CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA

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AN INTEGRATED VALORIZATION OF GRAPE STALKS FOR PRODUCTION OF VALUE-ADDED COMPOUNDS. CASE STUDY OF THE PRODUCTION OF SUGARS FOR SECOND GENERATION ETHANOL BIOREFENERIES

by
Tuğba Atatoprak

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Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa to fulfill the requirements of Master of Science degree in Applied Microbiology

by

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To Murat Atatoprak,

Sevgi Atatoprak,

Elif Su Atatoprak,

Mário Jorge Jesus Gonçalves.

Remember to look up the stars and not down at your feet.

Never give up work. Work gives you meaning and purpose and life is empty without it.

If you are lucky enough to find love, remember it is there and don't throw it away.

Stephen Hawking

Resumo

A biomassa lignocelulósica é um dos principais recursos energéticos alternativos para lidar com os problemas de aquecimento global e depleção dos recursos de combustíveis fósseis. O bagaço de uva é um exemplo de biomassa lignocelulósica que é composta por celulose, hemicelulose, lignina e outros compostos (ceras, taninos, etc.)..

Assim, a valorização integral da biomassa do bagaço de uva branca e vermelha foi realizada de forma a maximizar o seu valor económico, para a produção de bio-açúcares, lignina, compostos fenólicos e nanocelulose. O processo foi iniciado com o fracionamento dos extrativos por Soxlet no qual foi possível verificar que independentemente do processo de extração aplicado, a % de extrativos é consideravelmente maior em bagaço de uva branca do que em uva vermelha (ca. 20%). A remoção da lignina para aumentar a acessibilidade aos açúcares e digestibilidade foi realizada através de uma reação alcalina. As concentrações de lignina mais elevadas (ca. 50 %) foram obtidas utilizando 1 % NaOH durante 60 min. Posteriormente, de forma a obter açucares fermentáveis (bio-açúcares), foram testadas hidrólises ácida e enzimática (celluclast e β-glucosidase) no material deslignificado. A hidrólise com ácido sulfúrico a 3,5% permitiu obter uma concentração máxima de glicose (3,22 g/L), xilose (7,29 g/L) e arabinose (0,91 g/L). No entanto, as concentrações mais elevadas de açúcares foram obtidas por hidrólise enzimática do que por hidrólise ácida diluída, com valores de glicose com 6,06 g/L e xilose com 8,08 g/L. De forma, a validar a fermentabilidade dos açucares, estirpes de *Pichia stipitis* e *Saccharomyces cerevisiae* foram avaliadas, e caracterizadas quanto à produção de etanol em meios produzidos com açucares provenientes de ambas as hidrolises ácidas e enzimáticas. *Pichia stipitis* mostrou ser a estirpe de levedura com o melhor crescimento e produção de etanol nos meios de cultura preparados com os açucares derivados da hidrólise enzimática. Foram obtidos rendimentos de etanol de cerca de 0,22 g/L ao longo dos estudos de fermentação. *Saccharomyces cerevisiae* foi capaz de produzir etanol em ambos os tipos de meio produzidos com açúcares obtidos das hidrólises ácidas e enzimáticas, mas no geral o rendimento de etanol foi baixo ca. 0,14 g/L e 0,005 g/L respectivamente. Desta forma, foi possível concluir que a fermentação com os meios com açucares derivados da hidrolise enzimática e com a estirpe *Pichia stipitis,* foram as melhores condições encontradas para produzir etanol. Por fim, a parte cristalina da celulose que não sofreu hidrólise foi valorizada para produção de nanocelulose. Foi possível produzir nanocelulose com tamanhos de ca. 295nm e um potencial zeta de -37 mv. Pela primeira vez, a biomassa de bagaço de uva é reportada como uma fonte de nanocelulose, eventualmente potenciando o seu valor.

Palavras-chave: Bagaço de uva, Fermentação, Lignina, Composto fenólico, Nanocelulose.

Abstract

Lignocellulosic biomass is one of the potential and key alternative energy resources to deal with the problems of global warming and depletion of fossil fuel resources. Grape stalk is an example of lignocellulosic biomass which is composed of cellulose, hemicellulose, lignin and other compounds (waxes, tannins, etc.).

Thus, the integral recovery of the biomass of the white and red grape stalk was carried out in order to maximize its economic value for the production of bio sugars, lignin, phenolic compounds and nanocellulose. The process was started with the fractioning of the extractives by Soxhlet, in which it was possible to verify that, regardless of the extraction process applied, the% of extractives is considerably higher in white grape stalk than in red grape stalk (ca. 20 %). Removal of lignin to increase the accessibility to sugars and digestibility was performed by alkaline reaction. The highest lignin concentrations (ca. 50 %) were obtained using 1 % NaOH during 60 min. Subsequently, to obtain fermentable sugars (bio sugars), acid and enzymatic hydrolysis (celluclast and β-glucosidase) were tested in the delignified material. Hydrolysis with 3.5% sulfuric acid yielded a maximum concentration of glucose (3.22 g/L), xylose (7.29 g/L) and arabinose (0.91 g/L) during 60 min. However, higher concentrations of sugars were obtained by enzymatic hydrolysis than by dilute acid hydrolysis, with glucose values of 6.06 g/L and xylose with 8.08 g/L. In order to validate the fermentation of the sugars, strains of *Pichia stipitis* and S*accharomyces cerevisiae* were evaluated, and characterized in the ethanol production in media produced with sugars from both acidic and enzymatic hydrolysis. *Pichia stipitis* showed to be the yeast strain with the best growth and ethanol production in the culture media prepared with sugar derived from enzymatic hydrolysis. Ethanol yields of about 0.22 g/L were obtained throughout the fermentation studies. S*accharomyces cerevisiae* was able to produce ethanol from in both types of media produced with sugars from enzymatic and acid hydrolysis, but overall ethanol yields were lower ca. 0.14 g/L and 0.005 g/L respectively. In this way, it was possible to conclude that the fermentation with the media with sugars derived from enzymatic hydrolysis and with the strain *Pichia stipitis* were the best conditions found to produce ethanol.

Finally, the crystalline part of the cellulose that did not undergo hydrolysis was valued to produce nanocellulose. It was possible to produce nanocellulose with sizes ca. 295 nm and zeta potential of -37 mv. For the first time, grape stalk biomass is reported as a source of nanocellulose, eventually boosting its value.

Key words: Grape stalk, Fermentation, Lignin, Phenolic compound, Nanocellulose.

