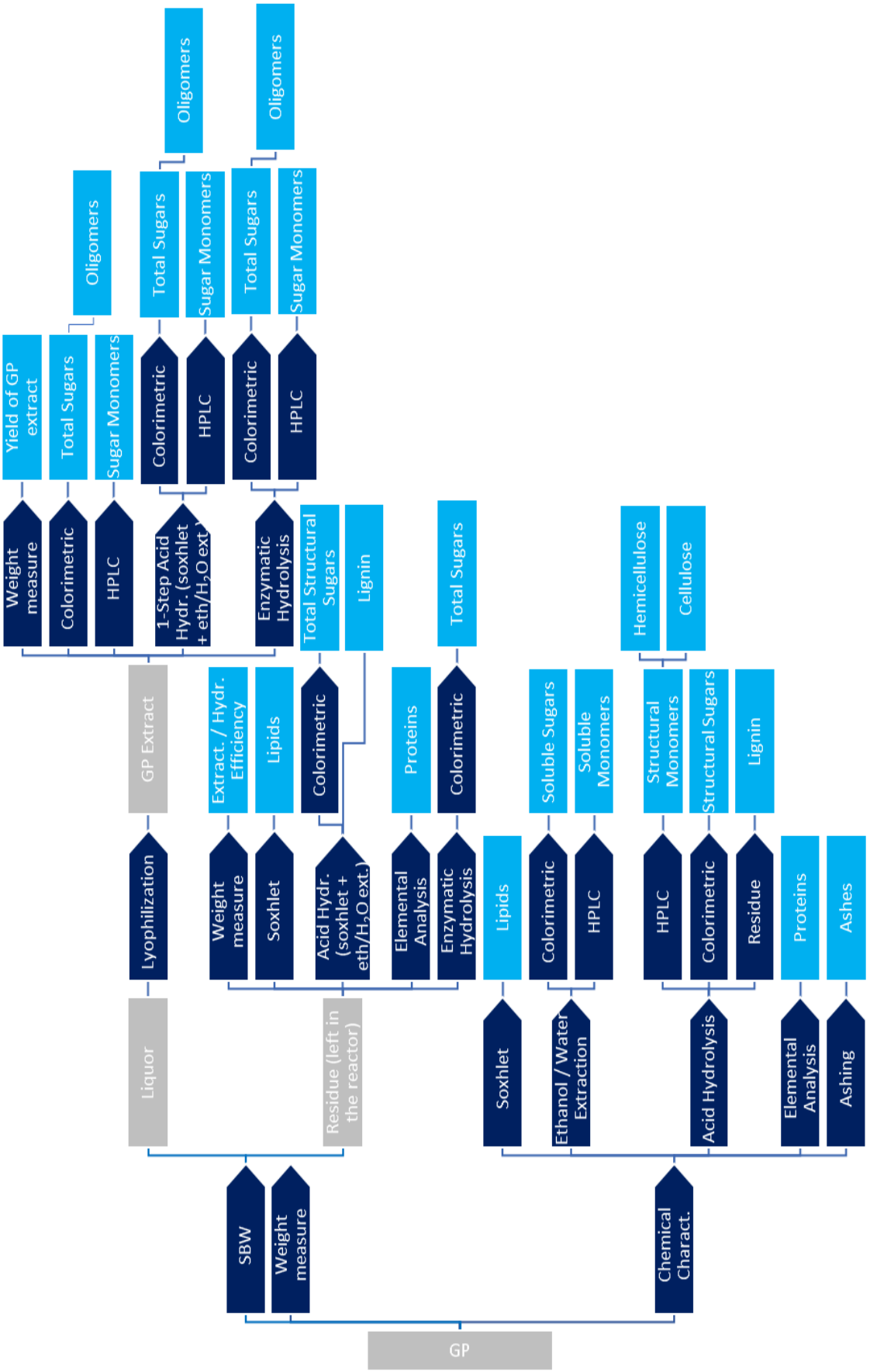

Figure 2.2 Grape pomace fractionation progression.

2.5. YEASTS GROWTH MEDIUM, INOCULUM & CELLS GROWTH

All the yeast strains used in this work were supplied by the Portuguese Yeast Culture Collection (PYCC), Caparica, Portugal (Table 2.1).

Yeast strains were maintained in a cold chamber at 4 °C on yeast extract peptone dextrose (YPD) medium containing: 20 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, and 20 g/L agar.

Table 2.1 *Yeast strains used in the present study.*

Yeast	Origin
Rhodotorula babjevae	Leaves
Rhodotorula mucilaginosa	Water
Lipomyces starkeyi	Soil

2.5.1. GROWTH MEDIUM

The growth medium used in control experiments for lipid production was optimized by Li et al.[49] The medium containing 70 g/L glucose, 0.1 g/L $(NH_4)_2SO_4$, 0.75 g/L yeast extract, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 0.4 g/L KH_2PO_4, was sterilized at 121 °C for 15 min, and supplemented with 1.91×10^{-6} mmol/L $ZnSO_4$, 1.50 mmol/L $CaCl_2$, 1.22×10^{-4} mmol/L $MnCl_2$, and 1.00×10^{-4} mmol/L $CuSO_4$. Carbon source were prepared ten times concentrated with 70g of glucose in 100 mL of sterile water, after which it was sterilized by filtration under vacuum. A minimal medium solution containing the main reagents was prepared in 90mL sterile water and pH was adjusted. A supplements solution containing the minor components ($ZnSO_4$, $MnCl_2$ and $CuSO_4$) was prepared 1000 times concentrated in 10mL sterile water. In a second experience, glucose amount was reduced to 35g/L. The amount of yeast extract and $(NH_4)_2SO_4$ were reduce accordingly.

GP extract medium was the same developed by Li et al, but with some modifications. The medium containing 39 g/L carbohydrates from the liquor, 0.1 g/L $(NH_4)_2SO_4$, 0.75 g/L yeast powder, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 0.4 g/L KH_2PO_4, was sterilized at 121 °C for 15 min, and was then supplemented with 1.91×10^{-6} mmol/L $ZnSO_4$, 1.50 mmol/L $CaCl_2$, 1.22×10^{-4} mmol/L $MnCl_2$, and 1.00×10^{-4} mmol/L $CuSO_4$, and centrifuged at 20000rpm for 15 min, to remove the pellet.

2.5.2. GROWTH

A 10 ml pre-inoculum was prepared in a 50mL and incubated overnight at 30ºC, under constant agitation stirring at 200 rpm Erlenmeyer. After 24 h, The pre-inoculum was then transferred to 90mL of the corresponding medium in a 1000mL erlenmeyer, and incubated overnight at 30ºC, under constant agitation stirring at 200 rpm. Two assays were done in a glucose supplemented medium, where different pH values (pH4 and pH6) were analyzed.

2.5.3. Optical Density

Growth was followed by optical density at 640nm (Amersham Biociences, Ultrospec 3100 Pro UV/Visible Spectrophotometer) until 144h.

2.5.4. Dry Weights

A calibration curve between Optical Density (OD) at 640nm and dry weights (DW) was used to correlate OD measured during growth assays with total biomass. For different dilutions, 15mL of yeast resuspension was filtrated under vacuum using pre-weighted 0.22µm filters, dried overnight in a hot chamber at 80ºC and the weight was measured.

2.5.5. Collecting Cells

After growing, oleaginous yeast cells were collected by centrifugation for 10 min at 9500 rpm (Sigma 3-16K Sartorius) and the supernatant was removed. PBS buffer solution (pH=7.4) was added to the pellet and samples were resuspended and centrifuged again, under the same conditions. Supernatant were removed and wet-cells mass was then weighted. After that, cells were lyophilized under vacuum at 770 mbar, during 48h (CHRIST ALPHA 1-4, Braun Biotec International) and weighted. Dry biomass was used for lipid analysis.

2.6. Yeasts Lipid Production

2.6.1. Analysis of lipid by Fluorimetry

Fluorescence spectrometry is based on the measurement of the intensity and wavelength distribution of the radiation emitted by a sample after being excited by radiation of a given wavelength. It is used to identify the presence and measure the amount of specific molecules in a medium.

Despite all its benefits, microbial lipid production is strongly dependent on environmental factors, such as the carbon source, C:N ratio, oxygen availability. Therefore, it is essential to monitor cell lipid content during the microbial lipid production process.[50]

To monitor cell lipid content, a lipid measurement has been previously proposed using the Nile Red fluorescent stain for quantification of microbial lipids. Its fluorescence is produced in highly hydrophobic environments and quenched in hydrophilic ones.[50] The fluorescence of Nile red by itself without lipids existed at around 600–605nm with a slight peak, but was negligible. Nile red is nearly insoluble in water and its fluorescence immediately quenches in aqueous solution.

While NR can stain most lipids, its fluorescence character varies depending on the lipid class. NR emits at a shorter wavelength when bound to neutral lipids, compared to polar compounds. Unsaturated fatty acids exhibit stronger fluorescence intensity than saturated fatty acids.[51]

The fluorescence intensity corresponding to the intracellular lipid amount was determined at the peak of the corrected spectrum. The value showed a linear relation with the lipid content of various oleaginous fungi and yeasts measured by the conventional method.[52]

Lipid detection by Nile red has been commonly measured with excitation at 480–490 and at 510–560 nm. The former target is neutral lipids to fluoresce and the latter target is polar lipids.[52]

Fluorescence spectroscopy technique, also known as fluorometry, was used to analyze yeast lipid production. Nile red (NR), a fluorescent probe of intracellular lipid droplets, was used as a fluorophore to quantify yeast's neutral and polar lipid content during yeast's growth. A 1mL final volume sample, containing 50µL of Nile Red 0.025µg/mL was used to obtain spectral data. The sample was previously diluted in PBS buffer and the cellular concentration was adjusted to OD 0.8 - 1.0 in all the samples. Spectral acquisition was performed before adding NR (control), and 2 minutes after addition since it corresponds to NR maximum emission peak.

Fluorescence spectra were acquired on a fluorimeter, using the software FluorEssence (Horiba Scientific). The excitation wavelength was set to 488nm and the emission spectrum was acquired from 500nm to 750nm. The 2nm slit was used for the excitation wavelength and the selected range of wavelengths. Polar lipids quantification was obtained by fluorescence values measured at 637 nm, and neutral lipids quantification by fluorescence values measured at 583 nm.

The software Origin Viewer v92 (OriginLab) was used to export results list to Excel - software where data processing was done.

2.6.2. Quantification of Lipid by Gravimetry

To measure total lipid content, gravimetric analysis was used following reference [51], as follows. Triplicate 100mg samples of freeze-dried cells (lyophilized) were transferred to 50mL vials, with 30mL Folch solvent (2:1 of CHCl3:MeOH, v/v), 0.5mm zirconia beads and 3.5mm glass beads. Cells were homogenized five times in a homogenizer (Vortex Mixer, Labnet) for 30s with 30s intervals on ice. After that, 6mL 0.9% NaCl was added for improved phase separation. Then, 15 mL of the chloroform-rich phase was evaporated gradually in a pre-weighted 25mL amber vial under a slow stream of nitrogen. Lipid weight after extraction was used to calculate the total lipid as a percent of cell dry weight.

2.6.3. Carotenoids Characterization by UV-Vis Spectrophotometry

Oil extract samples (10-25 mg) were dissolved in 1mL acetone and analyzed by UV-Vis spectrophotometry (452 nm) in a quartz cell, to quantify total carotenoids in biomass, using a β-carotene calibrating curve (0, 1, 5, 10, 15 ppm).[53]

3 RESULTS AND DISCUSSION

3.1. GRAPE POMACE CHEMICAL CHARACTERIZATION

As mentioned earlier, the GP feedstock was frozen the same day it arrived, and dried. Two GP drying methods were compared: drying in an oven at 60 ºC for two days, and drying in a lyophilizer for two days.[54] The two results obtained for the water content of GP after the drying step were in good agreement: 70.5 wt.% for drying in a lyophilizer, and 70.1 wt.% for drying in the oven at 60 ºC.

After the drying step, GP was milled to ca. 2 mm particles. The water content of GP powder was found by gravimetry: a sample was heated in the oven at 105 ºC overnight, and the reduction in weight determined. The GP powder obtained upon lyophilization had a water content of approximately 6 wt.%, whereas GP dried at 60 ºC had a water content of approximately 5 wt.%. Thus, GP as supplied had a water content of approximately 75%.

It must be noted that drying is required for the purpose of quantification in the studies to be performed, but would be avoided if the process were applied at an industrial site, since water has to be added for the SBW pretreatment. When applied, the drying method must not affect the carbohydrate content of the biomass. This is certainly the case of lyophilization and oven-drying at 60 ºC. For practical reasons, the chosen drying method for the subsequent experiments was lyophilization.

GP powder was used in all subsequent studies, and henceforth referred to as GP.

The second step was the chemical characterization of GP, using several methods described earlier. The major components identified in GP were carbohydrates, proteins, lipids, ash (resulting from inorganic salts), and lignin (Table 3.1; two replicate measurements, except in the case of protein).

Table 3.1 *GP composition.*

Component	Quantification (wt. %)	
	Lyophilized GP	**Oven dried GP**
Lipids	11.2 ± 2.4	10.7 ± 2.6
Total carbohydrates	32.7 ± 5.1 Soluble 7.1 ± 0.8 Structural 25.5 ± 4.3 Hemicellulose 14.0 ± 2.8 Cellulose 11.5 ± 3.1	28.3 ± 1.1 Soluble 6.8 ± 0.4 Structural 21.5 ± 1.4 Hemicellulose 11.2 ± 1.6 Cellulose 10.3 ± 2.2
Protein	15.2	15.2
Ash	8.0 ± 0.1	6.0 ± 1.0
Lignin	25.4 ± 3.5	27.8 ± 1.3

The results obtained for lyophilized GP and oven dried GP generally agree within the error associated to the measurements.

As shown in Table 3.1, GP has approximately 31 wt.% of total carbohydrates. The following most abundant constituent is lignin – 27 wt.% - followed by proteins – 15 wt.% - lipids – 11 wt. % - and ashes – 7 wt.%.

The quantification of carbohydrates requires the separation of free sugars (soluble) from the structural ones (insoluble). The free sugars are directly available in GP, unlike structural sugars, which are present as part of cellulose and hemicellulose structures.

Oven-dried GP yielded approximately 6.8% soluble carbohydrates, against 7.1% for lyophilized GP, as measured by the colorimetric method. HPLC analysis allowed the identification and quantification of fructose and glucose, in very similar amounts, which exist in grapes at the time of harvest (Fig. 3.1).

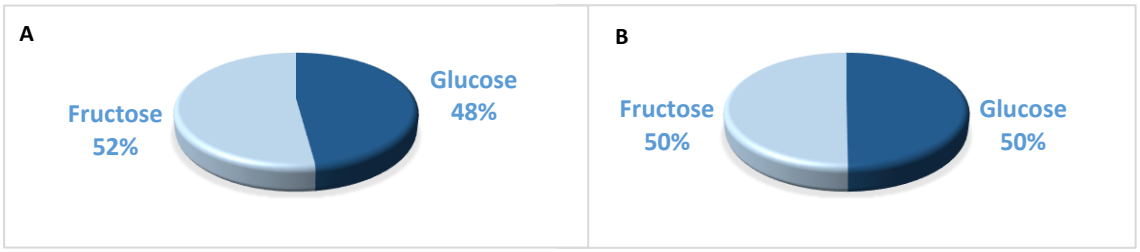

Figure 3.1 *Relative amounts of soluble sugars quantified in GP by HPLC analysis. A – Lyophilized GP. B – Oven-dried GP.*

GP submitted to acid hydrolysis for recovery of structural carbohydrates as sugar monomers led to approximately 23.5% structural carbohydrates, as measured by the colorimetric method. HPLC analysis allowed the identification and quantification of glucose, the constituent of cellulose, which makes up almost half of the total carbohydrates measured, as well as rhamnose, arabinose, galactose and xylose, from hemicellulose (Fig. 3.2).

The results obtained for GP characterization agree with results obtained in other studies.[3,13,55]

3.2. SBW Extraction / Hydrolysis

3.2.1. Process Efficiency and Yield of GP Extract

In the present work, it was studied the influence of temperature on the SBW extraction / hydrolysis efficiency of GP feedstock. Various assays were performed, reaching maximum temperatures of 190, 200, 220 and 240 °C. Two different types of assays were performed, as indicated earlier:

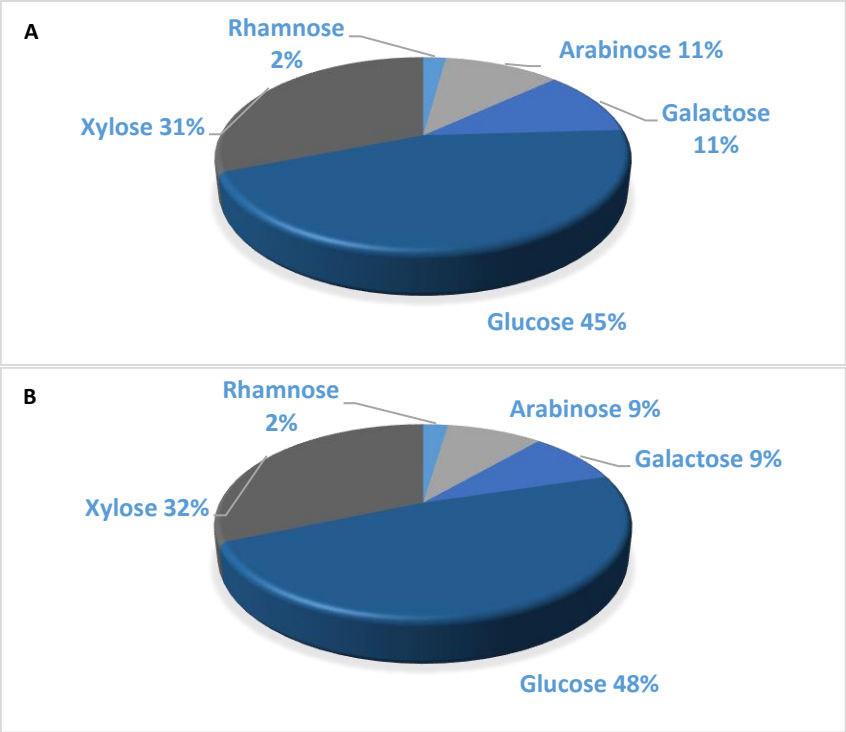

Figure 3.2 *Relative amounts of structural sugars quantified in GP by HPLC analysis. A – Lyophilized GP. B – Oven-dried GP.*

- In the first ones (SBW-200 and SBW-220), temperature increased continuously up to the target temperature. Samples were collected at temperature intervals of 50 ºC. For example, in the SBW-200 assay, a fraction was collected until the outlet temperature of water reached 50 ºC, another as that temperature varied from 50 to 100 ºC, and then from 100 to 150 ºC, from 150 to 200 ºC and, finally, a last sample was collected while the temperature of the outlet water remained at 200 ºC for 30 minutes.
- In the second ones (SBW-190p and SBW-240p), the objective was to progress in a series of plateaus (p stands for plateau). The first one was 130 ºC, aimed at recovering extractable material, not requiring dissolution of the biomass, the second one was 190 ºC, aimed at recovering hemicellulose, and the last one, in the case of the SBW-240p assay, at 240 ºC, aimed at achieving the maximum cellulose hydrolysis possible.[42]

In the first type of assays, there was a large temperature difference between the temperature of the water entering the reactor and the water leaving the reactor (Fig. 3.3). This was due to the poor heating of the system provided by both the heating wire and the oven. Only at the end of the assay did the two temperatures become equal. This means there was a temperature gradient throughout the length of the reactor.