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List of Abbreviation

$(NH_4)_2SO_4$ ... Ammonium Sulfate
1G ... First Generation
2G ... Second Generation
CBP ... Consolidated Bioprocessing
CFU ... Colony Forming Unit
CNC ... Cellulose Nanocrystal
d ... Days
DGS ... Delignified Grape Stalks
DLS ... Dynamic Light Scattering
DSMZ ... German Collection of Microorganisms and Cell Cultures
FPU ... Filter Paper Units
FTIR ... Fourier Transform Infrared Ray
g ... Gram
GS ... Grape Stalk
GSWE ... Grape Stalk Water Extracted
GSWEE ... Grape Stalk Water and Ethanol Extracted
h ... Hours
H_2SO_4 ... Sulphuric Acid
HGS ... Hydrolysed Grape Stalk
HMF ... Hydroxymethyl-furfural
HPLC ... High Performance Liquid Chromatography
IU ... International Unit
K_2HPO_4 ... Dibasic Potassium Phosphate
kg ... Kilogram
KL ... Klason lignin
L ... Liter
mg ... Milligram
$MgSO_4$ ... Magnesium Sulfate
Mhl ... Million Hectolitres
min ... Minute
ml ... Millilitre
mM ... Millimolar
mv ... Millivolts
NaOH ... Sodium Hydroxide
NCC ... Nanocrystalline Cellulose
NFC ... Nanofibrillated Cellulose
nm ... Nanometre
NREL ... National Renewable Energy Laboratory
ODW ... Oven Dry Weight
P. stipitis ... *Pichia stipitis*
pH ... Cologarithm of the Hydrogen Cation Concentration
PI ... Polydispersity Index
rpm ... Rounds Per Minutes
S. cerevisiae ... *Saccharomyces cerevisiae*
SD ... Standard Deviation
SHF ... Separate Hydrolysis and Fermentation
SSCF ... Simultaneous Saccharification and Co-fermentation
SSF ... Simultaneous Saccharification and Fermentation
UV-Vis ... Ultraviolet–Visible Spectrophotometry
w/v ... Weight/Volume Percentage

w/w .... Weight/Weight
ZP .... Zeta Potential

1. Introduction

1.1. Wine and Winery waste

Wine is an alcoholic beverage from fermentable carbon sources performed by yeasts mainly (but not exclusively) by strains of *Saccharomyces cerevisiae* (Jackson, 2008). It is the oldest and most economically important of all biotechnologies, that has been enjoyed from ancient times to modern times by many people for more than 7.5 thousand years (Bayrak, 2013). Even today wine industry constitutes an important part of the economy in several regions of the world. According to OIV (The International Organization of Vine and Wine), the world production of wine was of ca. 259 Million hl in 2016, especially by countries such as Italy (48,8 Mhl), France (41,9 Mhl) and Spain (37,8 Mhl), which are the main wine producers in Europe and worldwide.

Wine can be produced with honey, grains, rice, sugarcane and fruits usually grape. Grapes are one of the main agro-economic crops in the world, with more than 75 million tons produced every year and ca. 50 % is utilized in the wine-making process (FAO-OIV, 2016; Zhu et al., 2015).

Wine making process basically transforms sugar of grapes into ethanol and involves the generation of wastes that can be divided into two main categories, solid and liquid wastes (Figure 1.1). Solid wastes of grape are the stalk, pomace and lees. Grape stalk is obtained after the destemming process, while grape pomace obtained after the pressing process and consists of processed skins and seeds. On the other hand, lees are produced throughout the fermentation and sedimentation steps and contain dead yeast cells. Liquid waste is generated from the water used in the bottling steps of the wine making process (Zacharof, 2017; Oliveira et al., 2016). In terms of production, the solid by-products such as pomace are the ones that are produced in higher quantities, followed by stalks as shown in Figure 1.2.

Moreover, the production of 100 L of white wine gives rise to about 31.2 Kg of by-products (including 17 kg of skins and 4 kg of stalks) and about 25 kg of by-products arise from production of 100 L of red wine (including 13.2 kg of skins and 4 kg of stalks) (Prozil et al.,2012).

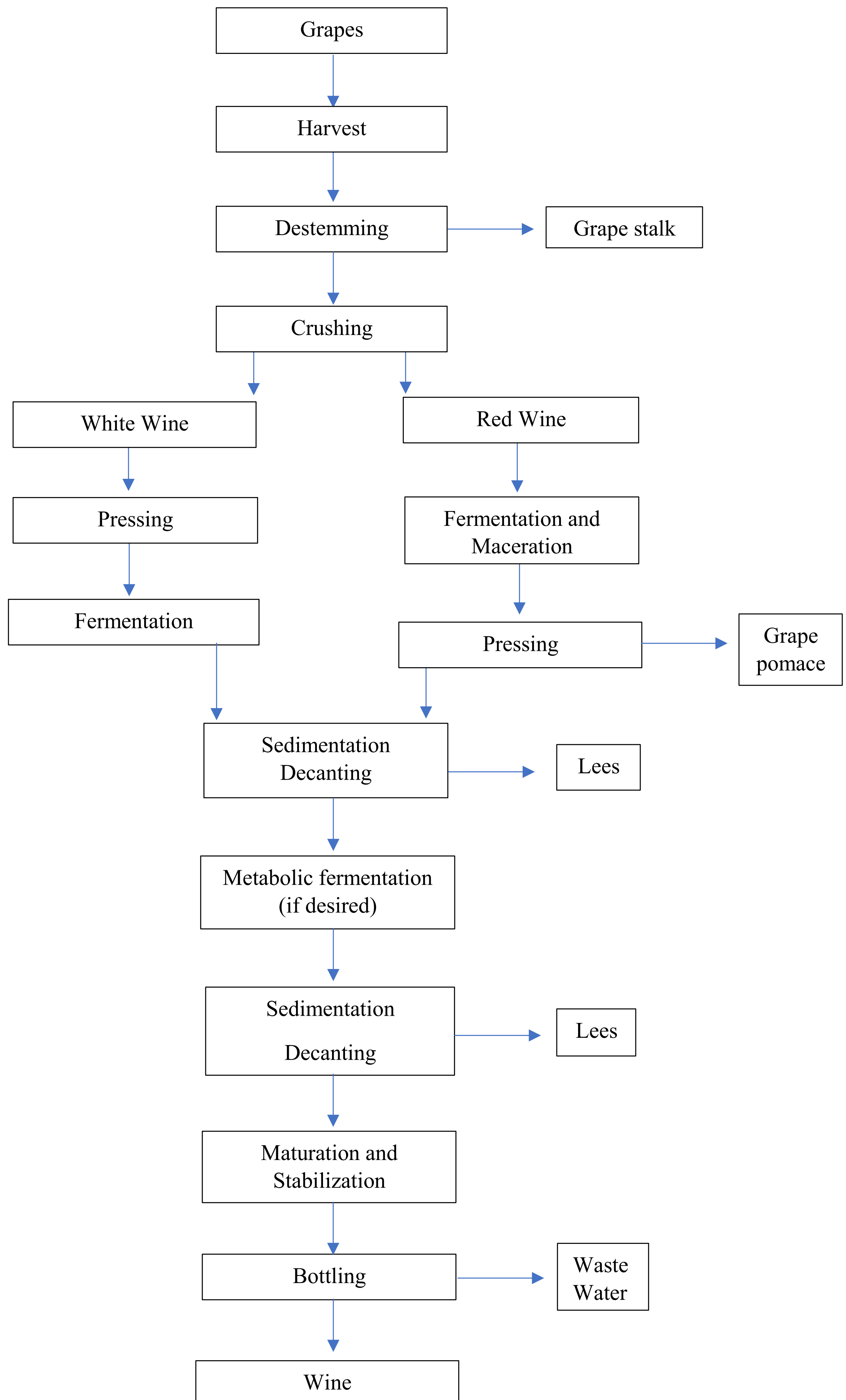


Figure 1. 1. *Diagram of the winemaking process and the different wastes generated along the process (adapted from Devesa-Rey et al., 2011; Zacharof et al., 2017; Oliveira et al., 2016)*

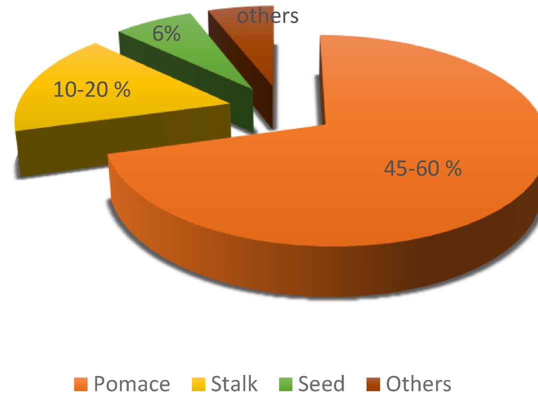


Figure 1. 2. *Solid wastes produced during wine process (adapted from Zacharof, 2017; Oliveria et al, 2016; González-Centeno et al., 2010).*

1.1.2. Valorization of winery waste

Winery waste production increases every year and per hectare of land per year it can be generated ca. 5 tons (Zacharof, 2017). As already mentioned before, the main by-products generated by wine making industry are grape pomace followed by grape stalks. Winery wastes are not intrinsically hazardous. However, they have high content of organic matter and the fact that production is concentrated in a period of the year poses potential pollution problems (Spigno et al., 2008). According to recent studies, discharging of winery wastes to soil originates inhibition of the germination properties of soil because of the biological oxygen demand, carbon and phenolic compounds (Lafka et al., 2007). For this reason, environmental and economically sustainable winery wastes management should be a priority for the industry worldwide. Therefore, valorization of winery wastes has become an important field of research for minimizing environmental impact. Until now, most of the research has been focusing on the exploitation of grape stalks as bio-sorbent material for the removal of toxic compounds and as bulking agent in composting processes, without any attention to the grape cultivar (Spigno et al, 2013). Only recently, the knowledge of grape stalk and other wastes composition has suggested that these wastes can be used as raw materials to process or extract new added value compounds (Table 1.1). Researchers have started investigating the use of grape stalk as a potential source of high added-value compounds, such as phenolic compounds with antioxidant activity, lignin, cellulose and hemicellulose (Spigno et al., 2013). In addition to that, grape pomace and lees represent a rich source of high-value by-products such as ethanol, tartrates and malates, citric acid, flavanol, and tannin (Yalçın et al., 2008). The grape pomace can be distilled

to recover ethanol and tartaric acid but the use of this waste for the extraction of antioxidant flavanols, used as a nutritional supplement, may provide an important economic advantage (Oliveira et al., 2016).

Table 1. 1. *Solid wastes and potential use already explored (Adapted from Oliveria et al, 2016)*

Waste	Uses	Reference
Grape pomace	Polyphenol production	Arvanitoyannis et al. (2006), Santamaría et al. (2002), Alonso et al. (2002), Sakata et al. (2010)
	Lactic acid production	Devesa-Rey et al. (2011), Silva et al. (2011)
	Ethanol and tartaric acid production	Yalcin et al. (2008)
	Tannins, polyphenols and oil production	Devesa-Rey et al. (2011), Silva et al. (2011)
Stalk	Plant substrate	Arvanitoyannis et al. (2006), Bustamante et al. (2009)
	Polyphenol production	Alonso et al. (2002)
Lees	Tartaric acid production	Yalcin et al. (2008)

1.2. Grape Stalk

Grape stalks are the skeletons of grape bunch and are composed mainly by lignocelluloses. Lignocelluloses contains polysaccharides such as cellulose and hemicelluloses and fiber as lignin. Other minor compounds and ash are also present as shown in Table 1.2 (Mood et al., 2013).

There are several works that explore stalks as source of different value-added compounds (Table 1.3). The reducing sugars are one example of compounds that can be obtained by the processing of the complex carbohydrates (cellulose and hemicellulose). Some processes such as polysaccharides acid and enzymatic hydrolysis have been studied and optimized along the last years. Nevertheless, the application of this process exclusively to produce ethanol with stalks sugars is certainly not attractive in terms of costs and gains. Hence, since stalks are rich in other valued compounds, an integrated valorization of these wastes and consequent generation of different compounds can be applied, increasing the gains and attracting the companies to explore these materials.

In this context, this thesis is focused on the valorization of two types of grape stalk (red and white) as biomass for biorefineries to produce second generation ethanol and value-added compounds (cellulose, hemicellulose, lignin, phenolic compounds, nanocellulose). There are several works that explore stalks composition and few with experimental optimization of processes to convert stalk cellulose and hemicelluloses in reducing sugars or other compounds with value (Spigno et al., 2008; Ping et al., 2011). None has explored an integrated valorization

of other compounds besides sugars. One example is nanocellulose, which consists of the crystalline part of cellulose that is resistant to hydrolysis processes and that is claimed for their interesting properties. Cellulose can be used for fiber production (Frederick et al., 2008), lignin can be hydrolyzed to obtain phenolic compounds and used as natural antioxidant and antimicrobial agent and hemicellulose can be further hydrolyzed to increase the concentration of fermentable sugars (Dogaris et al., 2009).

Table 1. 2. *General composition of grape stalks obtained by three authors.*

Compound	Spigno et al. (2008)	Ping et al. (2011)	Lorenzo et al. (2002)
Klason lignin	47.3	39.6	22.9
Soluble lignin		1.0	
Cellulose	25.3	36.3	29.9
Hemicellulose	13.9	24.5	35.3
Xylans	10.0	14.9	
Arabinans	3.9	3.7	
Mannans		2.0	
Galactans		3.9	
Ash	7.7	3.9	
Tanins		6.4	

[*] All data are yields of components (g) per 100g of oven-dried grape stalk.

Table 1. 3. *Explored uses/applications of grape stalks.*

Grape Cultivar	Valorization studies of grape stalks	Reference
Pinot Nero Mixture of Barbera and Bonarda	Lignocellulosic fractionation and chemical characterization	Spigno at al., (2008) Amendola et al. (2012)
Barbera Bobal	Antioxidants recovery	Spigno and De Faveri (2007); Garcia-Perez et al., (2010); Cárcel et al. (2010)
Bonarda and Barbera	Fermentable sugars via enzymatic treatment for biofuel production	Mazzaferro et al., (2011)
Not specified	Removal of toxic compounds (mainly metals)	Pocha and Villaescusa (2010) Wilson et al. (2012)
Not specified	Production of activated carbon	Martínez et al. (2009)
Not specified	Fermentation substrate	Lorenzo et al. (2002); Khot et al. (2012)

1.3. Added-value Compounds of Grape Stalk

1.3.1. Lignocellulose

Lignocellulose is composed of cellulose, hemicelluloses and lignin. Cellulose is the main component of plant cell walls and it consists of D-glucose subunits which are linked together by β-1,4-glycosidic bonds (Sakimoto et al., 2017). The chemical formula of cellulose is $(C_6H_{10}O_5)_n$ and the structure of one chain of the polymer is presented in Figure 1.3. Hence, cellulose is an excellent source of glucose for fermentation processes.

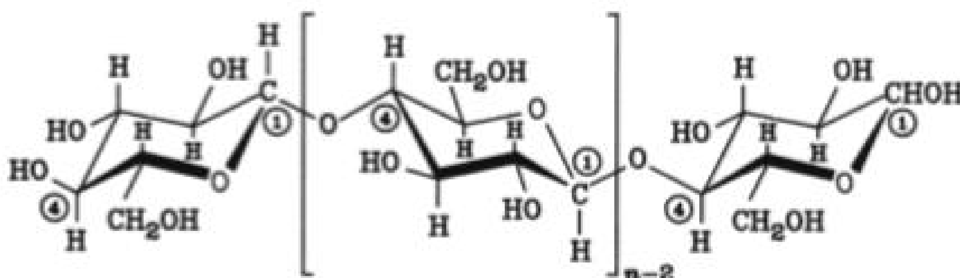


Figure 1. 3. *Structure of single cellulose molecule. Reprinted from Harmsen et al., 2010.*

On the other hand, hemicellulose is group of polysaccharides which consist of hexoses (D-glucose, D-galactose and D-mannose) as well as pentose (D-xylose and L-arabinose) and small amount of D-glucuronic, D-galacturonic and methylgalacturonic acids. Xylan is the most abundant hemicellulose in grape stalk. It is followed by arabinan, galactan and mannan (Spigno et al., 2008).