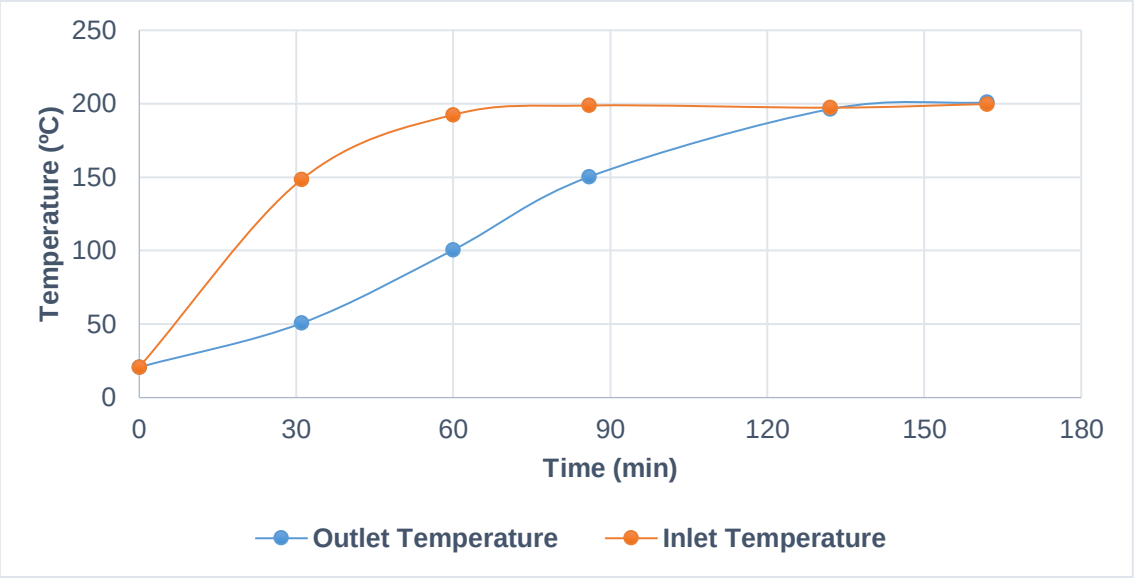

Figure 3.3 *Difference between the water inlet temperature and the water outlet temperature in assay SBW-200, at a water flow rate of 10 mL/min, at 70 bar.*

Quite the opposite happened in assays performed according to the plateau scheme, which led to very good agreement between the water inlet and outlet temperatures (Fig. 3.4), being the most indicated for further studies.

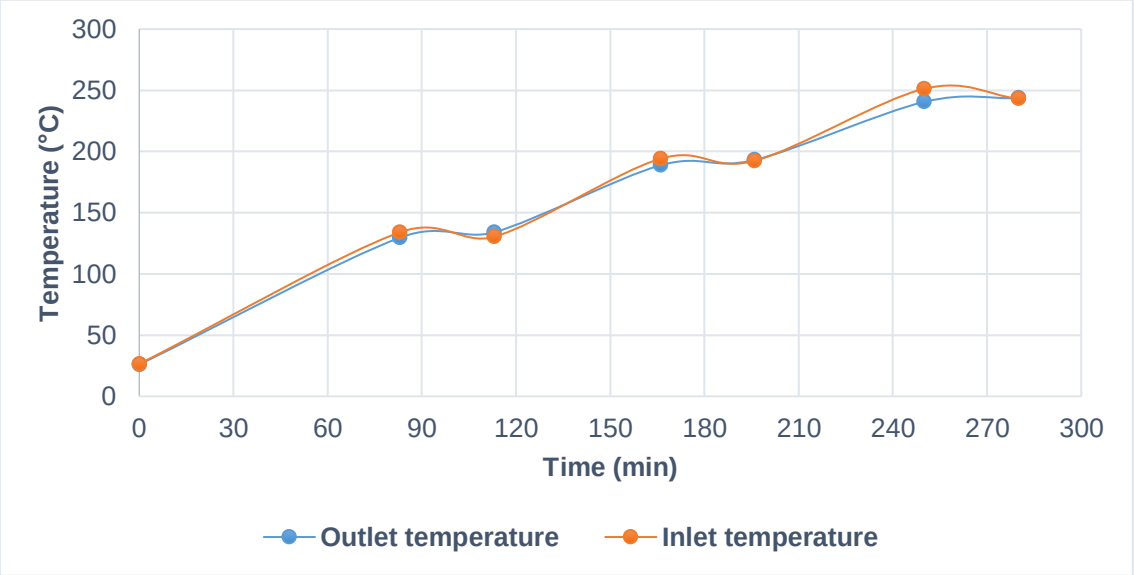

Figure 3.4 *Difference between the water inlet temperature and the water outlet temperature in assay SBW-240p, at a water flow rate of 10 mL/min, at 70 bar.*

In the assays carried out, a color change was observed in all the samples collected (Fig. 3.5). The first samples were clearer, slightly red. As the extraction / hydrolysis went on, the color accentuated, turning into purple-red. When temperature increased to its maximum value, the samples gained turbidity and became brownish. This should be due to the fact that, in the beginning, anthocyanins extraction occurs, leading to a red color; but at higher temperatures the Maillard reaction takes place since proteins are also present in the samples, leading to the

brownish color. [56]

Figure 3.5 *Samples collected along the SBW-200. Samples were collected at 50 ºC intervals, up to a temperature of 200 ºC (1 – ambient to 50 ºC; 2 – 50 to 100 ºC; 3 –100 to 150 ºC; 4 –150 to 200 ºC; 5 – at 200 ºC).*

The degree of biomass extraction / hydrolysis (Table 3.2) is given by the weight difference of GP loaded into the reactor and GP residue recovered from the reactor at the end of an experiment, as follows:

$$Extraction\ and\ hyrolysis\ of\ GP\ (\%) = \frac{GP\ (input) - GP\ (reactor)}{GP\ (input)} * 100 \quad \textbf{(Equation 1)}$$

The yield of GP extract (Table 3.2) is measured by the amount of extract obtained, by lyophilizing the sugar-rich liquor collected throughout the whole assay. It can be calculated as follows:

$$Yield\ of\ GP\ extract\ (\%) = \frac{GP\ (extract)}{GP\ (input)} * 100 \quad \textbf{(Equation 2)}$$

Table 3.2 *Degree of extraction/hydrolysis of GP with SBW, and yield in GP extract, in assays carried out at different temperatures, at a water flow rate of 10 mL/min and 70 bar.*

Assay	Temperature (ºC)	Extraction/hydrolysis (%)	Yield of GP extract (%)
SBW-190p	190	59	25
SBW-200	200	56	48
SBW-220	220	60	31
SBW-240p	240	71	31

As temperature increases, the ionic product of water also increases, turning water into a strong catalyst to hydrolyze biomass. As such, the efficiency of the extraction / hydrolysis process tends to increase directly with the temperature. The results obtained (Table 3.2) generally obey this trend. The first three values agree within the error associated to these measurements, and the fourth value is higher.

The way the assays were conducted influenced their duration. The heating time is directly proportional to the target temperature, and thus assays performed at higher temperatures were

generally longer (Fig. 3.6, 3.7).

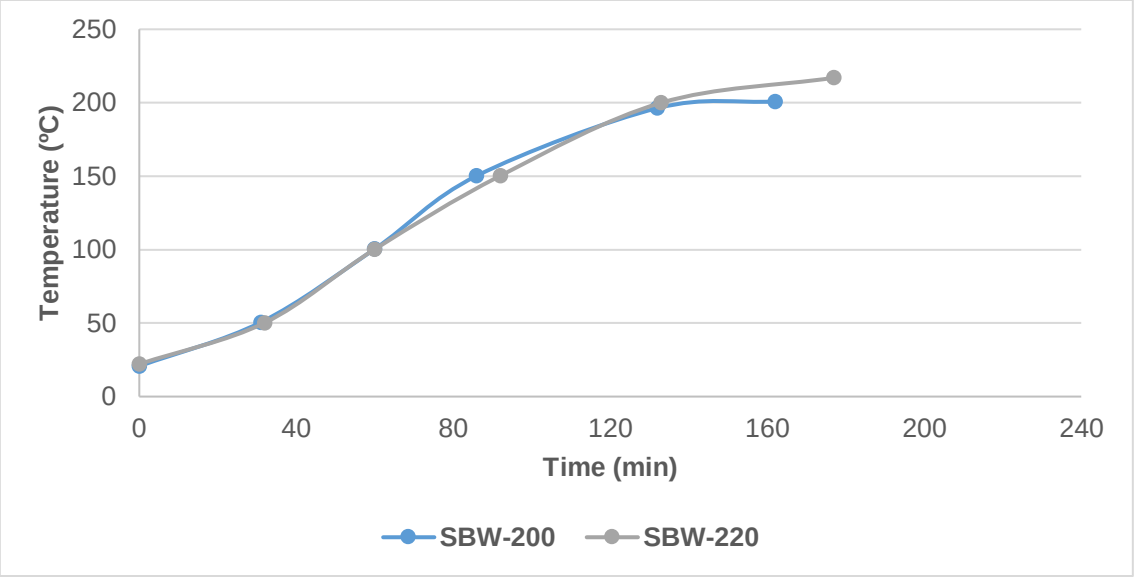

Figure 3.6 *Water outlet temperature in assays SBW-200 and SBW-220, at a water flow rate of 10 mL/min, at 70 bar.*

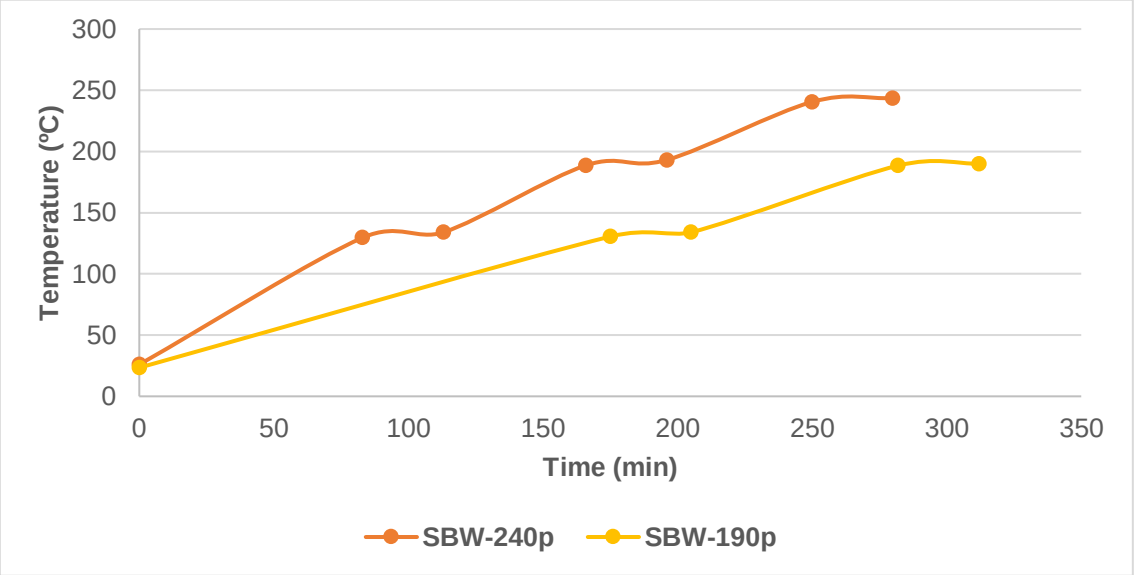

Figure 3.7 *Water outlet temperature in assays SBW-190p and SBW-240p, at a water flow rate of 10 mL/min, at 70 bar.*

Even so, it was expected that the yield of GP extract would not vary significantly up to the temperature at which cellulose starts to depolymerize (near 230 ºC)[42], and then increase, depending on the extension of cellulose dissolution. The maximum temperature reached was 240 ºC in assay SBW-240p. It is not very high, and thus it was expected to lead to a low degree of cellulose depolymerization.[42]

A factor to keep in mind is that only assays SBW-190p and SBW-240p were performed with a constant temperature throughout the reactor. When looking at the results obtained for these two

assays, the prediction holds true, with both a higher extension of GP extraction/hydrolysis and higher yield in GP extract in assay SBW-240p than in assay SBW-190p.

Looking at assays performed without plateaus, the value obtained for the yield of GP extract was extremely high in the SBW-200 assay, compared with the SBW-220 assay.

Table 3.2 also shows that there is a discrepancy between the amount of biomass that disappears from the reactor, measured by percentage of biomass extraction / hydrolysis, and the yield of GP extract. This difference could be partly explained by the production of volatile compounds that are released during the process, as well as loss of mass as the system cools down at the end of the assay. Any material extracted but not recovered as GP extract accentuates the difference between the degree of biomass extraction/hydrolysis, and the yield of GP extract. In any case, the differences observed should be lower. They are reasonably low for the SBW-200 assay, but compared to previous studies with the same apparatus, too low.[57]

Replicate measurements would be needed to clarify these findings.

3.2.2. Yield of Carbohydrates and Sugar Profile

As mentioned earlier, carbohydrates (total reducing sugars) were quantified using a colorimetric method, and monosaccharides were identified and quantified using HPLC.

The results obtained (Table 3.3) suggest similar amounts of total carbohydrates recovered in all the assays, of approximately 26 g / 100 g GP. This agrees with the rationale behind these experiments. Up to 130 ºC, only extractives should be recovered, followed by hemicellulose constituents at temperatures up to 220 ºC, and then some hydrolysis of cellulose.[42]

Table 3.3 *Total carbohydrates and monosaccharides recovered in assays carried out at different temperatures, at a water flow rate of 10 mL/min and 70 bar.*

Assay	Temperature (ºC)	Carbohydrates recovered (g/100 g GP)	Monosaccharides recovered (g/100 g GP)
SBW-190p	190	24	n.d.*
SBW-200	200	26	5.6
SBW-220	220	16	n.d.*
SBW-240p	240	27	4.1

*n.d. means not determined

In this respect, the amount of carbohydrates recovered in assay SBW-220 is too low. This can be clearly seen when representing the carbohydrates recovered as a function of temperature (Fig. 3.8, 3.9).