Finally, lignin is a complex phenolic polymer that is present in lignocellulosic compounds arounds 15-20%. It is also known as the substance that makes up the woody structure of the root and stem of the plant. As a heteropolymeric aromatic compounds include coniferyl, sinapyl and p-coumaryl alcohols and due to the presence of functional group, lignin has antioxidant and antimicrobial properties (Watkins et al., 2015). Pan et al. (2006) showed that lignin of hybrid poplar at elevated temperature, longer reaction time, increased level of catalyst and diluted ethanol presented higher antioxidant activities based on 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Also, Nada et al. (1999) studied the antimicrobial activities of the different lignin's precipitated from the pulping liquor and the results showed that prepared lignin had no antimicrobial effects against the Gram-negative bacteria (*Escherichia coli*) and filamentous fungi (*Aspergillus niger*) but was effective against the Gram-positive bacteria (*Bacillus subtilis* and *Bacillus mycoides*). Lignin has been used for several applications as raw material, such as

syngas production, hydrocarbons, phenols, oxidized products and macromolecules (Holladay et al. 2007).

1.3.2. Other compounds

1.3.2.1. Phenolic compounds

Grape stalk contains large amounts of phenolic compounds and most of them are originated from lignin. These phenolic compounds are chemical compounds that can be used as natural antioxidants and microbial growth inhibitors.

The antioxidant activities of the winery byproducts derived extracts largely depend on the extraction efficiency of bioactive components, which is mostly phenolics and their profile and diversity. The extraction yields of phenolic compounds from winery by-products is affected by different factors, such as the extraction techniques, solvents, time, temperature and many others. Previous studies have tested solvents, such as methanol, ethanol, acetone, ethyl acetate and their combinations for the extraction of phenolic compounds in winery by-products. Flavonoids are most common phenolic compounds found in grape stalks.

1.3.2.2. Nanocellulose

Nanocellulose can be obtained from cellulose and has a rigid rod-shaped structure, is 1-100 nm in diameter, and is tens to hundreds of nanometers in length. Nanocellulose is categorized into nanocrystalline cellulose (NCC) and nanofibrillated cellulose (NFC). Both types of nanocellulose are chemically similar, but dissimilar as physical characteristic since NFC has "spaghetti" and NCC has "rice" form (Lee et al.,2014). Nanocellulose has various superior characteristics which are nanoscale dimension, high surface area, unique optical properties, high crystallinity, and stiffness together with the biodegradability and renewability of cellulose. Because of their properties, nanocellulose could be used for several applications, immobilized enzyme/protein (Edwards et al., 2012), blood vessel replacement (Brown et al., 2013), drug delivery (Jackson et al., 2011), nucleus pulposus replacement (Eyholzer et al., 2011), food packaging (Ferrer et al., 2017) and antimicrobial nanomaterials (Fortunati et al., 2014)

The extraction of nanocellulose includes always the removal of the amorphous part of cellulose leaving the crystalline part using acid hydrolysis, or alkaline hydrolysis, delignification via oxidation, organosolv pretreatment and ionic liquids pretreatment to cellulose depolymerization for nanocellulose synthesis (Ng et al., 2015).

Acid hydrolysis treatments promotes the breakage of β-1,4 glycosidic bonds of cellulose and is a process with lowering energy consumption. Cellulose has crystalline and amorphous forms and the amorphous regions will break up releasing the individual crystallites when subjected to acid treatment (Figure 1.4) (Mondal, 2017). The amorphous part of cellulose is composed of

the sugars, especially glucose. Hence, in this thesis considering that the amorphous part of cellulose is first removed by similar processes that are used to obtain nanocellulose, the final crystalline part that is generated was used to produce nanocellulose.

In addition, enzymatic process for nanocellulose synthesis offers the potential for higher yields, higher selectivity, lower energy costs and milder operating conditions than chemical processes.

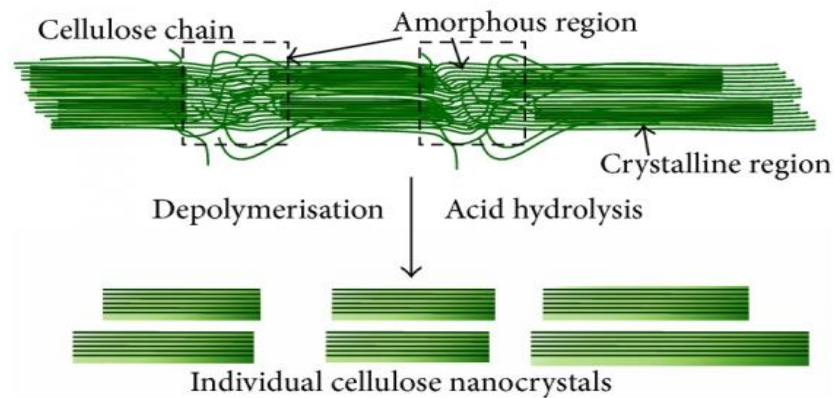


Figure 1. 4. *Depolymerization cellulose to nanocellulose. Reprinted from Lee et al.,2014.*

1.4. Deconstruction of lignocelluloses in cellulose, hemicelluloses and lignin

Cellulose of grape stalk residues are not available for hydrolysis due to its binding to hemicellulose and enclosure by lignin. Therefore, a pretreatment of the residue must be employed to remove lignin. These pretreatment processes are crucial steps for the biochemical conversion of lignocellulosic biomass into e.g. bioethanol. It is required to alter the structure of cellulosic biomass to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars (Mosier et al., 2005).

There are several pretreatment methods which consist of physical (grinding and milling, microwave and extrusion), chemical (alkali, acid, organosolv, ozonolysis and ionic liquid), physicochemical pretreatment (steam explosion, liquid hot water, ammonia fiber explosion, wet oxidation and CO_2 explosion) or biological pretreatments (Figure 1.5) (Mood et al., 2013).

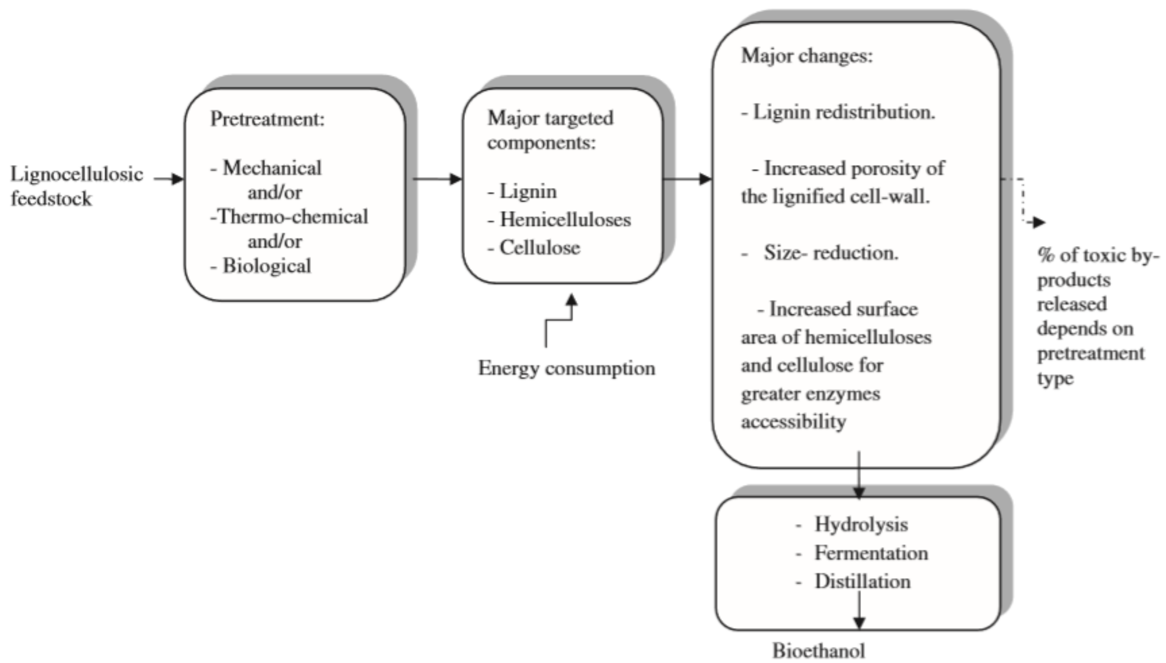


Figure 1. 5. *Pretreatment upstream processes. Reprinted from Limayem et al. (2012).*

In this thesis, the chemical treatment approach based on alkaline solvents was used, by means of optimized parameters by other researchers (Feist et al., 1970; Sun and Cheng, 2002; Pujol et al., 2013). The reasons are related to the advantages that were found and described below, not only for wine wastes pretreatment, but also for other residues.

In terms of alkaline solvents, the most common are sodium hydroxide (NaOH), potassium hydroxide (KOH) and calcium hydroxide ($CaOH_2$) (Bali et al., 2015). Alkaline pretreatment increases the porosity and surface area of cellulose, decreases the degree of polymerization and crystallinity of cellulose, destroying the structural linkages between lignin and carbohydrate by saponification of intermolecular ester bonds and disrupting the lignin structure by breaking its glycosidic ether bond (Figure 1.6) (Fan et al., 1987; Soccol et al., 2011). In addition, compared to other pretreatment processes, the alkaline process brings several advantages such as low operation costs, reduced degradation of holocellulose, high solubilization of lignin and low formation of fermentation inhibitors (Cheng et al., 2010).

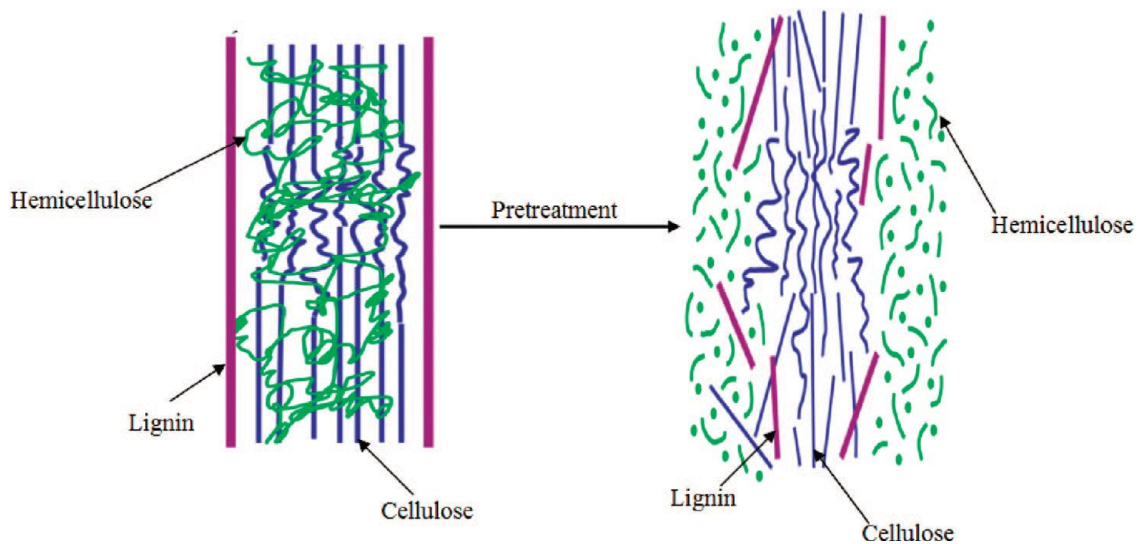


Figure 1. 6. *Schematic presentation of effects of pre-treatment on lignocellulosic biomass. Reprinted from Kumar et al. (2009).*

1.4.1. Hydrolysis

1.4.1.1 Enzyme Hydrolysis

To obtain bioethanol from lignocellulosic biomass, the biomass is subjected to various processes, such as pretreatment, saccharification and ethanol fermentation. Pretreatment is essential to make cellulose accessible to enzymes. Enzymes processes are preferred over chemical treatments such as acid or alkaline hydrolysis, because of the minimized loss of monomers, decreased production of by-products during hydrolysis and higher conversion efficiencies (Waldron, 2010; Bon et al, 2007). Besides that, it has a disadvantage that enzyme process is slower than chemical hydrolysis (Cardona et al., 2010).

There are several enzymes for the hydrolysis of biomass such as cellulase, xylanase, ligninase, pectinase, etc. Cellulase is the most important enzyme if the biomass contains about 40% or above cellulose. Cellulase is a multi-enzyme complex of three different enzymes; exoglucanase, endoglucanase and beta-glucosidase (Singhania et al., 2013). Firstly, endoglucanase cleaves between cellulose fibers and it releases small cellulose fragments with free reducing and then non-reducing ends, which are attacked by exoglucanase to release small oligosaccharides and finally cellobiose is hydrolyze into glucose monomers by beta-glucosidase. Beta-glucosidase is limiting enzyme which is complete the final step of hydrolysis by converting the cellobiose to glucose (Singhania et al., 2013).

Overall, the success of the conversion of lignocellulose into fermentable sugars relies in the feedback properties, pretreatment type and conditions, and the type of substrate and substrate concentration, enzyme combination and its dosage and process conditions (temperature, pH,

residence time, etc.) (Pengilly, 2013). When fermentation takes place, it is important to maintain high carbohydrate concentrations during hydrolysis to achieve high ethanol concentrations during fermentation (Cortez et al., 2010). For this reason, following the route of enzymatic hydrolysis, there are four different fermentation-hydrolysis configurations for pretreated lignocellulose, namely: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and lastly, consolidated bioprocessing (CBP) (Olsson et al., 2005). In this study, a separate hydrolysis and fermentation (SHF) was carried out.

1.4.1.2. Dilute acid hydrolysis

The main advantages of the dilute hydrolysis process are the low amount of acid required (2-5%) and low amounts of produced fermentation inhibitors (Mood et al., 2013). Several types of acids can be used, such as sulphuric, hydrocloric, acetic, phosphoric, nitric and formic acid (Galbe and Zacchi, 2002). Sulphuric and hydrochloric acids are the most commonly used catalysts for hydrolysis of lignocellulosic biomass (Lenihan et al., 2010).

Dilute-acid hydrolysis has become cost-effective alternative to enhance biomass separation to isolate cellulose, hemicellulose, and lignin which can further be used for nanocellulose synthesis and chemical production. Generally, dilute acid can dissolve and recover most of the hemicellulose as dissolved sugars up to 100% conversion under low process severity (low temperature and low acid concentration) (Lee et al., 2014).