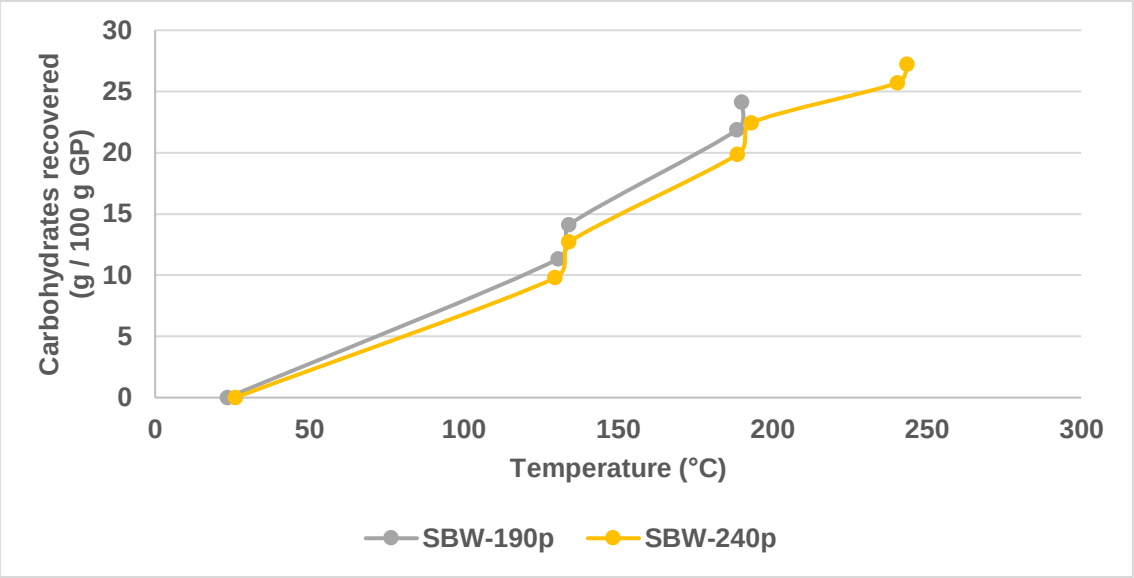

Figure 3.8 *Carbohydrates recovered in assays SBW-190p and SBW 240p, performed at a water flow rate of 10 mL/min, at 70 bar.*

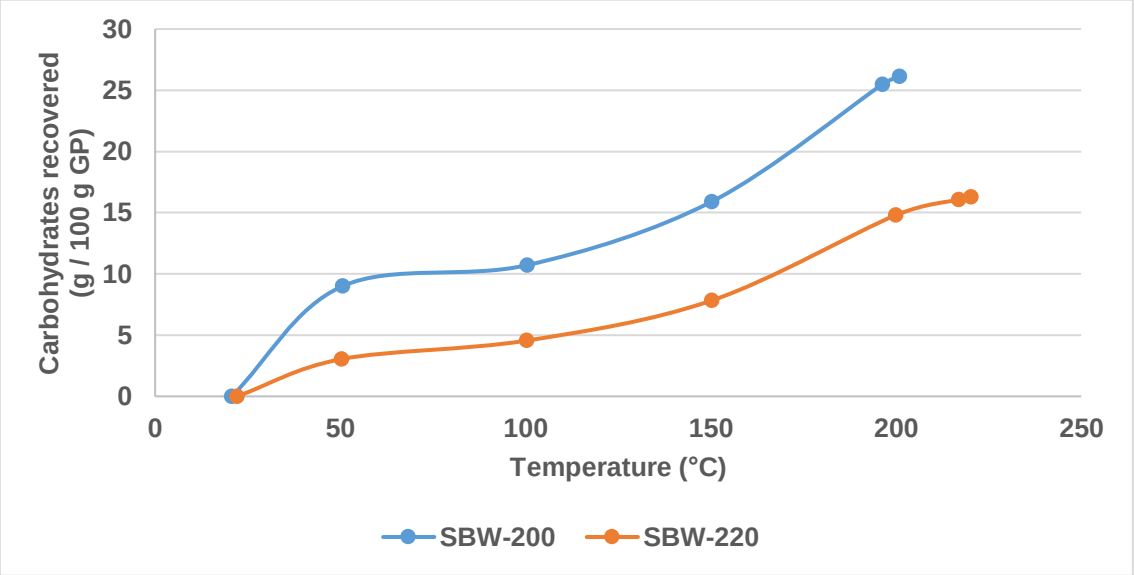

Figure 3.9 *Carbohydrates recovered in assays SBW-200 and SBW 220, performed at a water flow rate of 10 mL/min, at 70 bar.*

Assay SBW-200 and the assays in plateaus led to similar results (assay SBW-220 again seems an outlier). Up to 130 ºC, the yield of GP extract is about 15%. This must correspond to the extraction of soluble sugars (approximately 7 g/ 100 g GP) and other extractives, such as waxes, lipids, some protein or inorganic material. It is known that the liquors have phenolic compounds in their composition, but their amounts are insignificant in comparison with the carbohydrate content.[3]

To clarify this, the residue that remained in the reactor at the end of the SBW-240p assay was submitted to more detailed analysis (Table 3.4). As seen earlier, GP had approximately 27 wt.% of lignin, 15 wt.% of protein and 11 wt.% of lipids, whereas the residue left in the reactor had

approximately 26 wt.% of lignin, 8 wt.% of protein and 19 wt.% of lipids. Considering the amounts of GP and GP residue weighed, the above percentages correspond to an amount of material that exceeds that collected in the form of carbohydrates. This means that if all the protein, lignin and lipids not found in the residue left in the reactor were collected as GP extract, these three quantities together would represent a higher amount of material than the amount of carbohydrates collected.

Only assay SBW-200 yielded an amount of GP extract that was almost double in weight as the amount of carbohydrates, and it has already been pointed out that in some respects, the value for the yield of GP extract in this assay seems to be in error.

Table 3.4 *Composition of the residue left in the reactor after SBW treatment in the SBW-240p assay.*

Component	Quantification (g / 100 g residue left in reactor)
Lipids	18.5
Total carbohydrates	6.2
Protein	7.6
Lignin	25.6

Again, replicate measurements would be needed to clarify these aspects.

Focusing now on the shape of the progress curve for the SBW-240p assay, as temperature increases above 130 ºC but before it reaches the temperature at which cellulose starts to decompose, the target is hemicellulose. Considering that GP has approximately 11% of hemicellulose, a jump in carbohydrates recovered of approximately 11% would be expected in this temperature range, and is indeed observed.

As for the decomposition of cellulose, looking at the SBW-240p assay, a small increase of approximately 4% in carbohydrates recovered is observed as temperature increases from the 190 ºC plateau to the 240 ºC plateau and until the end of the experiment. This value is not very high, but other authors refer higher temperatures for the recovery of glucose from cellulose and therefore it was not expected to be able to recover 100 % of the total existing glucose, also due to the partial degradation of released glucose molecules in the process.[42]

Given that the most aggressive conditions reached in the assays were not expected to be able to decompose the whole amount of cellulose of GP, as indeed the progress curve in Figure 3.10 evidences, it was also not expected to be able to practically close a mass balance for carbohydrates considering the amount of carbohydrates recovered as GP extract and the amount of carbohydrates left in the residue remaining in the reactor, as is observed. The residue remaining in the reactor has approximately 6.2 wt.% of carbohydrates, which represent approximately 1.8 g/100 g GP. GP has about 11 wt.% cellulose. Admitting that those 1.8 g were glucose from cellulose, about 9 g of glucose from cellulose should have been released and

extracted from GP.

The amount of carbohydrates obtained (Fig. 3.10, 3.11) is directly correlated with the amount of GP extract, with a similar progression. The amount of GP extract in the SBW-200 is too high, as referred earlier. If it were similar to the values obtained in the other assays, the curves for the yield of GP extract and the yield of carbohydrates would be more similar to those in assay SBW-240p.

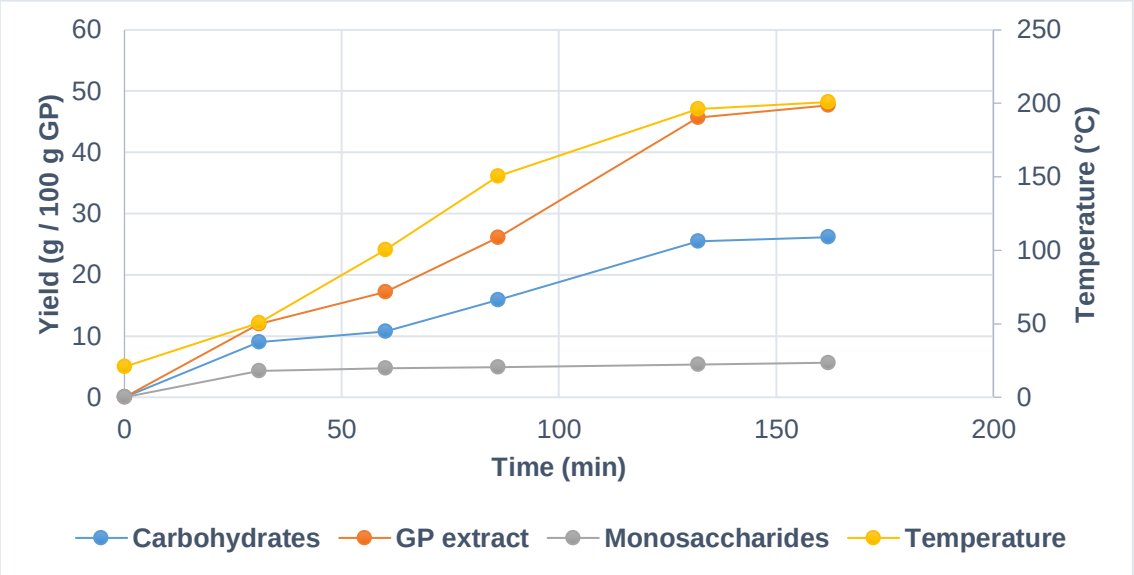

Figure 3.10 *Yield of GP extract, of total carbohydrates and of monosaccharides in assay SBW-200, performed at a water flow rate of 10 mL/min, at 70 bar.*

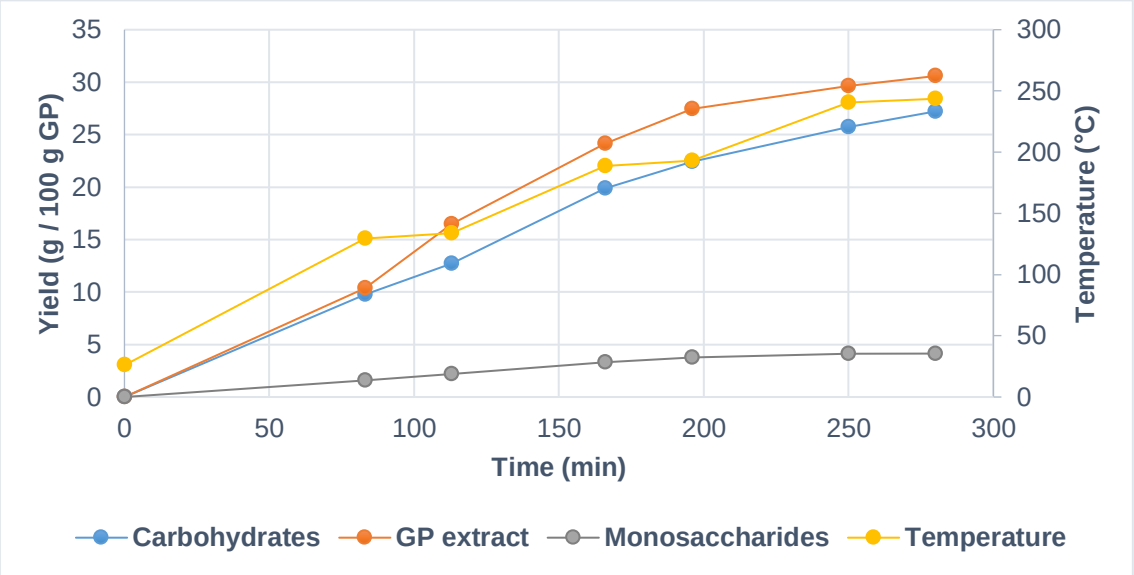

Figure 3.11 *Yield of GP extract, of carbohydrates and of monosaccharides in assay SBW-240p, performed at a water flow rate of 10 mL/min, at 70 bar.*

The result for the amount of monosaccharides seems to be in error. In fact, GP has approximately 7 wt.% of soluble sugars, and this amount should be present in the liquors collected, at least those

collected at the lowest temperatures. The results obtained by HPLC indicate that the monosaccharides in the sugar-rich liquors never exceed 4-6 wt.%. Also, this value does not change as temperature increases and the hydrolysis of GP starts taking place. It is known that at higher temperatures, the degradation of the monosaccharides occurs, to give compounds such as 5-HMF and furfural.[3] But before this is observed, the amount of monosaccharides should increase to some extent.

In any case, the marked difference between the progress curves for total carbohydrates and for monosaccharides (Fig. 3.10 – 3.11) indicates that when hydrolysis of biomass takes place, it produces a much greater amount of oligosaccharides than of monosaccharides.

Evidence that the amount of monosaccharides measured by HPLC is not entirely correct comes also from the sugar profile obtained in the GP extracts. The SBW-200 assay (Fig. 3.12) showed that before temperature reached 150 ºC, only the monosaccharides available in GP as soluble sugars were recovered, in similar amounts, as expected. But from that temperature on, arabinose, resulting from the hydrolysis of hemicellulose, was also detected. So were minor amounts of galactose, xylose and mannose, also resulting from the hydrolysis of hemicellulose, not represented in Fig. 3.12. The relative amount of fructose was very similar to that of glucose, as expected.[58]

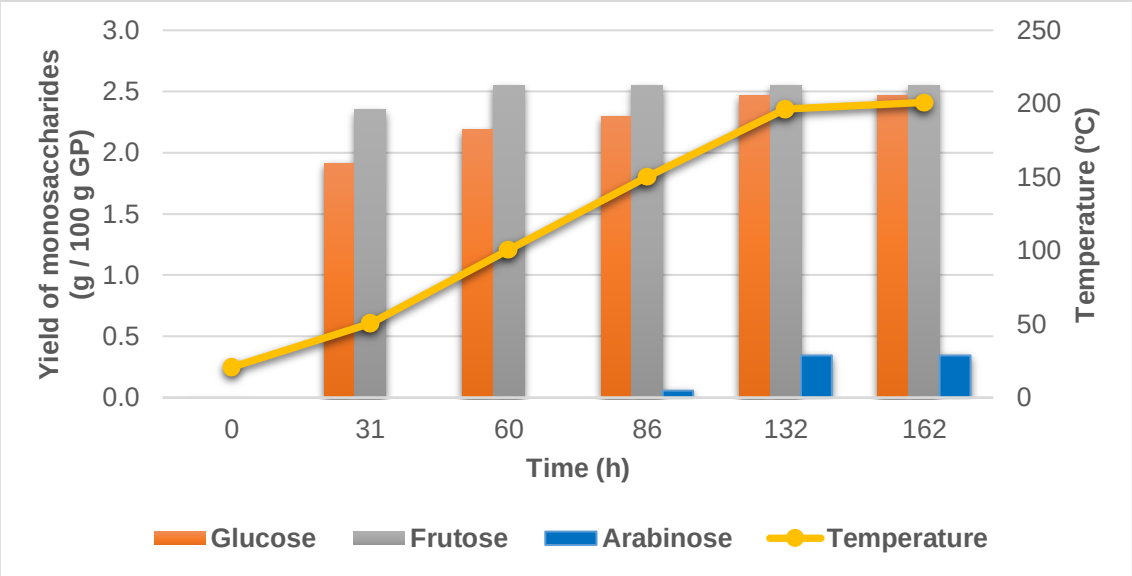

Figure 3.12 *Yield of monosaccharides and temperature profile in assay SBW-200, performed at a water flow rate of 10 mL/min, at 70 bar.*

In the SBW-240p assay (Fig.3.13), the results obtained were similar. The amounts of glucose and fructose were not represented because HPLC analysis yielded values that were too low and undoubtedly in error. These two monosaccharides exist in GP as soluble sugars, in similar amounts, as shown in Fig.3.12, and are extracted from the start of the assay. It was chosen to represent only arabinose, xylose and galactose. The absolute amounts of these monosaccharides may also be in error, but their presence was detected. Arabinose was the most

abundant monosaccharide from hemicellulose found in GP extract, as found also in the SBW-200 assay, followed by xylose and galactose. Again, evidence of the dissolution of hemicellulose was found only after temperature reached 150 ºC, in minute amounts, and more expressively as temperature rose to near 190 ºC, as expected.[42]

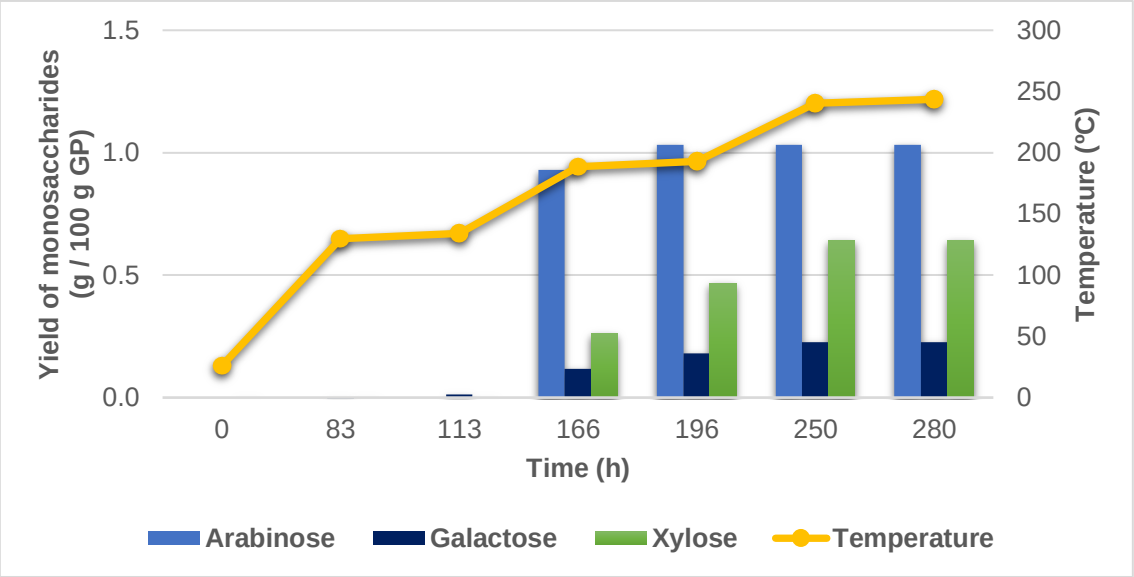

Figure 3.13 *Yield of monosaccharides and temperature profile in assay SBW-240p, performed at a water flow rate of 10 mL/min, at 70 bar.*

In summary, and selecting assays SBW-200 (Fig. 3.14) and SBW-240p (Fig. 3.15) as reference, it was possible to recover about 84% of the total amount of carbohydrates of GP as GP extract, corresponding to about 27 g /100 g GP. Of these carbohydrates, about 20% were in the form of monosaccharides, the remaining 80% being in the form of oligosaccharides.

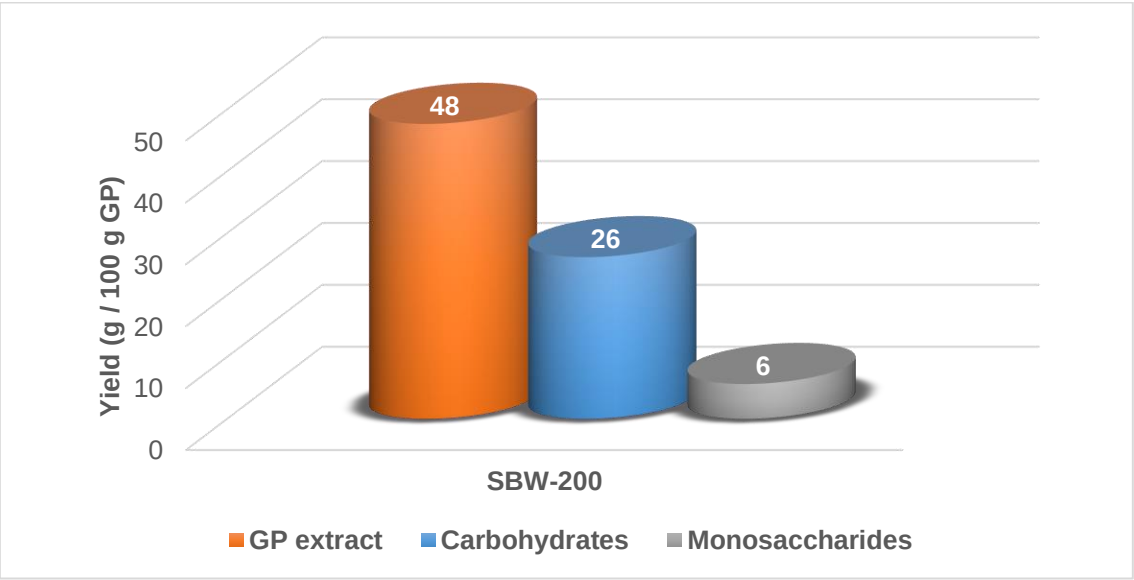

Figure 3.14 *Yield of GP extract, of carbohydrates and of monosaccharides in assay SBW-200, performed at a water flow rate of 10 mL/min, at 70 bar.*

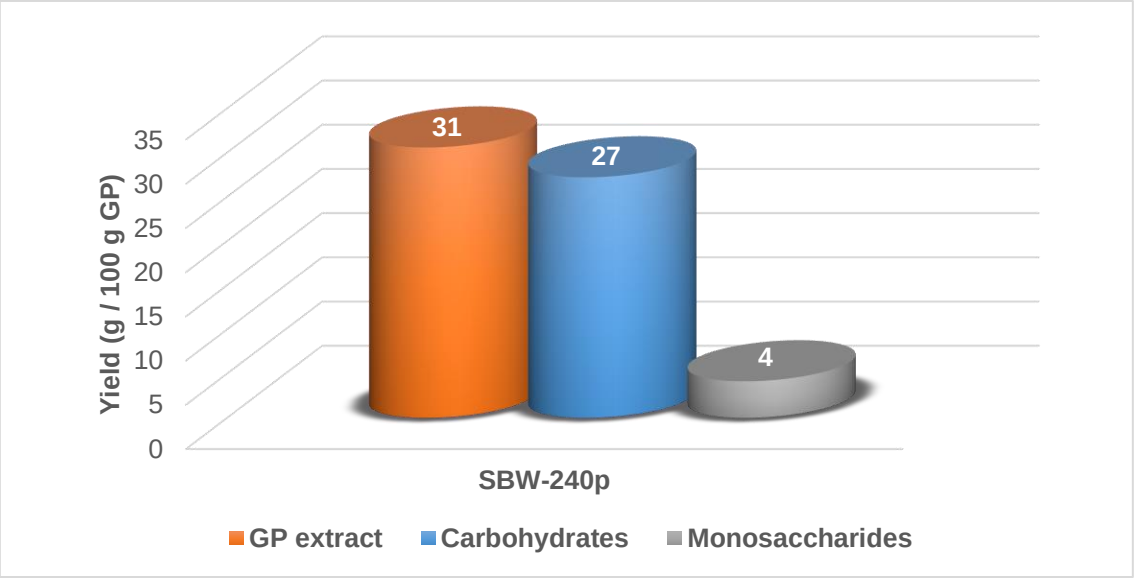

Figure 3.15 *Yield of GP extract, of carbohydrates and of monosaccharides in assay SBW-240p, performed at a water flow rate of 10 mL/min, at 70 bar.*

3.3. Enzymatic Hydrolysis

3.3.1. Yield of Immobilization of Viscozyme on Glutaraldehyde-activated Chitosan

After immobilizing the enzymes, they were washed with buffer solutions to eliminate unbounded and ionic and hydrophobic bounded enzymes.[44] The washing solutions were analyzed for protein according to the Lowry method.[46] Use of a calibration curve built for a model protein allowed the spectroscopic determination of the protein content of the washing solutions. This was compared with the protein content of the Viscozyme solution used in the immobilization procedure, after taking into the density of Viscozyme, and its protein content (7 wt.%), as specified by the supplier.

It was concluded that of all the protein that contacted with the glutaraldehyde-activated support, approximately 79% remained attached to it. This yield of immobilization is a little lower than the values obtained by other authors for this type of immobilization procedure.[44,59]

3.3.2. Hydrolysis of a model substrate with free and immobilized enzyme

In order to evaluate the potential of Viscozyme, in free and immobilized form, in the hydrolysis of lignocellulosic material, a model biopolymer consisting of arabinose and galactose – arabinogalactan – was used as substrate, since it mimics more accurately the carbohydrate content of GP extract (composed by about 80% oligosaccharides).

The free enzyme released 14.5 g monosaccharides/ 100 g arabinogalactan in 72 hours, at an average rate of 22 mg/h.FBG unit, while the immobilized enzyme hydrolyzed

arabinogalactan at a rate of 13 mg/h.FBG unit. If the yield of immobilization had been 100%, the results obtained would indicate that approximately 59% of enzyme activity was kept upon immobilization. Considering the yield of immobilization, this value increases to about 75%. Covalent immobilization often brings about a more pronounced decrease in specific enzyme activity, compensated by an extremely high operational stability,[44,45] not tested in the current thesis. The value obtained for the yield of immobilization of Viscozyme is probably a little low, as already mentioned. More assays should be done to confirm these findings.

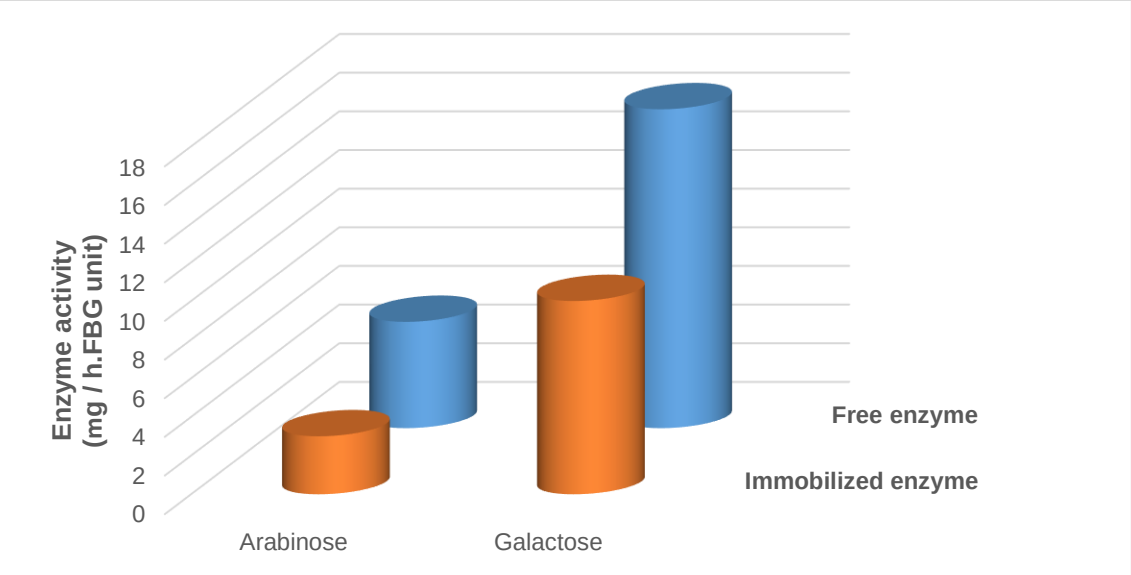

Figure 3.16 *Comparison between the activity of free and immobilized Viscozyme towards arabinogalactan.*

The amount of arabinose obtained was about one third of the amount of galactose (Fig. 3.16). The composition of arabinogalactan depends on its source, and can vary between approximately 3 and 6.[60] Residual amounts of glucose, xylose and fructose were also detected, in agreement with information on the sugar composition of commercially available arabinogalactan containing approximately 5 wt.% of other monosaccharides in addition to arabinose and galactose.[60,61]

3.3.3. HYDROLYSIS OF GP MATERIAL WITH FREE ENZYME

An enzymatic hydrolysis was performed of the GP extract obtained from pretreatment with SBW-200 of the GP residue that remained in the reactor after hydrolysis, and of the biomass itself, GP. Free Viscozyme was used. The reaction was followed for 48 h (Fig.3.17).

Viscozyme has approximately 23 wt.% sucrose.[47] The amount of carbohydrate introduced by Viscozyme in the reaction medium was subtracted from all quantifications based on the colorimetric method. For comparison, carbohydrate amounts are indicated on a GP basis, using the amount of GP used in the assay, together with the amounts of GP extract and GP residue left in the reactor.

GP was submitted to the enzymatic process to verify if Viscozyme alone can degrade the lignocellulosic matrix without the aid of SBW pretreatment. GP has approximately 7 wt.% soluble carbohydrates. As seen in Fig.3.17, the amount of carbohydrates detected in the reaction medium increases along the assay to approximately 12 g / 100 g GP, and then stabilizes. Assuming that during the process all the soluble carbohydrates were extracted, these results indicate that, after 48 h, about 5 wt.% of the structural carbohydrates were recovered as well. This represents about 20% of the total amount of structural carbohydrates (23.5 wt.%; Table 3.1). Thus, the enzymatic hydrolysis of GP as supplied is not very efficient, leading to high process costs when scaling-up.

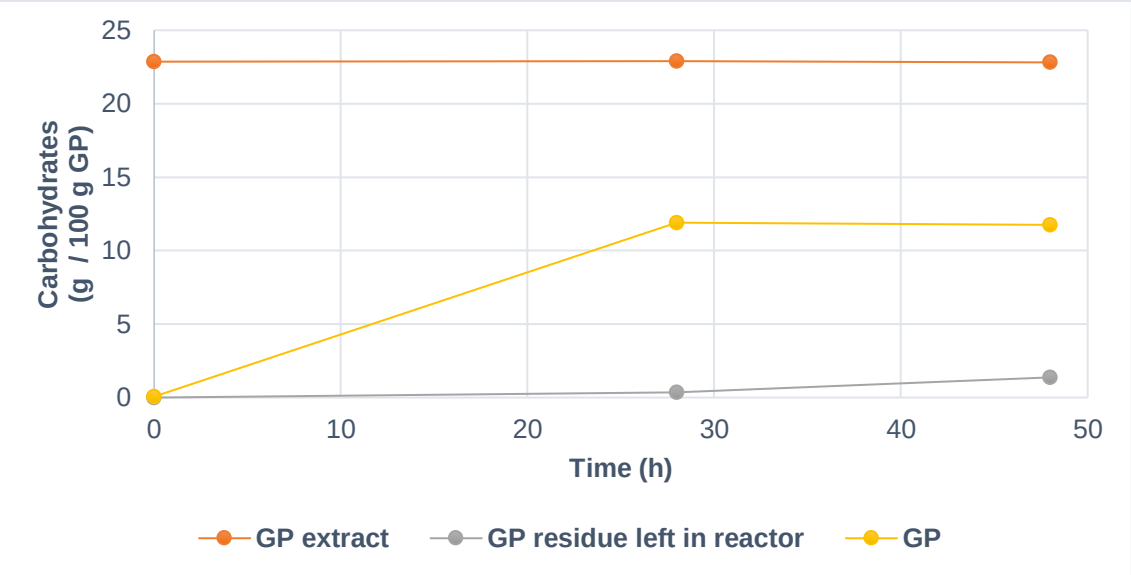

Figure 3.17 *Carbohydrates obtained in the course of enzymatic hydrolysis of GP, GP extract obtained in the SBW-200 assay, and GP residue left in the reactor after performing that experiment. Carbohydrates are indicated on a GP basis, for comparison.*

The result obtained for GP extract is as expected. The enzyme should be able to break down oligosaccharides into monosaccharides, but the colorimetric method for the quantification of total sugars does not distinguish between the two types of structures. Therefore, there was no temporal variation in the amount of sugars. HPLC analysis of the reaction medium was essential to understand the possible advantage of combining SBW pretreatment with enzymatic treatment towards the production of growth medium for oleaginous yeasts, in a strategy earlier identified as liquor B (Fig.1.10). However, once again this analytical method was difficult to implement, and yeast growth assays were conducted with GP extract resulting solely from SBW treatment. As for the 1-step acid hydrolysis, an alternative approach to convert GP extract oligosaccharides into monosaccharides, the results obtained were not satisfactory. Unlike what has been reported by other authors,[43] this was not an efficient methodology for GP extract, and HPLC analysis revealed only traces of carbohydrates.

The GP that remained in the reactor was analyzed as referred earlier. Table 3.4 indicates that after the SBW-240p assay, the residue left in the reactor had approximately 6 wt.% of carbohydrates. The SBW-200 assay did not go beyond the point of cellulose dissolution, and

therefore the residue produced should have a higher amount of cellulose, up to the maximum value of 11 wt.% (Table 3.1). The extraction of hemicellulose and some lignin should make access to the cellulose structure easier, and therefore hydrolysis of cellulose easier too. However, the experiment performed suggests that these carbohydrates are difficult to digest by the enzyme, undergoing little hydrolysis. When using immobilized enzyme, with a lower specific activity than the free enzyme, no evidence of hydrolysis was obtained.

3.4. OLEAGINOUS YEASTS

Oleaginous yeasts were used to confirm the viability of GP hydrolysates as an assimilable carbon source. The growth and production of lipids under the conditions of pH and glucose concentration close to that found in GP hydrolysates were analyzed in a glucose medium; the best performer yeast was selected and growth and lipid accumulation on GP hydrolysate were characterized and quantified.

3.4.1. PH EFFECT ON *R. BABJEVAE* GROWTH AND LIPID PRODUCTION, USING GLUCOSE AS CARBON SOURCE

The liquor obtained from the SBW hydrolysis of GP has a rather low pH, around pH4. Certain yeast strains are known to produce more lipids at lower pH,[62] hence the impact of pH on cell growth and lipid production was evaluated for *R. babjevae* on a growth medium with glucose as carbon source. The pH values tested were - pH4 and pH6, the former being a pH value closer to the pH value of liquor and the latter an optimum pH.

3.4.1.1. *R. BABJEVAE* GROWTH ON GLUCOSE SUPPLEMENTED MEDIUM AT PH4 AND AT PH6

R. babjevae growth was followed for 144h, on a medium supplemented with glucose as sole source of carbon and limiting nitrogen (control medium), designed to improve lipid production,[49] so that it could later be compared with the medium containing GP extract as carbon source.

The effect of the pH was not significant and both growth curves pH4 and pH6 were very similar (Fig. 3.18). *R. babjevae* had an exponential phase until 24h growth. After that, it entered the stationary phase. The biomass reached 4.4 g (dry weight)/L for pH4 and 4.5 g(dry weight)//L for pH6.

The pH measured in the medium at the end of the growth was, in average, pH 3.0 for both assays due to the medium acidification caused by yeasts metabolism. However, we could conclude that medium at pH4 allows a good yeast growth.

An analysis of glucose consumption over time was also carried out. Since the stationary phase was achieved when plenty of glucose was still present, it may reflect the limitation of another nutrient, most probably nitrogen.[49] Elementary analysis should be done to confirm that.

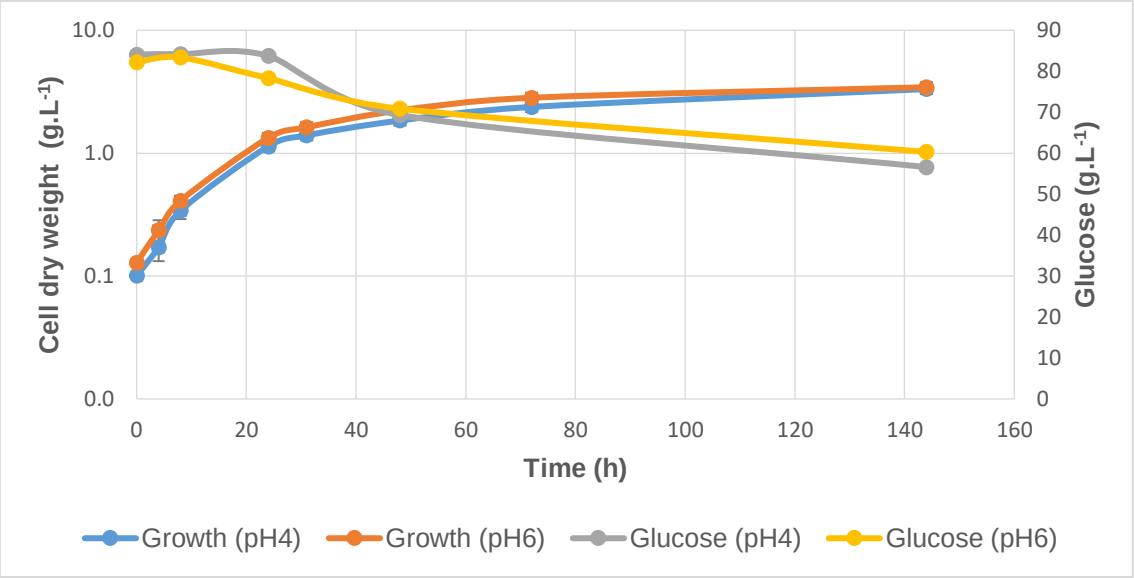

Figure 3.18 *R. babjevae growth curve and glucose consumption, on a glucose supplemented medium at initial pH=4 and initial pH=6. Average for two growth assays.*

3.4.1.2. LIPID PRODUCTION BY *R. BABJEVAE*

Nile red can stain most lipids, its fluorescence character varies depending on the lipid polarity. Nile red emits at a shorter wavelength when bound to neutral lipids, compared to polar ones. Unsaturated fatty acids exhibit stronger fluorescence intensity than saturated fatty acids.[51] Lipid detection by Nile red has been commonly measured with excitation at 480–490 and at 510–560 nm. The former targets neutral lipids to fluoresce and the latter the polar lipids.[52] The excitation wavelengths of the neutral lipids (583nm) and the polar lipids (637nm) were measured at different growth times for the assays at pH4 and at pH6. The total lipids were also determined by the sum of neutral and polar lipids. Fluorescence unit is in scintillation per second (SPS).

Emission spectra with excitation at 488 nm, 0.5 µg/mL of the Nile Red fluorophore and spectral acquisition time of 2 min were acquired with the purpose of monitoring lipid production along several stages of yeast growth. Experimental conditions, such as Nile Red concentration and spectra acquisition time were defined initially.

The evolution of total lipid amount during the growing period is represented (Fig. 3.19). The fluorescence increases with time, being that at 144h it reaches a maximum of 4.29×10^6 SPS / $g.L^{-1}$ for pH4, and a maximum of 3.89×10^6 $g.L^{-1}$ for pH6.