Dilute acid hydrolysis consists of two chemical reactions. One reaction converts cellulosic materials to sugar and the other converts sugars into other chemicals, many of which inhibit the growth of downstream fermentation yeasts. The same conditions that cause the first reaction to occur, simultaneously cause over-degradation of sugars and lignin, creating inhibitory compounds such as organic acids, furans, and phenols (Zhang and Shahbazi, 2011).

1.4.1.3. Production of inhibitors

Comparing to cellulose, hemicellulose can be easily hydrolyzing by diluted acid, alkali, or enzymes under mild conditions. However, during hemicellulose hydrolysis, pentose sugar monomers dehydrate to the inhibitor furfural. Also, hexose sugars degrade to the toxic hydroxymethyl-furfural (HMF). Those unwanted coproducts (furfurals and hydroxymethyl furfurals) and some other inhibitors (Figure 1.7) that have toxic effects on the fermenting organisms and reducing the ethanol yield and productivity during fermentation. However, it does not affect nanocellulose synthesis.

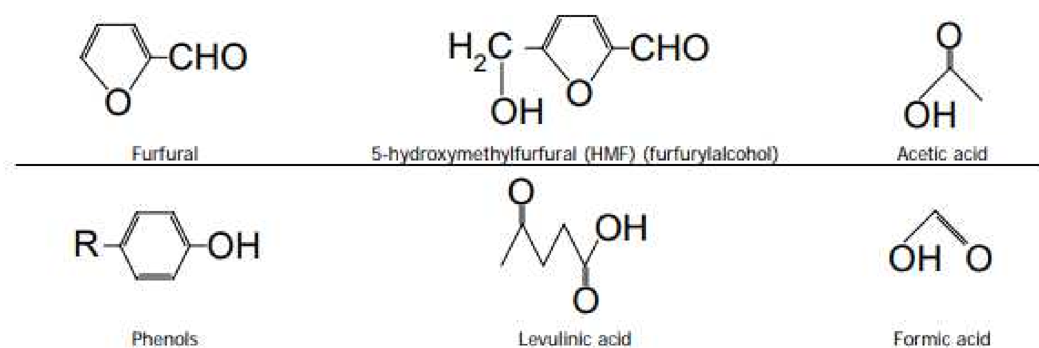


Figure 1. 7. *Major types of inhibitors for fermentation and their chemical structure. Reprinted from Mussatto and Roberto, 2004.*

1.5. Production of second-generation ethanol

The burning of fossil fuels contributes to the emissions of greenhouse gases as well as global warming that causes climate change, rise of sea level, loss of biodiversity and urban pollution. Bioethanol is one of the most promising alternatives to fossil fuels, which can be produced from various renewable sources rich in carbohydrates (Zabed et al., 2017).

During the last decade, the production of bioethanol from biomass materials received more attention in the worldwide. In the U.S., bioethanol is primarily produced from corn starch feedstocks (1G) while in Brazil biofuel is mainly produced from sugarcane juice and molasses (1G). Together, these countries account for 89% of the current global bioethanol production (Limayem et al., 2012).

In addition, bioethanol can be produced from different types of lignocellulosic biomass fermentation which is called second generation ethanol (2G) (Figure 1.8).

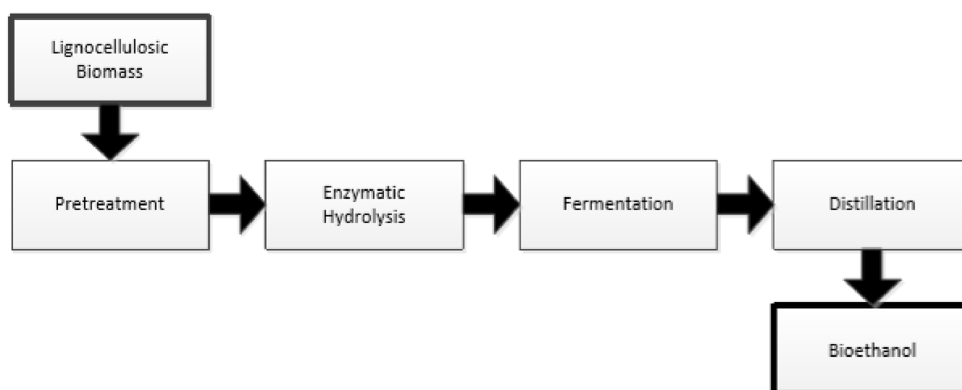


Figure 1. 8. *A simplified flow sheet to produce ethanol from lignocellulosic biomass. Reprinted from (Pengilly, 2013).*

The major limitations of lignocellulosic fermentation are the high costs of enzymes and slow hydrolysis rates of lignocellulose to obtain degradation results that are reasonable (Banerjee et al., 2010; Bansal et al., 2012, 2009) A recent NREL report stated that enzymes can contribute up to 25 % of the conversion cost (which excludes the cost of the feedstock) (Bansal et al., 2012; Pengilly, 2013). Additionally, it has several advantages when compared to sugar- or starch-derived bioethanol (1G). For instance, the cellulosic materials are renewable, low cost and are available in large quantities. 2G biofuels can be generated from forest and agricultural residues and by-products present a promising alternative to the current problem of resource competition with the food production from agriculture (Pengilly, 2013; Sarkar et al., 2012). The utilization of other plant residues and feedstocks could help to mitigate the limitation of land and biomass availability, and thus represents a better alternative for large scale bioethanol production than 1G technologies (Pengilly, 2013).

Kim and Dale (2004) reported that 442 billion liters of bioethanol can be produced from lignocellulosic biomass and that total crop residues and wasted crops can produce 491 billion liters of bioethanol per year, about 16 times higher than the actual world bioethanol production (Gupta et al., 2015).

Hence, bioethanol production could be the route to the effective utilization of agricultural wastes. For this reason, grape stalks are chosen as bioresource for bioethanol production. Nevertheless, in order to have viability as already mentioned, it should be included in an integrative process of valorization, in a concept of biorefinery, with the production of other compounds beside bioethanol.

1.6. Fermentation

The polysaccharides that are hydrolyzed into fermentable sugars i.e. glucose and xylose, are converted by yeasts into ethanol and carbon dioxide, with the generation of heat. The main goal during fermentation to reach an ethanol yield closest to the maximum theoretical value of 0.51 $g_{\text{ethanol}} \cdot g^{-1}_{\text{consumed sugar}}$ (Olsson et al,2005). The fermentation is usually carried out in large, closed stainless-steel tanks, which are temperature controlled to lower the fermentation temperature as appropriate.

Saccharomyces cerevisiae is one of the widely studied and used yeast in starch- and sucrose-based ethanol production, which can produce high ethanol yields (> 0.45 g.g-1), has high tolerance to ethanol (> 100 g.L-1) and can tolerate to inhibitors presence that are produced during biomass pretreatment, as previously mentioned (Cortez, 2010; Olofsson et al., 2008).

Moreover, *S. cerevisiae* is GRAS (generally recognized as safe) for human consumption which enhances its advantageous utilization more than other yeasts and microorganisms.

During chemical pretreatment, pentose and hexose carbon sugars are produced. *S. cerevisiae* can only ferment hexose sugars such as glucose, mannose and galactose (Cortez, 2010). It cannot ferment pentose sugars e.g. xylose and arabinose which are being the main building blocks of hemicelluloses (Cortez, 2010). The development of microorganisms that efficiently ferment these hemicellulose sugars is, xylose-fermenting yeasts, e.g. *Pichia stipitis* and *Candida shehatae*, can therefore be beneficial for use in the fermentation of materials that have high xylan content, such as lignocellulosic biomass (Olofsson et al., 2008).