As was concluded for yeast growth at pH 4, the total lipid production kinetics is identical for both mediums. It increases exponential during the exponential growth and slowly thereafter (Fig. 3.19).

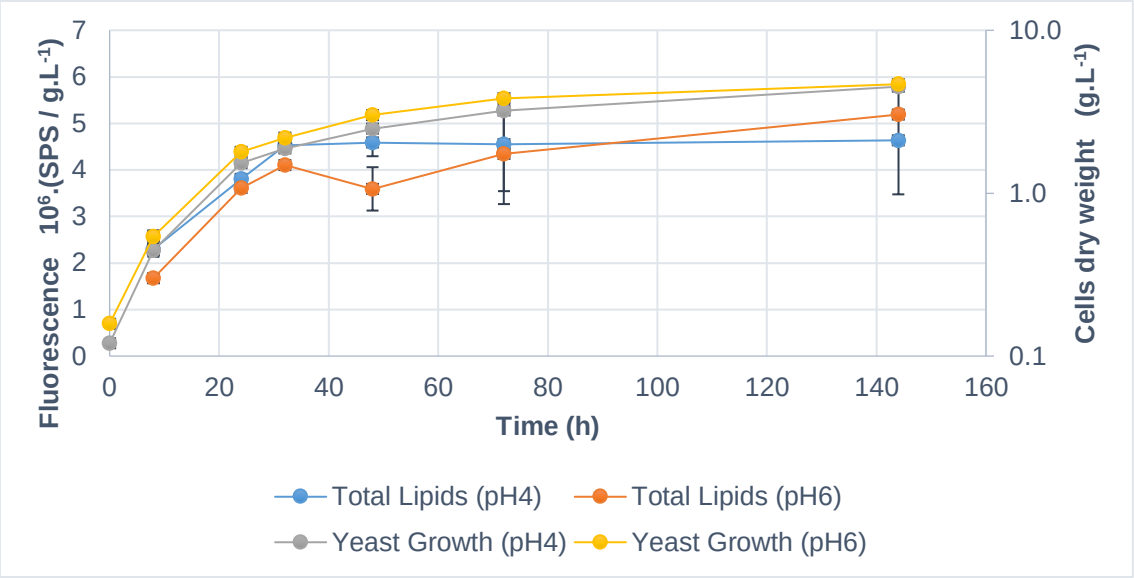

Figure 3.19 *R. babjevae growth curve and lipid production on a glucose supplemented medium at initial pH=4 and initial pH=6, measured by NR fluorescence. Average for two growth assays.*

When analyzing lipid production by *R. babjevae* (Fig. 3.20), we found more neutral lipids at pH4 relatively to pH6. Therefore, to obtain neutral lipids, pH 4 seems more favorable pH value.

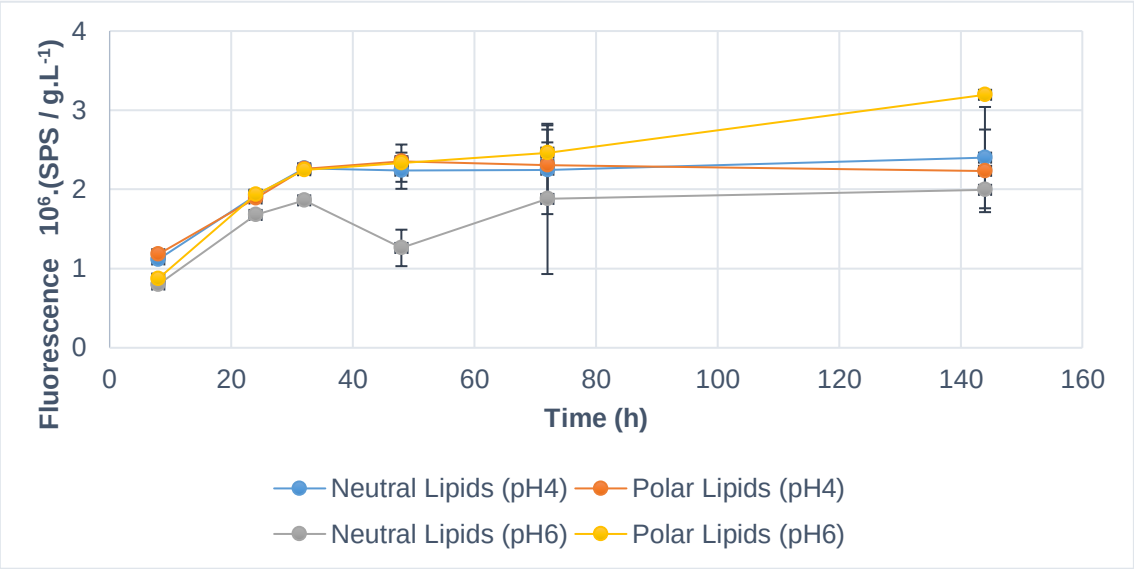

Figure 3.20 *R. babjevae polar and neutral lipid production on a glucose supplemented medium at initial pH=4 and initial pH=6, measured by NR fluorescence. Average for two growth assays.*

Lipid production was compared with glucose consumption for both pH values (Fig. 3.21). Glucose was slowly used during the stationary phase (after 40h) in agreement with slightly increment in lipid production.

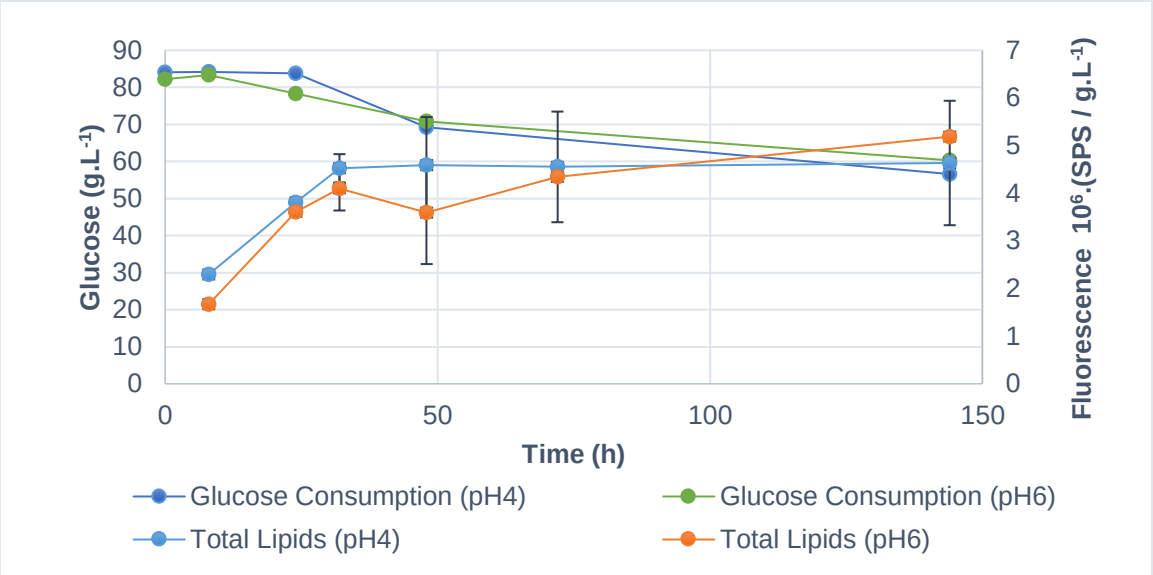

Figure 3.21 *R. babjevae lipid production and glucose consumption on a glucose supplemented medium at initial pH=4 and initial pH=6, measured by NR fluorescence. Average for two growth assays.*

To measure total lipid content in the cells, gravimetric analysis was used according to Sitepu et al.[51] For that, cells were recovered after 144h of growth, lyophilized and used for lipid extraction. Lipid weight after extraction was considered as total lipid and represented as a percent of cell dry weight (table 3.5).

In line to what was observed with fluorescence (Fig. 3.21), the lipid production is practically the same for growth at pH4 and pH6.

Table 3.5 *Biomass and lipid production at pH=4 and pH=6 by R. babjevae.*

Cell dry weight ($g.L^{-1}$)		Total lipids (%)		Total lipid weight ($g.L^{-1}$)	
pH4	pH6	pH4	pH6	pH4	pH6
4.5 ± 0.7	5.1 ± 0.6	22.3 ± 2.8	23.7 ± 3.3	1.0 ± 0.2	1.2 ± 0.2

3.4.2. Growth and Lipid Production by *R. babjevae*, *R. mucilaginosa* and *L. starkeyi* using Glucose as Carbon-source

Three yeasts, *Rhodotorula babjevae*, *Rhodotorula mucilaginosa* and *Lipomyces starkeyi* were tested to see which are the best to produce and consequently accumulate lipids at pH4 medium supplemented with 35 g/L glucose.

The amount of glucose in the medium was reduced to 35 g/L, instead of the 70 g/L proposed by Li et al.[49] and used in the previous assays, because yeasts did not used all the sugar available and a lower glucose concentration will allow a better comparison with GP extracts – these extracts have low amounts of monosaccharides and it is difficult to obtain high sugar concentrations. The nitrogen was adjusted in accordance to maintain the same C:N and N-limiting conditions.

3.4.2.1. *R. babjevae, R. mucilaginosa* and *L. starkeyi* Growth on Glucose supplemented Medium at pH4

R. babjevae, R. mucilaginosa, and L. starkeyi growth were followed for 144 h, on a glucose medium (Fig. 3.22), so that it could later be used as a control for the growth on a medium containing GP extract as carbon source.

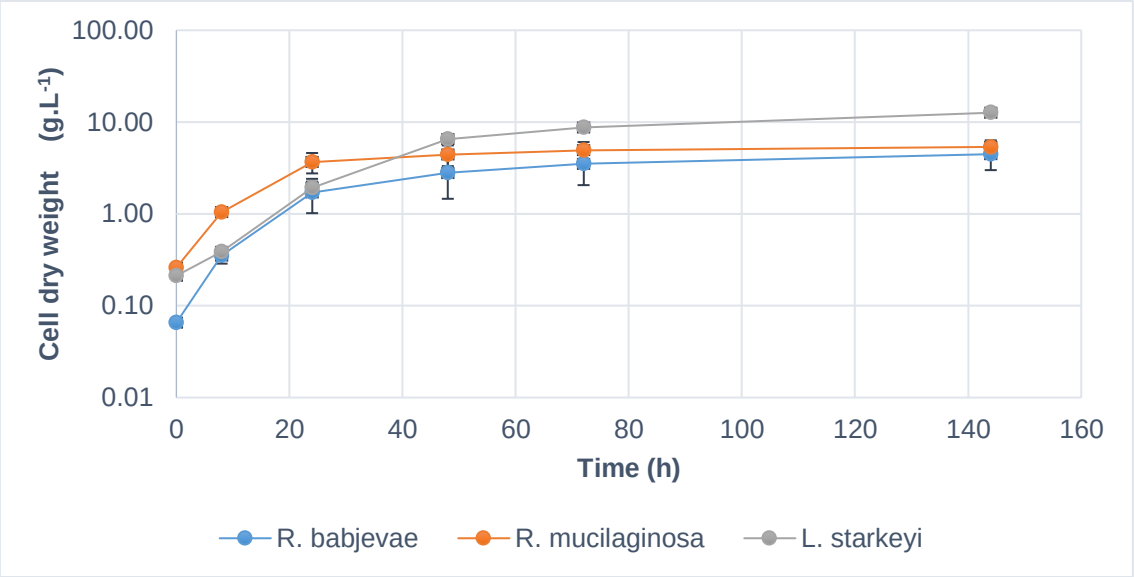

Figure 3.22 *R. babjevae, R. mucilaginosa and L. starkeyi growth curves, on a glucose supplemented medium at initial pH=4. Average for two growth assays.*

Growth curves are represented in Fig. 3.23 – 3.25. Biomass reaches a concentration of approximately 4.5 g/L in a dry weight basis for *R. babjevae*, followed by *R. mucilaginosa*, which reaches a biomass concentration of approximately 5.4 g/L. *L. starkeyi* is, by far, the yeast which biomass reaches the highest concentration - approximately 12.7 g/L.

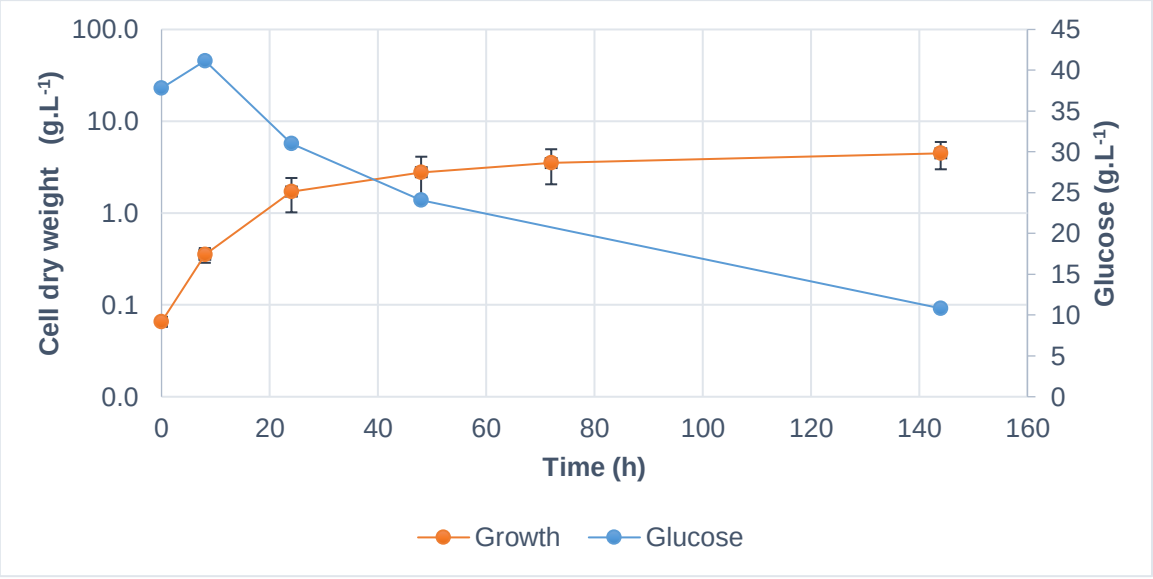

Figure 3.23 *R. babjevae growth curve and glucose consumption, on a glucose supplemented medium at initial pH=4. Average for two growth assays.*

Glucose consumption profiles reveals differences between yeasts (Fig. 3.23 - 3.25). *R. mucilaginosa* stopped to consume glucose immediately after reaching the stationary phase while both *R. babjevae* and *L. starkeyi* continued to consume glucose at similar rates

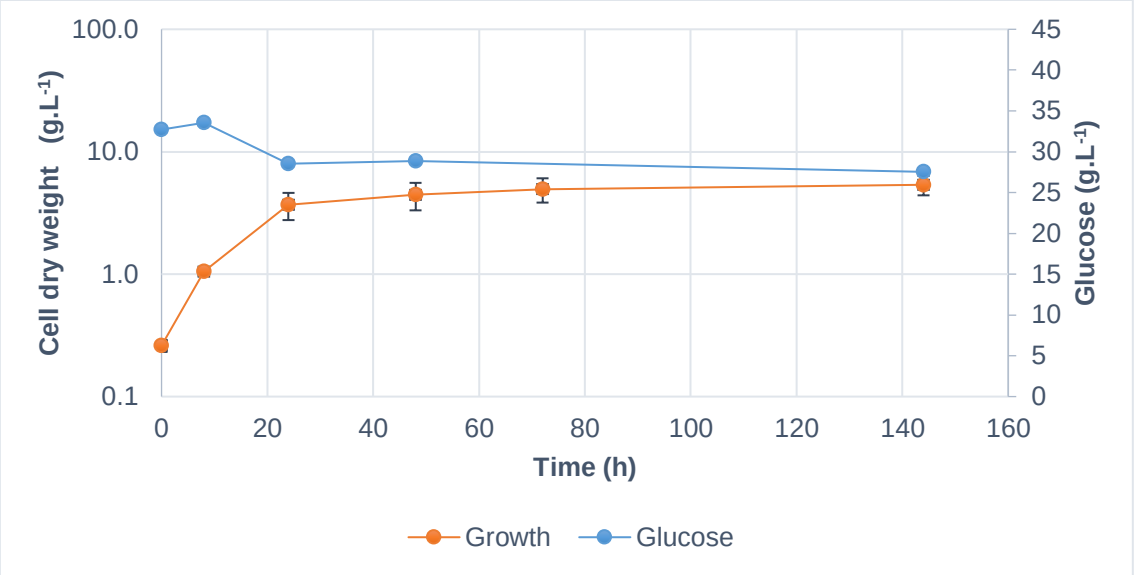

Figure 3.24 *R. mucilaginosa growth curve and glucose consumption, on a glucose supplemented medium at initial pH=4. Average for two growth assays.*

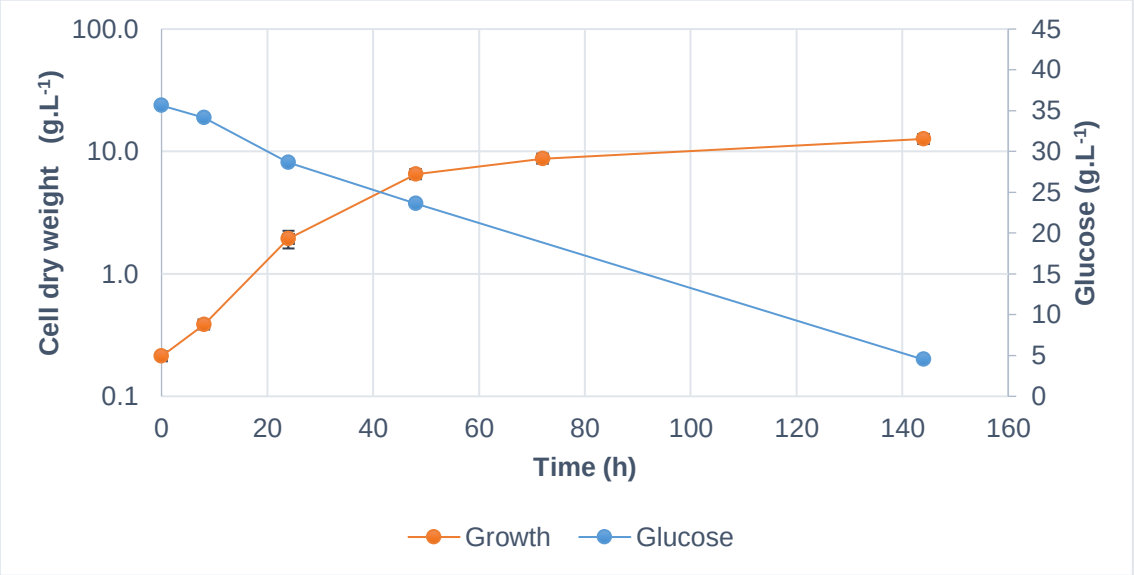

Figure 3.25 *L. starkeyi growth curve and glucose consumption, on a glucose supplemented medium at initial pH=4. Average for two growth assays.*

At the end, cells were recovered and lyophilized. The average mass of the lyophilized cells obtained in the two growths is higher for *L. starkeyi*, 9.6 g/L, followed by *R. babjevae* with 5.7 g/L, and *R. mucilaginosa* with 4.1 g/L (Table 3.6), which corroborates the results obtained by cells dry weight.

3.4.2.2. *Lipid Production by R. babjevae, R. mucilaginosa and L. starkeyi*, on Glucose Supplemented Medium

As was done for *R. babjevae* (see chapter 3.4.1.2), lipid production was followed by NR fluorescence (Fig. 3.26 – 3.28).

At 144h, *R. babjevae* produced 3.93*10^6 SPS / g.L^{-1} total lipids, from which 1.87*10^6 SPS / g.L^{-1} were neutral lipids and 2.06*10^6 SPS / g.L^{-1} were polar lipids. Nearly half of the total lipids are lipids of interest – neutral lipids, like TAG or carotenoids. These values corroborate the ones found in the first assay with higher glucose concentration.

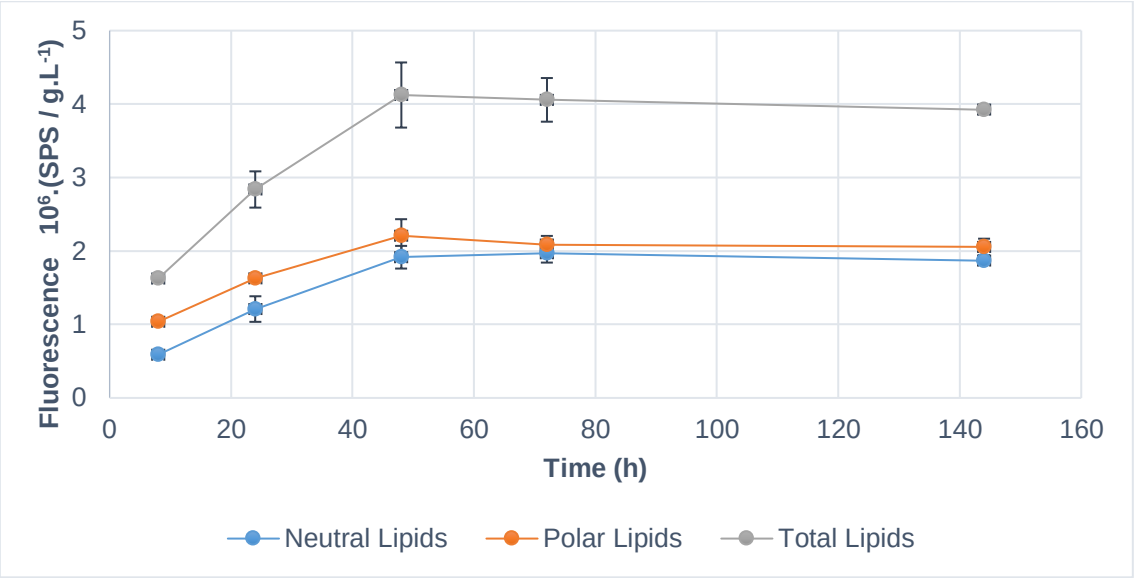

Figure 3.26 *R. babjevae total, neutral, and polar lipid production on a glucose supplemented medium at initial pH=4, measured by NR fluorescence. Average for two fluorimetry assays (one for each growth).*

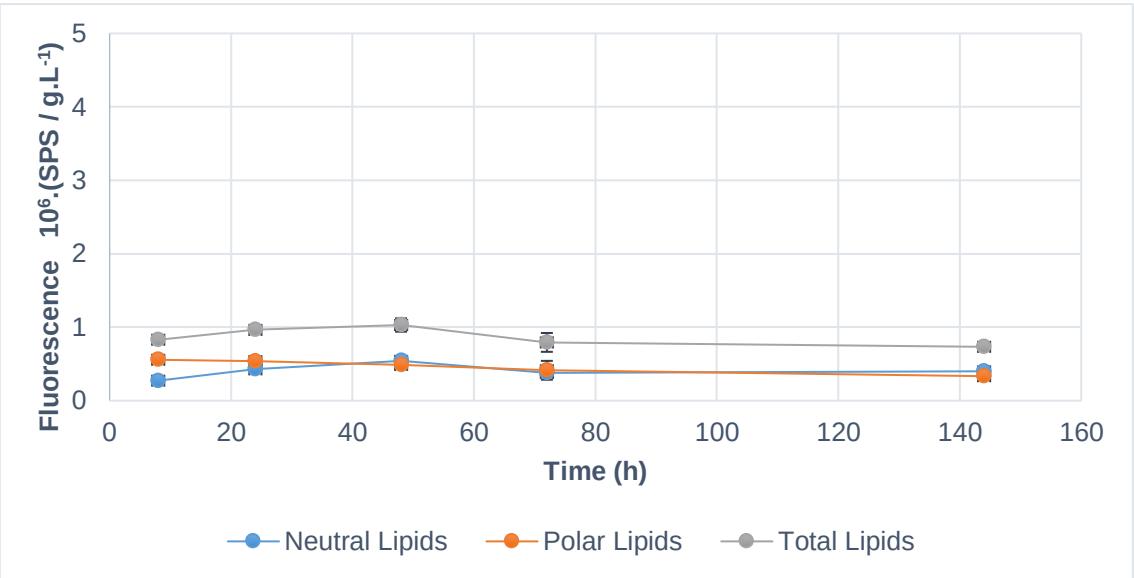

Figure 3.27 *R. mucilaginosa total, neutral, and polar lipid production on a glucose supplemented medium at initial pH=4, measured by NR fluorescence. Average for two fluorimetry assays (one for each growth).*

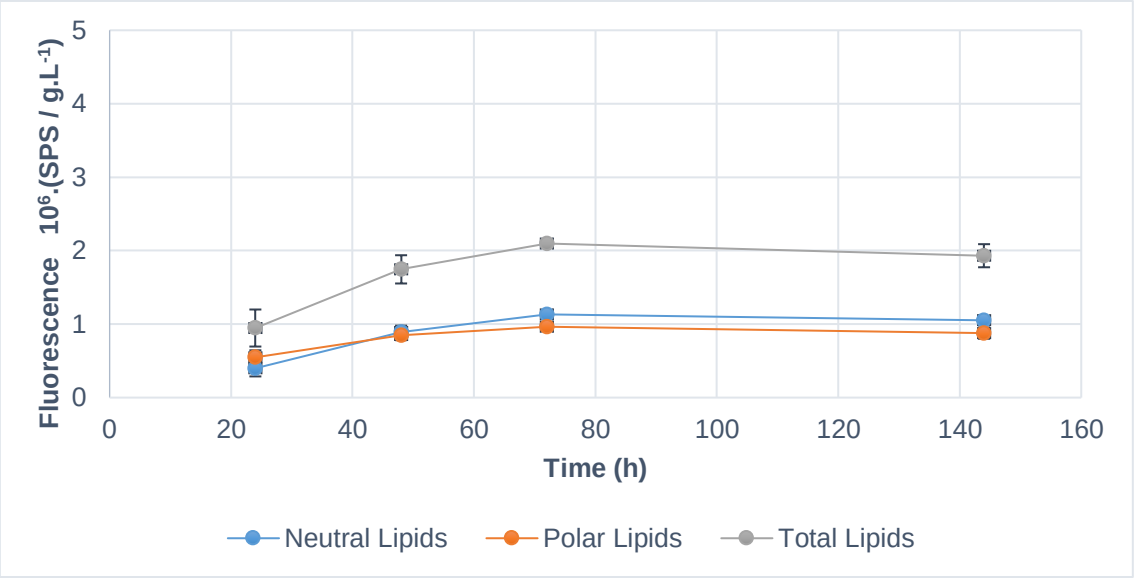

Figure 3.28 *L. starkeyi total, neutral, and polar lipid production on a glucose supplemented medium at initial pH=4, measured by NR fluorescence. Average for two fluorimetry assays (one for each growth).*

L. starkeyi produced 1.93*10^{6} SPS / g.L^{-1} total lipids, from which 1.05*10^{6} SPS / g.L^{-1} are neutral lipids and 8.77*10^{5} SPS / g.L^{-1} are polar lipids. *R. mucilaginosa* was the worse lipid producer with a value of 7.34*10^{5} SPS / g.L^{-1} total lipids, from which 4.00*10^{5} SPS / g.L^{-1} are neutral lipids and 3.34*10^{5} SPS / g.L^{-1} are polar lipids. Moreover, these yeasts show a decrease in total lipids in the late-stationary phase.

3.4.2.3. *R. babjevae*, *R. mucilaginosa*, and *L. starkeyi* Total Lipids Content and Yields, on Glucose Supplemented Medium

The amount of total lipids produced by each one of the three yeasts is represented in Table 3.6. *Lipomyces starkeyi* has produced more than the other yeasts, followed by *Rhodotorula babjevae*, and at last by *Rhodotorula mucilaginosa*.

Table 3.6 *Yeasts cells and total lipids dry weights, in a glucose supplemented medium.*

Oleaginous Yeast	Cell dry weight (g.L^{-1})	Total lipids (%)	Total lipid weight (g.L^{-1})
Rhodotorula babjevae	5.7	25.5	1.45
Rhodotorula mucilaginosa	4.1	18.5	0.77
Lipomyces starkeyi	9.6	55.5	5.33

In what concerns to yields, L. starkeyi is, by far, the most rentable yeast (Table 3.7).

Table 3.7 *Yeasts growth rate (μ), biomass per substrate yield (Yx/s), product (lipids) per biomass yield (Yp/x), and product per substrate yield (Yp/s), in a glucose supplemented medium.*

Yeast	μ (h^{-1})	Biomass (g)	Lipids (g)	Yx/s (gX / gS)	Yp/x (gP/gX)	Yp/s (gP/gS)
R. babjevae	0.13	0.63	0.16	0.16	0.25	0.04
R. mucilaginosa	0.11	0.46	0.08	0.12	0.18	0.02
L. starkeyi	0.11	1.06	0.59	0.28	0.55	0.15

The carotenoids content within the lipid fraction was analyzed by UV-spectrophotometry. *R. babjevae* has the highest amount, being, in average, 6.3 mg carotenoids / g cells dry weight, followed *by R. mucilaginosa* with nearly 0.9 mg carotenoids / g cells dry weight. *L. starkeyi* do not produce carotenoids.

3.4.3. Growth and Lipid Production by *R. babjevae* and *L. starkeyi* using GP Extract as Carbon-Source

R babjevae and *L. starkeyi* were selected for the next step in which GP extract will be the sole carbon source in the growth medium. The extract used was the one obtained from assay. SBW-240, a mixture comprehending from monosaccharides to oligosaccharides (Fig. 3.29), with 39 g/L carbohydrates and pH4.

Figure 3.29 *R. babjevae (left) and L. starkeyi (right) in a mix of carbohydrates supplemented medium obtained from the GP extract.*

3.4.3.1. R. babjevae and L. starkeyi Growth on GP Extract Medium

R. babjevae and *L. starkeyi* growth were followed for 144h, on GP extract medium. Both *R. babjevae* and L. starkeyi were capable to growth on GP extract (fig 3.30 - 3.31). The biomass reaches a concentration of approximately 1.0 g/L, in a dry weight basis, for *R. babjevae* and, once

again, a higher value for *L. starkeyi*, of 4.7 g/L. Although they were able to growth GP, the biomass attained was much less than on glucose medium. Replicates should be done to confirm this result.

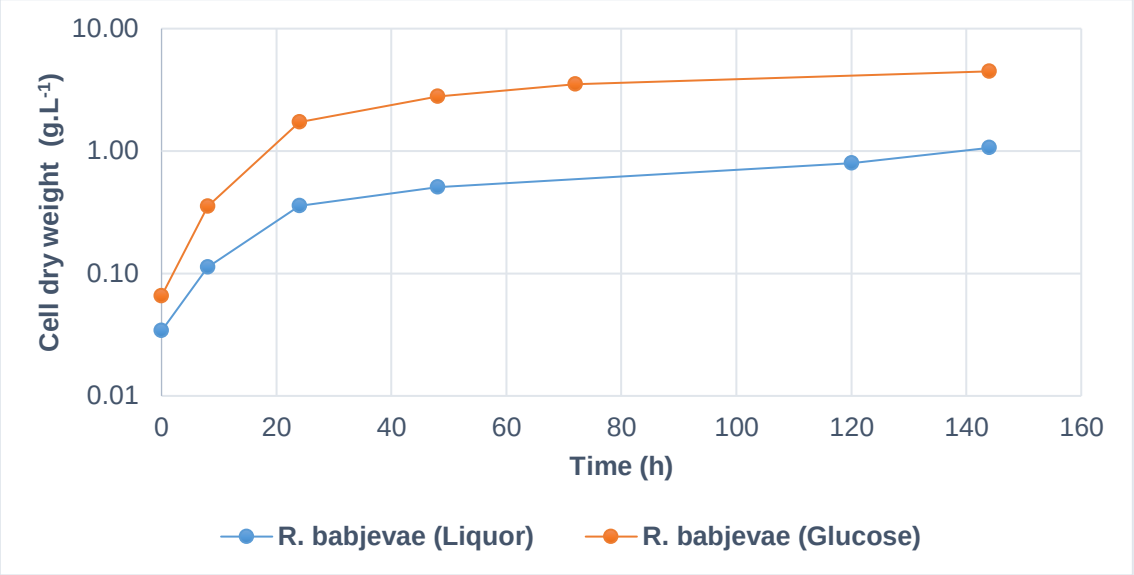

Figure 3.30 *R. babjevae growth curves on a glucose supplemented medium and on a GP extract medium at initial pH=4.*

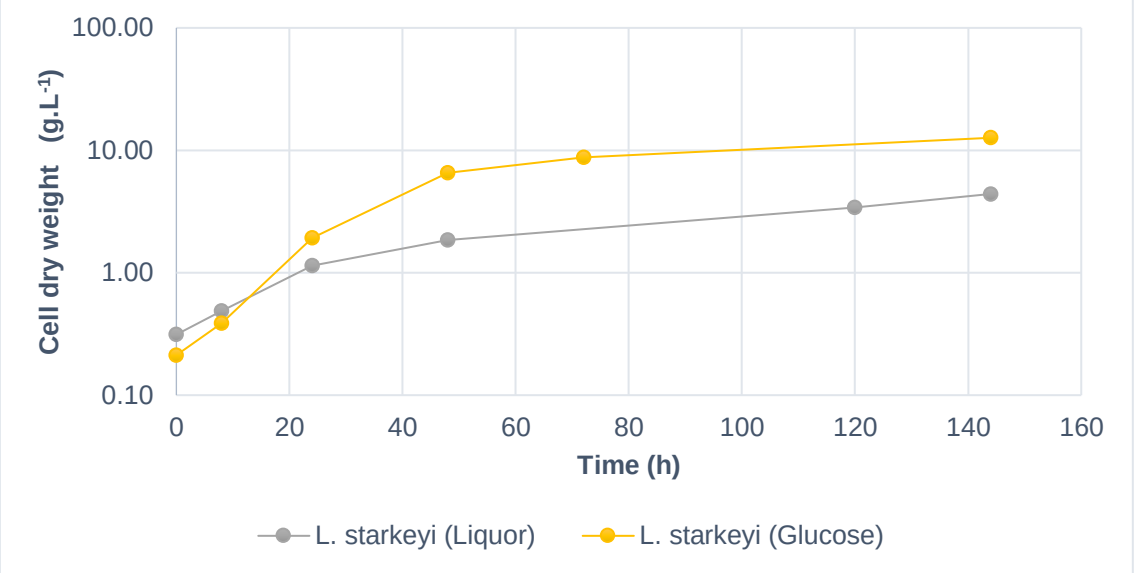

Figure 3.31 *L. starkeyi growth curves on a glucose supplemented medium and on a GP extract medium at initial pH=4.*

In opposition to growths in glucose supplemented medium, in which there are an accentuated sugar consumption during the exponential phase and the beginning of stationary phase, the GP extract medium reveals little or none monosaccharides consumption since its value maintains constant along the growth (Fig 3.32). It reveals that yeasts resort mainly to other sources, or is simply due to the difficulties encountered in HPLC analysis.

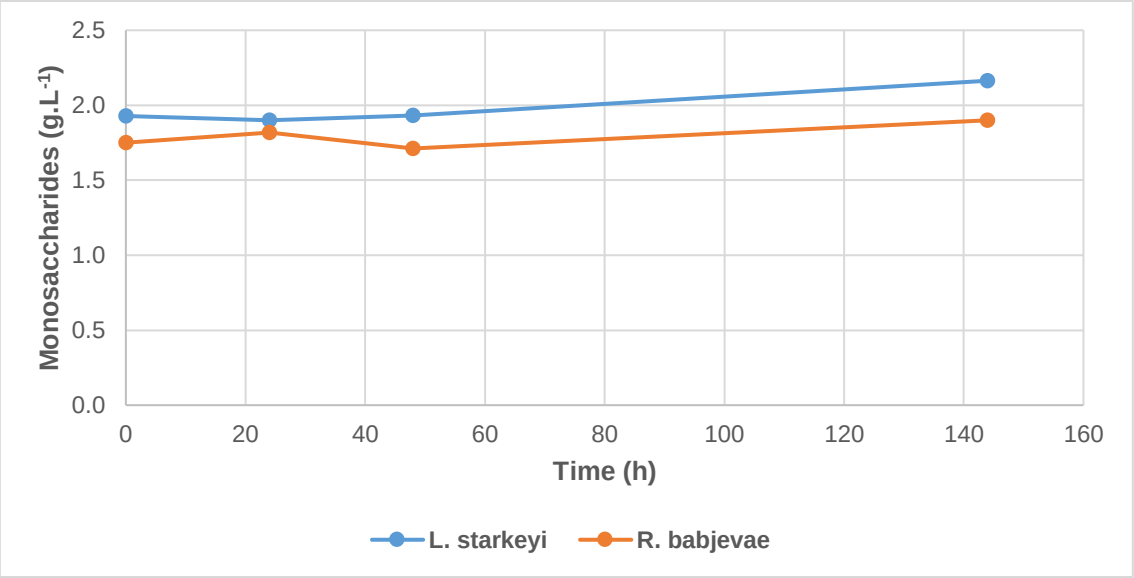

Figure 3.32 *R. babjevae and L. starkeyi monosaccharides consumption, on a GP extract medium.*

The pH measured in the medium at the end of the *R. babjevae* growth was pH 3.2, and for L. starkeyi growth, was pH 3.5.