1.7. Work objectives

Considering the exposed in introduction, the main objective of this thesis was to evaluate the grape stalks as a potential source of compounds with value for use in several applications, employing the concept of a biorefinery. For such, the optimization of methods was performed to achieve the deconstruction of grape stalks biomass to obtain phenolic compounds extracts, lignin, fermentable sugars and nanocellulose. Finally, the sugars extracted were tested in fermentation processes for production of 2G ethanol. (Figure 1.9).

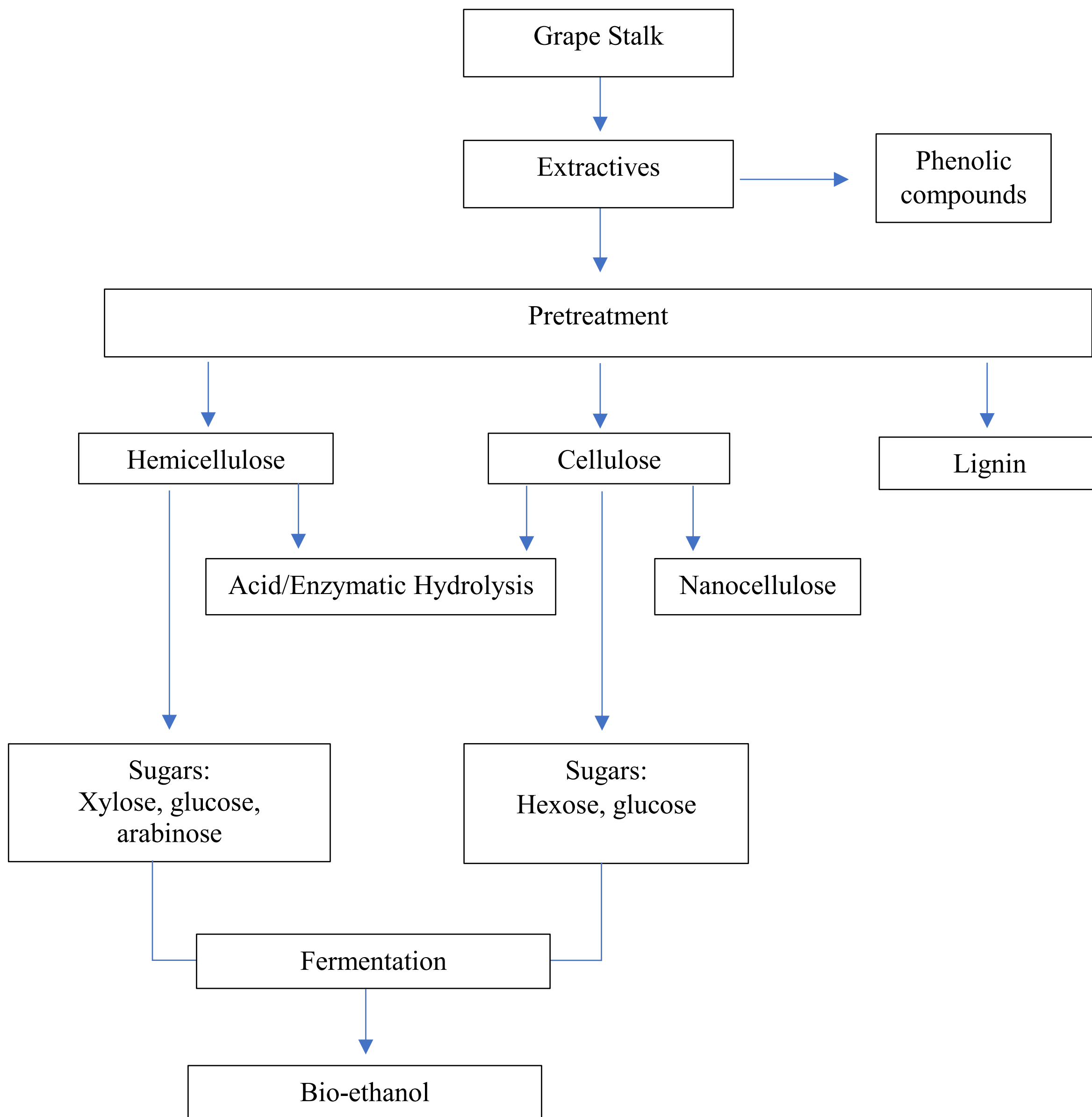


Figure 1. 9. *Work objectives of thesis. Evaluation of the potential grape stalks to be used in a biorefinery.*

2. Materials and Methods

2.1. Materials

Red Grape stalk (Vinhão variety) was provided by Quinta do Mascate in Braga and white grape stalk (Loureiro variety) from Sogrape company located in Barcelos (Portugal). After their arrival they were kept at -80°C in sealed plastic bags. Both varieties material was dried in an oven at 60 °C for 24 h and then milled with a small kitchen grinder. The milled material was called GS red or white.

Figure 2. 1. *(A) Grape stalk; (B) Grape stalk after milling.*

2.2. Fractioning processes

2.2.1. Extractives fractioning

Extractives were separated from the two GS materials (red and white) owing to their solubility in water or neutral organic solvents following the protocols described in NREL (2008).

Two processes were tested to decrease the time of extraction and save costs. The first process with deionized water was performed for 6 h, while the second one was the recommended by the protocol, which consisted of a successive solvent extraction with water and ethyl alcohol (Carlo Erba, Vel De Reul, France) by 6 and 16 h, respectively. Both were performed in a Soxhlet extractor equipment. The extractives contents solubilized by each solvent were determined using the difference between the mass values of the original material and solid residue after drying at 100°C for 24 h. After extraction, the liquid phase (extract) was kept at -20 °C. Results were reported according to equation 1, as a percentage of the oven dry weight (ODW). The final solid material without extractives was called GSWE (grape stalk water extracted) and GSWEE (grape stalk water and ethanol extracted) red or white.

$$Extractives\ \% = \frac{Weight_{flask\ plus\ extractives} - Weight_{flask}}{Weight_{initial\ sample}} \times 100 \qquad (1)$$

Where, the weight $_{flask\ plus\ extractives}$ corresponds to the mass of the sample after treatment in grams (g), the weight $_{flask}$ to the mass of the empty flask in grams (g) and weight $_{initial\ sample}$ to the mass of the initial samples in grams (g).

2.2.2. Alkaline pretreatment of grape stalk

Red and white grape stalks without extracts were pretreated following optimized parameters by other researchers (Egüés et al., 2013; Pujol et al., 2013). These were soaked with 1 % NaOH (Eka, Bohus, Sweden) solution at the ratio of 1: 10 (solid: liquid) for 15 and 60 min in autoclave. After this process, samples were centrifuged and cleaned with deionized water until the black color disappearance and achievement of neutral pH values. All supernatants were collected and join in a final solution called black liquor, which was stored at refrigerated conditions until further use.

The resulting delignified solid material was further subject to drying at 100ºC during 24 h and was called DGS.

$$Delignification\ \% = \frac{Weight_{flask\ plus\ pretreated\ sample} - Weight_{flask}}{Weight_{initial\ sample}} \times 100 \qquad (2)$$

Where, the weight $_{flask\ plus\ pretreated\ sample}$ corresponds to the mass of the sample after alkaline treatment in grams (g), the weight $_{flask}$ to the mass of the empty flask in grams (g) and weight $_{initial\ sample}$ to the mass of the initial samples in grams (g).