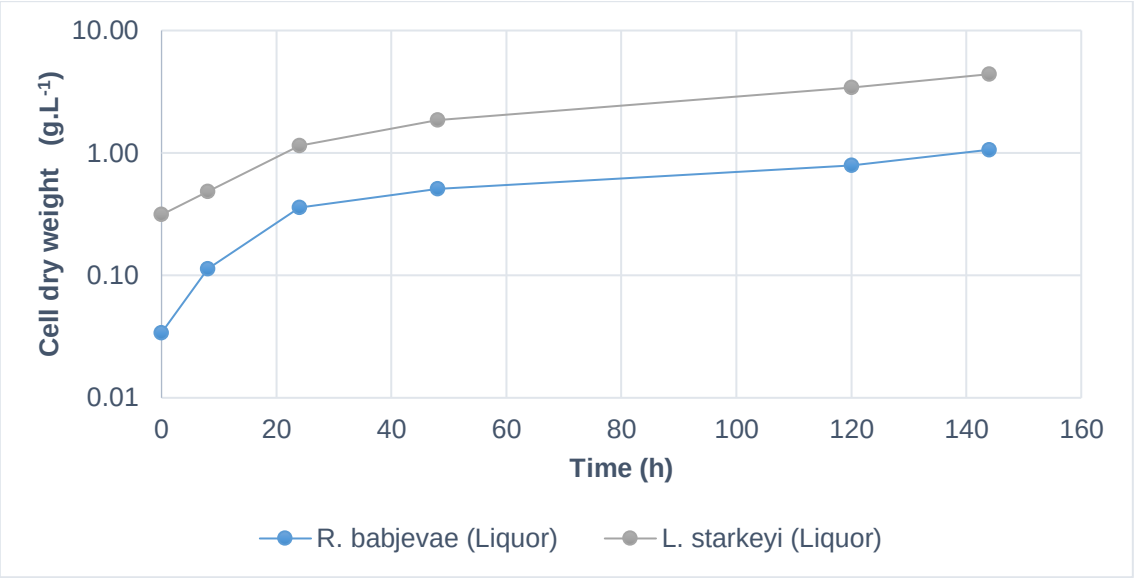

Figure 3.33 *R. babjevae and L. starkeyi growth in a liquor medium, at pH4.*

The mass of the lyophilized cells obtained in the two growths is higher for *L. starkeyi*, 0.9 g/L, followed by *R. babjevae* with 0.8 g/L (Table 3.9).

4 CONCLUSIONS

Initially, one of the objectives of this thesis was using two types of alternative media for yeast growth: one in which the carbon source consisted in carbohydrates resulting from the hydrolysis of grape pomace (GP) with subcritical water (SBW) – liquor A and corresponding GP extract – and another resulting from liquor A after the corresponding GP extract was submitted to enzymatic hydrolysis, in order to break down oligosaccharides into monosaccharides – liquor B. The goal was to grow yeasts on GP extract so as to obtain lipids and carotenoids, since these have commercial value and allow the valorization of GP.

The first step was the chemical characterization of GP. SBW treatment does not require drying GP. But if GP cannot be processed right away, and water must be removed to avoid microbial proliferation, then drying in air does not affect its carbohydrate content.

GP was extracted / hydrolyzed by SBW by applying two different temperature programs: one in which temperature increased continuously from the beginning to the end of the assay, and another in which temperature increased stepwise, with each step consisting in a ramp and a plateau. It was found that in the former assays there was a temperature gradient along the length of the reactor, and that in the second type of assays the water inlet and outlet temperatures coincided. Plateau assays are thus more accurate.

The best results as regards the extraction / hydrolysis efficiency, yield in GP extract and yield in carbohydrates, was the plateau-type assay that reached the highest temperature: 240 ºC. In this assay, approximately 84 wt.% of the total carbohydrates of GP were recovered, indicating that in addition to extensive hydrolysis of hemicellulose, some hydrolysis of cellulose occurred as well.

Most of the carbohydrates recovered (80%) were in the form of oligosaccharides. This led to a study of the enzymatic hydrolysis of these structures in order to increase the amount of monosaccharides, with a view to obtaining a GP extract that microorganisms could assimilate more easily, or at all. A commercial enzyme complex – Viscozyme – was used. Immobilization of Viscozyme on glutaraldehyde-activated chitosan microparticles was achieved with high yield of immobilization and moderate loss of enzyme activity.

It was observed that Viscozyme could degrade GP to a certain extent, but very slowly, and that it was not effective for the GP residue that remained in the reactor after the SBW treatment. But the most useful result from the point of view of the initial work program would be to carry out the enzymatic hydrolysis of GP extract. However, the colorimetric method for the quantification of sugars does not distinguish between monosaccharides and polymers of these, and difficulties when applying HPLC analysis did not allow drawing any conclusion.

Therefore, yeast growth experiments were carried out using as carbon source GP extract from liquor A, and no experiments were performed using GP extract from liquor B. Three yeast species were tested and found to be able to grow and produce lipids under conditions of low pH (pH=4) in a glucose medium, mimicking the pH conditions found in SBW hydrolysates. *R. babjevae*, which was the one that produced the highest amount of carotenoids, and L. starkeyi, which, despite not producing carotenoids, produced the highest amount of lipids, were chosen to

test the GP extract obtained in the course of the plateau-type assay reaching 240 ºC. Although the concentration of monosaccharides available was rather low, both yeasts were able to grow in those media, which is a good prognostic for a future utilization of GP extracts generated by SBW treatment.

5 FUTURE WORK

To improve carbohydrate extraction / hydrolysis with subcritical water, the experimental apparatus should allow work at higher temperatures, such as 280 ºC, for a greater degree of cellulose conversion. Plateau-type experiments are more accurate, but take longer. To overcome this problem, a different reactor with thinner walls, or a furnace with good wall to wall contact with the reactor could be implemented. It would also be interesting to test other water flow rates that affect the time it takes for compounds released at one point in the reactor to exit the reactor, and therefore possible compound degradation.

Regarding the enzymatic hydrolysis, it would be interesting to mount a reactor downstream of the high-pressure reactor, to degrade the oligosaccharides into monomers. In addition to the immobilization of Viscozyme on glutaraldehyde-activated chitosan, following the same method proposed by Lorenzoni et al.[44], other immobilization approached could be applied, such as those proposed by Zhang et al.[63] or by Shi-Kuo et al.[64]. HPLC analysis is essential to characterize GP extracts and follow enzymatic hydrolysis. Many problems were encountered when applying this technique, and must be solved in future work. A higher concentration of monosaccharides is preferred for yeast growth, since these species assimilate these more easily than oligosaccharides.

As for yeast growth, if the objective is the production of fatty acids*, L. starkeyi* is recommended, but if it is the production of carotenoids, *R. babjevae* is recommended. Carotenoids and fatty acid profiles should be drawn for these yeasts, but due to time constraints, it was not possible to achieve that goal when using GP extract medium.

REFERENCES

1. Clark, J. H., Luque, R. & Matharu, A. S. Green Chemistry, Biofuels, and Biorefinery.pdf. *Annu. Rev. Chem. Biomol. Eng.* 183–207 (2012).

2. Baig, M. N. *et al.* Evaluation and modelling of continuous flow sub-critical water hydrolysis of biomass derived components ; lipids and carbohydrates. *Chem. Eng. Res. Des.* **91,** 2663–2670 (2013).

3. Pedras, B. M. da S. Valorization of grape pomace through hot compressed water extraction / hydrolysis. (New University of Lisbon - Faculty of Sciences and Technology, 2015).

4. Zhu, G., Zhu, X., Fan, Q. & Wan, X. Resources , Conservation and Recycling Recovery of biomass wastes by hydrolysis in sub-critical water. *"Resources, Conserv. Recycl.* **55,** 409–416 (2011).

5. Bhatia, L., Johri, S. & Ahmad, R. An economic and ecological perspective of ethanol production from renewable agro waste : a review. *AMB Express* **2,** 1 (2012).

6. Stephen K. Ritter. Lignocellulose: A Complex Biomaterial. *Chemical & Engineering News (c&en)* 15 (2008). at <http://cen.acs.org/articles/86/i49/Lignocellulose-Complex-Biomaterial.html>

7. Haghighi Mood, S. *et al.* Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. *Renew. Sustain. Energy Rev.* **27,** 77–93 (2013).

8. Aachary, A. A. & Prapulla, S. G. Xylooligosaccharides (XOS) as an Emerging Prebiotic: Microbial Synthesis, Utilization, Structural Characterization, Bioactive Properties, and Applications. *Compr. Rev. Food Sci. Food Saf.* **10,** 2–16 (2011).

9. Vergara-salinas, J. R., Vergara, M., Altamirano, C., Gonzalez, Á. & Pérez-correa, J. R. Characterization of pressurized hot water extracts of grape pomace : Chemical and biological antioxidant activity. *Food Chem.* **171,** 62–69 (2015).

10. Food and Agriculture Organization of the United Nations (FAO). Crops Statistics. (2017). at <http://www.fao.org/faostat/en/#data/QC>

11. Dwyer, K., Hosseinian, F. & Rod, M. The Market Potential of Grape Waste Alternatives. *J. Food Res.* **3,** 91–106 (2014).

12. Nair, S. & Pullammanappallil, P. VALUE ADDED PRODUCTS FROM VINEYARD WASTES - A REVIEW. (2003).

13. Koutinas, A. a *et al.* Valorization of industrial waste and by-product streams via fermentation for the production of chemicals and biopolymers. *Chem. Soc. Rev.* **43,** 2587–627 (2014).

14. Llobera, A. & Caellas, J. Dietary fibre content and antioxidant activity of Manto Negro red grape (Vitis vinifera): pomace and stem. *Food Chem.* **101,** 659–666 (2007).

15. Arvanitoyannis, I. S., Ladas, D. & Mavromatis, A. Wine waste treatment methodology. *Int. J. Food Sci. Technol.* **41,** 1117–1151 (2006).

16. Miyazawa, T., Ohtsu, S. & Funazukuri, T. Hydrothermal degradation of polysaccharides in a semi-batch reactor: Product distribution as a function of severity parameter. in *Journal of Materials Science* **43,** 2447–2451 (2008).

17. Brunner, G. Supercritical fluids: Technology and application to food processing. *J. Food Eng.* **67,** 21–33 (2005).

18. Möller, M., Nilges, P., Harnisch, F. & Schröder, U. Subcritical water as reaction environment: Fundamentals of hydrothermal biomass transformation. *ChemSusChem* **4,** 566–579 (2011).

19. Kruse, A. & Dinjus, E. Hot compressed water as reaction medium and reactant. 2. Degradation reactions. *J. Supercrit. Fluids* **41,** 361–379 (2007).

20. Park, J.-N., Shin, T.-S., Lee, J.-H. & Chun, B.-S. Production of Reducing Sugars from Laminaria japonica by Subcritical Water Hydrolysis. *APCBEE Procedia* **2,** 17–21 (2012).

21. Sohail, S., Rosendahl, L. & Rudolf, A. Hydrothermal liquefaction of biomass : A review of subcritical water technologies. *Energy* **36,** 2328–2342 (2011).

22. Watchararuji, K., Goto, M., Sasaki, M. & Shotipruk, A. Value-added subcritical water hydrolysate from rice bran and soybean meal. *Bioresour. Technol.* **99,** 6207–6213 (2008).

23. Prado, J. M. *et al.* Obtaining sugars from coconut husk, defatted grape seed, and pressed palm fiber by hydrolysis with subcritical water. *J. Supercrit. Fluids* **89,** 89–98 (2014).

24. Rostagno, M. A., Prado, J. M., Vardanega, R., Forster-Carneiro, T. & Meireles, M. A. A. Study of recovery of fermentable sugars with subcritical water and carbon dioxide from palm fiber and grape seed. *Chem. Eng. Trans.* **37,** 403–408 (2014).

25. Mohamad, N. R., Marzuki, N. H. C., Buang, N. A., Huyop, F. & Wahab, R. A. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnol. Biotechnol. Equip.* **29,** 205–220 (2015).

26. Wang, R., Wang, J., Xu, R., Fang, Z. & Liu, A. Oil production by the oleaginous yeast Lipomyces starkeyi using diverse carbon sources. *BioResources* **9,** 7027–7040 (2014).

27. Lamers, D. *et al.* Selection of oleaginous yeasts for fatty acid production. *BMC Biotechnol.* **16,** 1–10 (2016).

28. Butinar, L., Spencer-Martins, I. & Gunde-Cimerman, N. Yeasts in high Arctic glaciers: The discovery of a new habitat for eukaryotic microorganisms. *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* **91,** 277–289 (2007).

29. Beopoulos, A. *et al.* Yarrowia lipolytica as a model for bio-oil production. *Progress in Lipid Research* **48,** 375–387 (2009).

30. Probst, K. Single Cell Oil Production Using Lipomyces Starkeyi: Fermentation, Lipid Analysis and Use of Renewable Hemicellulose-Rich Feedstocks. (2014).

31. Sitepua, I. R. *et al.* Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeasts species. *Bioresour. Technol.* **4,** 1–23 (2013).

32. Probst, K. V, Schulte, L. R., Durrett, T. P., Rezac, M. E. & Vadlani, P. V. Oleaginous yeast: a value-added platform for renewable oils. *Crit. Rev. Biotechnol.* **36,** 942–955 (2015).

33. Ratledge, C. & Wynn, J. P. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv. Appl. Microbiol.* **51,** 1–51 (2002).

34. Vorapreeda, T., Thammarongtham, C., Cheevadhanarak, S. & Laoteng, K. Alternative routes of acetyl-CoA synthesis identified by comparative genomic analysis: Involvement in the lipid production of oleaginous yeast and fungi. *Microbiology* **158,** 217–228 (2012).

35. Beopoulos, A. & Nicaud, J. Yeast: A new oil producer? *Oilseeds Fats Crop. Lipids* **19,** 22–28 (2012).

36. Zhang, H., Damude, H. G. & Yadav, N. S. Three diacylglycerol acyltransferases contribute to oil biosynthesis and normal growth in Yarrowia lipolytica. *Wiley Online Libr.* 25–38 (2012). doi:10.1002/yea

37. Zoz, L., Carvalho, J. C., Soccol, V. T., Casagrande, T. C. & Cardoso, L. Torularhodin and torulene: Bioproduction, properties and prospective applications in food and cosmetics - A review. *Brazilian Arch. Biol. Technol.* **58,** 278–288 (2015).

38. Chang, Y. H. *et al.* A comparative study on batch and fed-batch cultures of oleaginous yeast Cryptococcus sp. in glucose-based media and corncob hydrolysate for microbial oil

production. *Fuel* **105,** 711–717 (2013).

39. Rodrigues, F., Ludovico, P. & Leão, C. Sugar Metabolism in Yeasts : an Overview of Aerobic and Anaerobic Glucose Catabolism. *Biodivers. Ecophysiol. Yeasts* 101–121 (2006). doi:10.1007/3-540-30985-3_6

40. Silva, L. T. Evaluating the potential of yeast strains to produce added value products for the food and / or pharmaceutical industries. (Universidade Nova de Lisboa, 2015).

41. Sitepu, I., Selby, T., Lin, T., Zhu, S. & Boundy-Mills, K. Carbon source utilization and inhibitor tolerance of 45 oleaginous yeast species. *J. Ind. Microbiol. Biotechnol.* **41,** 1061–1070 (2014).

42. Yu, Y., Lou, X. & Wu, H. Some recent advances in hydrolysis of biomass in hot-compressed water and its comparisons with other hydrolysis methods. in *Energy and Fuels* **22,** 46–60 (2008).

43. Duarte, L. C., Silva-Fernandes, T., Carvalheiro, F. & Gírio, F. M. Dilute acid hydrolysis of wheat straw oligosaccharides. *Appl. Biochem. Biotechnol.* **153,** 116–126 (2009).

44. Lorenzoni, A. S. G., Aydos, L. F., Klein, M. P., Rodrigues, R. C. & Hertz, P. F. Fructooligosaccharides synthesis by highly stable immobilized β-fructofuranosidase from Aspergillus aculeatus. *Carbohydr. Polym.* **103,** 193–197 (2014).

45. Klein, M. P. *et al.* Effect of the support size on the properties of β-galactosidase immobilized on chitosan: Advantages and disadvantages of macro and nanoparticles. *Biomacromolecules* **13,** 2456–2464 (2012).

46. Waterborg, J. H. The Lowry Method for Protein Quantitation. *Springer,The Protein Protoc. Handook* 7–10 (2002). doi:10.1385/1-59259-169-8:7

47. Sheet, P. D. Viscozyme ®. (2012).

48. Selig, M., Weiss, N. & Ji, Y. *Enzymatic Saccharification of Lignocellulosic Biomass*. *NREL* (2008).

49. LI, Y. H., LIU, B., ZHAO, Z. B. & BAI, F. W. Optimization of Culture Conditions for Lipid Production by Rhodosporidium toruloides. *Chin. J. Biotechnol.* **22,** 650–656 (2006).

50. Da Silva, T. L., Feijão, D. & Reis, A. Using multi-parameter flow cytometry to monitor the yeast rhodotorula glutinis CCMI 145 batch growth and oil production towards biodiesel. *Appl. Biochem. Biotechnol.* **162,** 2166–2176 (2010).

51. Sitepu, I. R. *et al.* An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. *J Microbiol Methods* **91,** 321–328 (2012).

52. Kimura, K., Yamaoka, M. & Kamisaka, Y. Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence. doi:10.1016/j.mimet.2003.10.018

53. Lichtenthaler, H. K. & Buschmann, C. Chlorophylls and Carotenoids : Measurement and Characterization by UV-VIS Spectroscopy. *Curr. Protoc. Food Anal. Chem.* 1–8 (2001).

54. Hames, B. *et al. Preparation of Samples for Compositional Analysis*. *NREL* (2008).

55. Sousa, E. C. *et al.* Chemical composition and bioactive compounds of grape pomace (Vitis vinifera L.), Benitaka variety, grown in the semiarid region of Northeast Brazil. *Food Sci. Technol.* **34,** 135–142 (2014).

56. Hedegaard, R. V, Liu, L. & Skibsted, L. H. Quantification of radicals formed during heating of β-lactoglobulin with glucose in aqueous ethanol. *Food Chem.* **167,** 185–90 (2015).

57. Pedras, B. *et al.* Valorization of white wine grape pomace through application of subcritical water: Analysis of extraction, hydrolysis, and biological activity of the extracts obtained. *J. Supercrit. Fluids* (2017). doi:10.1016/j.supflu.2017.05.020

58. Amerine, M. a & Thoukis, G. The glucose-fructose ratio of California grapes. *Vitis* **1,** 224–

229 (1958).

59. Adriano, W. S., Filho, E. H. C., Silva, J. A., Giordano, R. L. C. & Gonçalves, L. R. B. Stabilization of penicillin G acylase by immobilization on glutaraldehyde-activated chitosan. *Brazilian J. Chem. Eng.* **22,** 529–538 (2005).

60. Prescott, J. H., Groman, E. V. & Gulyas, G. New molecular weight forms of arabinogalactan from Larix occidentalis. *Carbohydr. Res.* **301,** 89–93 (1997).

61. Willför, S., Sjöholm, R., Laine, C. & Holmbom, B. Structural features of water-soluble arabinogalactans from Norway spruce and Scots pine heartwood. *Wood Sci. Technol.* **36,** 101–110 (2002).

62. Santamauro, F., Whiffin, F. M., Scott, R. J. & Chuck, C. J. Low-cost lipid production by an oleaginous yeast cultured in non-sterile conditions using model waste resources. *Biotechnol. Biofuels* **7,** 34 (2014).

63. Zhang, D., Hegab, H. E., Lvov, Y., Snow, L. D. & Palmer, J. Immobilization of cellulase on a silica gel substrate modified using a 3 - APTES self - assembled monolayer. *Springerplus* **5,** (2016).

64. Li, S. K. *et al.* Simple and efficient synthesis of copper(II)-modified uniform magnetic Fe3O4@SiO2 core/shell microspheres for immobilization of cellulase. *J. Nanoparticle Res.* **15,** (2013).

65. Blanchette, C., Lacayo, C. I., Fischer, N. O., Hwang, M. & Thelen, M. P. Enhanced Cellulose Degradation Using Cellulase- Nanosphere Complexes. *PLoS One* **7,** (2012).

66. Ding, Z., Zheng, X., Li, S. & Cao, X. Immobilization of cellulase onto a recyclable thermo-responsive polymer as bioconjugate. *J. Mol. Catal. B Enzym.* **128,** 39–45 (2016).

67. Hartono, S. B. *et al.* Functionalized Mesoporous Silica with Very Large Pores for Cellulase Immobilization Functionalized Mesoporous Silica with Very Large Pores for Cellulase Immobilization. *J. Phys. Chem. C* **114,** 8353–8362 (2010).

68. Khoshnevisan, K. *et al.* Immobilization of cellulase enzyme on superparamagnetic nanoparticles and determination of its activity and stability. *Chem. Eng. J.* **171,** 669–673 (2011).

69. Mubarak, N. M. *et al.* Immobilization of cellulase enzyme on functionalized multiwall carbon nanotubes. *J. Mol. Catal. B Enzym.* **107,** 124–131 (2014).

70. Roth, H.-C., Schwaminger, S. P., Peng, F. & Berensmeier, S. Immobilization of Cellulase on Magnetic Nanocarriers. *ChemistryOpen* **5,** 183–187 (2016).

71. Podrepsek, G. H., Primozic, M., Knez, Z. & Habulin, M. Immobilization of cellulase for industrial production. *Chem. Eng. Trans.* **27,** 235–240 (2012).

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APPENDIX

A. Subcritical Water Hydrolysis – Carbohydrates Quantification

Colorimetric Method – Sugars Calibration Curve

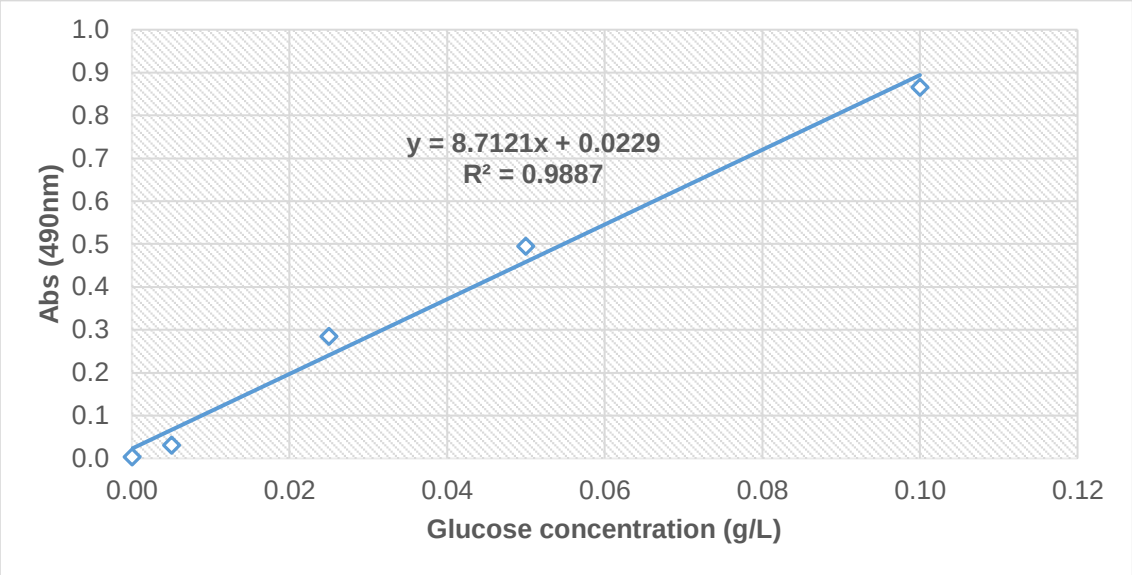

Figure A.1 *Glucose calibration curve for grape pomace hydrolysates.*

B. Enzymatic Hydrolysis – Protein Quantification

Lowry Method – BSA (Bovine Serum Albumin) Calibration Curve

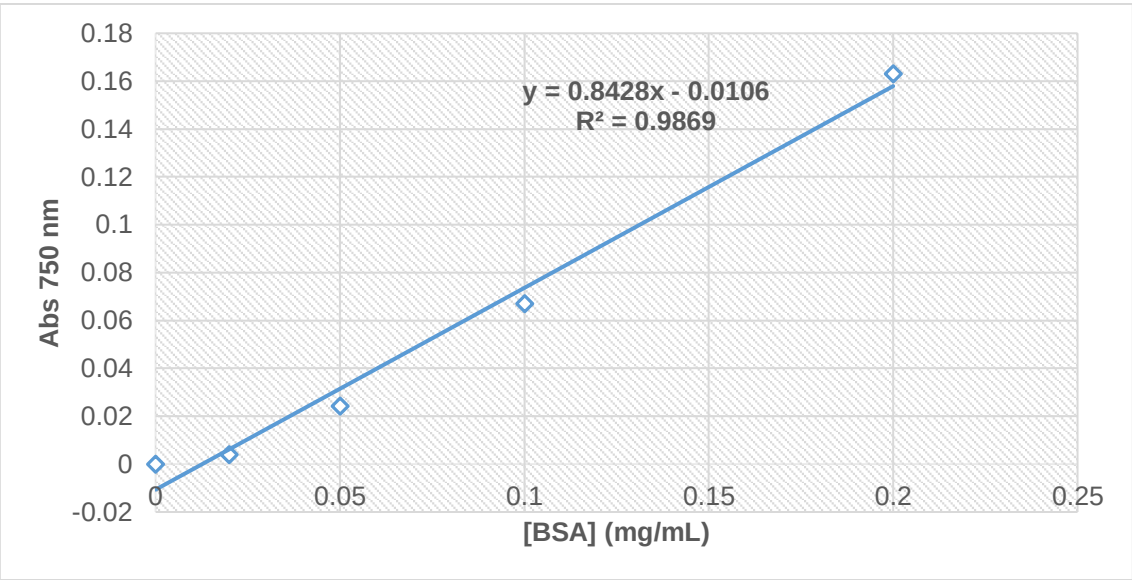

Figure B.1 BSA protein calibration curve for enzymatic hydrolysis.

C. Oleaginous Yeasts – Biomass Quantification:

***Rhodotorula babjevae* Calibration Curve**

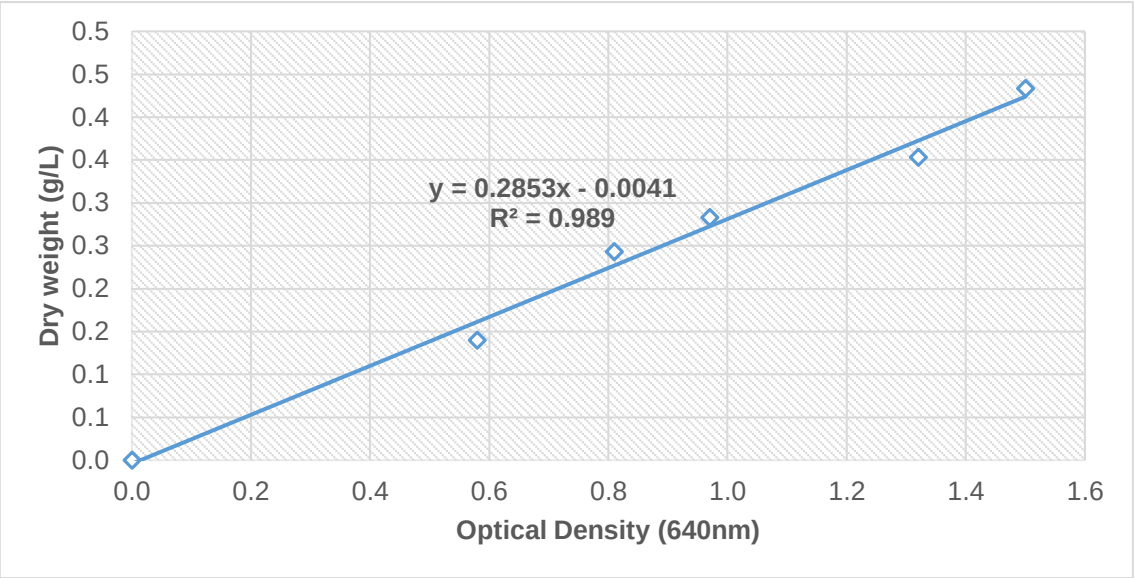

Figure C.1 *Biomass calibration curve for Rhodotorula babjevae growth.*

***Rhodotorula mucilaginosa* Calibration Curve**

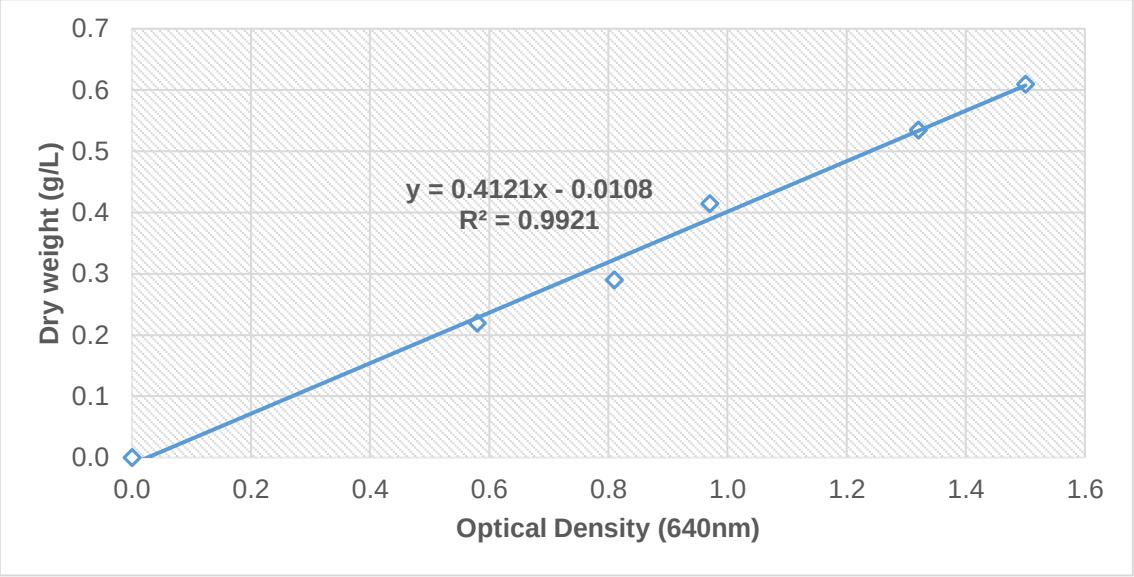

Figure C.2 *Biomass calibration curve for Rhodotorula mucilaginosa growth.*

***Lipomyces starkeyi* Calibration Curve**

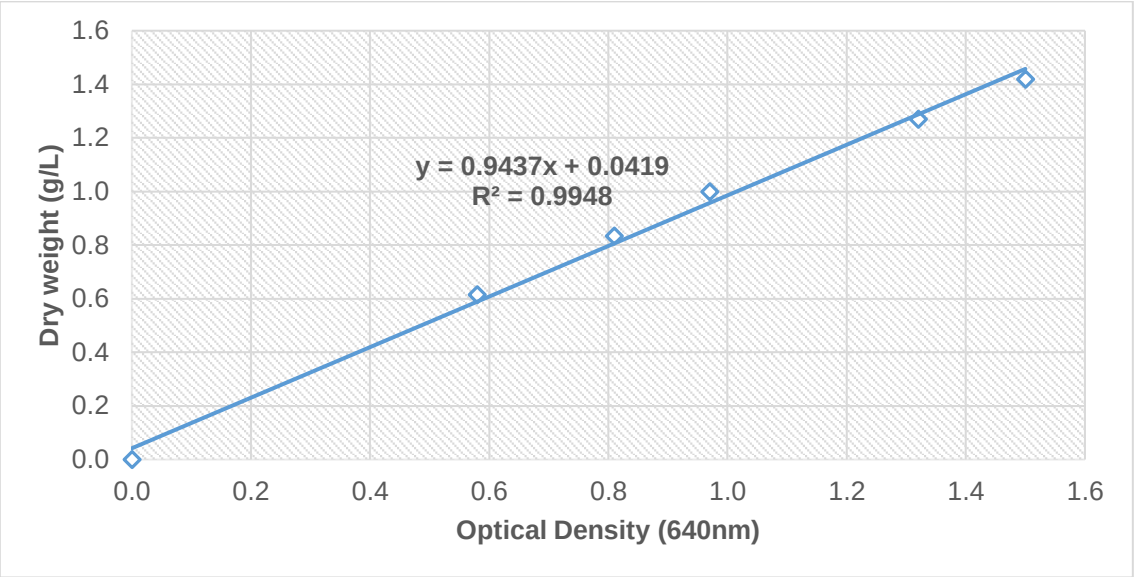

Figure C.3 Biomass calibration curve for Lipomyces starkeyi growth.

D. Oleaginous Yeasts – Carotenoids Quantification

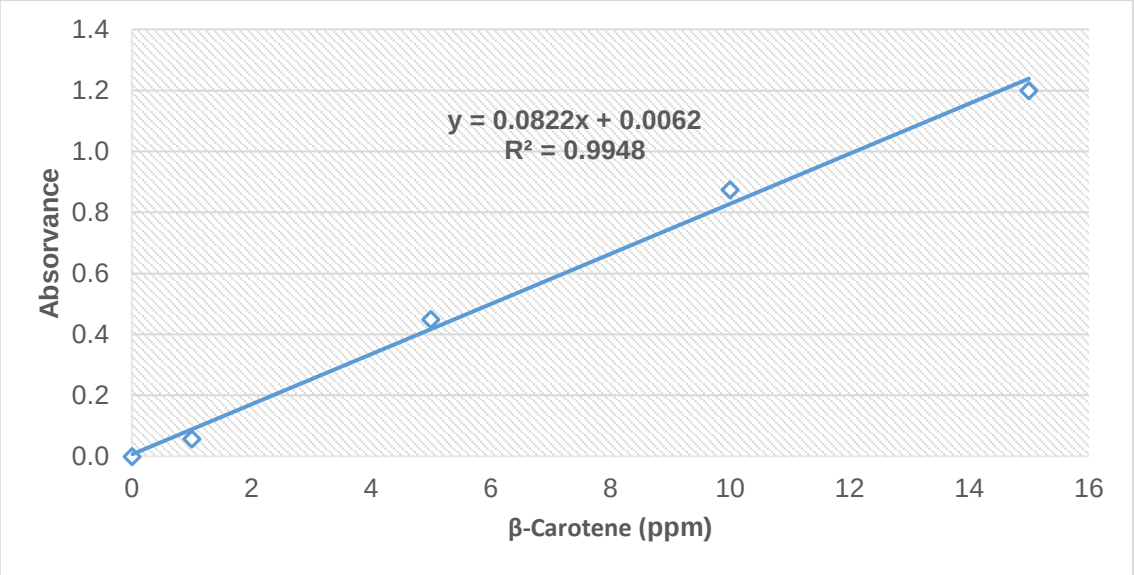

Figure D.1 *β-carotene calibration curve for carotenoids quantification.*