2.2.2.1. Extraction of lignin derivatives from black liquors

The black liquors previously obtained were subject to the concentration of the soluble lignin using two methods. The first method tested was freeze drying the liquor (Zhang et al., 2016), while the second method was a controlled precipitation of the lignin compounds of the liquor by acidification with sulfuric acid (95%) until pH 2. After lowering the pH, solutions were kept for 24 h to allow the sedimentation of the precipitated lignin. The next steps were to centrifuge the samples at 8000 rpm for 20 min and wash them with distilled water twice to discard possible impurities such as sugars or inorganic particles. Finally, the samples were dried at 60 °C in an oven for 48 h (Domínguez-Robles et al., 2017). The yields and purity of the rich lignin fractions were calculated according to equation 3 and 4:

$$Lignin\ yield\ \% = \frac{Weight_{lignin\ after\ dry}}{Weight_{lignin\ on\ black\ liquor}} x100 \qquad (3)$$

Where, the weight $_{\text{lignin after dry}}$ means mass of dry lignin after treatment (g) and the weight $_{\text{lignin on black liquor}}$ is the mass of lignin on black liquor (g).

$$Purity\ of\ lignin\ \% = \frac{Mass\ of\ the\ pure\ lignin}{Mass\ of\ total\ lignin} x100 \qquad (4)$$

Where, mass of the pure lignin are the grams of total pure lignin obtained after treatment and mass of total lignin are the grams of total lignin in sample.

2.2.3. Biosugars production from the delignified materials

2.2.3.1. Dilute acid hydrolysis

The effect of the type of acid, concentration and reaction times was tested in the saccharification process. Red delignified grape stalks (DGS) were subject to weak hydrolysis using H_2SO_4 (Honeywell, Seelze, Germany) and acetic acid (Sigma, Steinhelm, Germany) at low concentrations (0.5 %, 2 %, 3.5 % w/w based on the dry matter), in a ratio solid to liquid of 1:10 and processed at 121˚C for two periods of time (15 min and 60 min) in an autoclave. At the end of the treatment, the samples were centrifuged at 5000 rpm during 10 min. The supernatant which has the higher yield of sugars was used for fermentation and the pellet was dried and stored for further use for production of nanocellulose which was called HGS.

2.2.3.2. Enzymatic hydrolysis

Enzymatic hydrolysis was performed using the enzymatic celluclast mixture preparation (Novozymes,Bagsvaerd, Denmark) and β-glucosidase (Sigma, Saint Louis, USA). The quantity of enzymatic activity units used were estimated considering that red DGS had 43.79 % of cellulose. Samples were suspended in citrate buffer (pH 5.0, 0.05 M) (Gama et al., 2015) with 20 FPU (filter paper units) of celluclast and 40 IU (international unit) of beta-glucosidase per gram cellulose (Ping et al., 2011). The hydrolysis was carried out at 50 ºC for 24 h and at the end solutions were centrifuged at 5000 rpm for 10 min (Verardi et al., 2012). After analytical procedures to estimate the sugars released a second hydrolysis was carried for 48 h to obtain a higher yield of sugars. Sugar rich supernatants were used for fermentation procedures described below, while the pellet was used for production of nanocellulose and was called HGS.

For both hydrolysis processes (acid and enzymatic) the saccharification % of sugars were calculated according to equation 5:

$$Saccharification\ \% = \frac{Reducing\ sugar \times 0.9}{Cellulose\ content\ in\ pretreated\ subsrate} x100 \qquad (5)$$

Where, reducing sugars is the concentration of reducing sugars after saccharification in mg/ml and the cellulose content in pretreated substrate is concentration of sugar content before saccharification in mg/ml.

Taking into the account the production of fermentation inhibitors compounds such as acetic acid, furfural and 5-hydroxymethylfurfural (HMF) (Lu et al., 2009; Klinke et al., 2004) during these processes. Standard curves were performed for all inhibitors (0.3125-5 g/L) to quantity them by HPLC.

2.3. Nanocellulose extraction

2.3.1. Extraction of nanocellulose

HGS-red fractions were subject to two processes. One was subjecting them to strong acid hydrolysis. The hydrolysis was performed at 50 °C, for 30 min, under vigorous and constant stirring (Madureira et al., 2018). For each gram of HGS, 20 mL of a solution of H_2SO_4 64 % (w/w) was used. Immediately following the hydrolysis, the suspension was diluted 10-fold with cold water to stop the hydrolysis reaction and centrifuged twice for 10 min at 7000 rpm to remove the excess acid.

Since after this process the resulting fraction rich in nanocellulose still presented color and components that could affect the purity of nanocellulose, a second process was applied bleaching the enzyme HGS-red. The first solution was an acetate buffer with 27 g NaOH + 75 ml of glacial acetic acid in 1 L of water and second solution was a solution of 1.7 % (m/v) sodium chlorite; sample was covered with equal volumes of both solutions, at 80 ºC (Madureira et al., 2018) and applying again the same process of hydrolysis as explained before.

In both cases, the precipitates obtained were dialyzed with deionized water to remove non-reactive sulphate groups, salts and soluble sugars, until a neutral pH value was achieved (5-7 days). Subsequently, the resulting suspension of the dialysis process was sonicated for 5 min at 70 % intensity in a VCX 130 ultrasonicator (Sonics & Materials, Newtown, USA), with sample tubes immersed in an ice bath to prevent heating. The colloidal suspensions were stored in a refrigerator at 4 °C, with the addition of some drops of chloroform to avoid any bacterial growth until the freeze-drying process. The freeze-drying process was performed using a Vacuum Freeze Drier (Model FT33, Armfield, UK), under a vacuum pressure of 100 millitorr; the temperature in the freezing chamber was -46 °C, and the temperature in the sample chamber was 15 °C. The nanocellulose was labelled as CNC acid or CNC enzymatic. The CNC % was calculated according to equation 6:

$$CNC\ \% = \frac{Weight_{flask\ plus\ treated\ sample} - Weight_{flask}}{Weight_{initial\ sample}} \times 100 \qquad (6)$$

Where, the weight $_{\text{flask plus treated sample}}$ corresponds to the mass of the sample after freeze-drying process in grams (g), the weight $_{\text{flask}}$ to the mass of the empty flask in grams (g) and weight $_{\text{initial sample}}$ to the mass of the initial samples in grams (g).

Table 2. 1. *Resume of the fractions obtained along the process and designations attributed.*

Initial sample	Process stage	Final sample	Designation
Red grape stalks (GS)	Extractives separation	Stalks without extractives	Red GSWE/GSWEE
White grape stalks (GS)			White GSWE/GSWEE
Red and white GSWE	Delignification process (NaOH)	Solid residue without lignin	Red DGS White DGS
Liquid residue from delignification	Freeze-drying Precipitation	Technical lignin	
Red DGS	Acid hydrolysis Enzymatic hydrolysis	Supernatants	Acid Biosugars media
			Enzymatic Biosugars media
		Solid residues	HGS
HGS	Extraction	Inorganic fraction	Acid CNC/ Enzymatic CNC

2.4. Analytical methods

2.4.1. Sugars and lignin quantification

Monosaccharides (glucose, xylose, arabinose) that can be used to estimate cellulose (glucose) and hemicelluloses (xylose, arabinose) were quantified in the GS, GSWE, GSWEE and DGS fractions, following the protocols described in NREL (2012). Briefly, samples were hydrolyzed using 3 ml of 72 % H_2SO_4 (Honeywell, Seelze, Germany) per 300 mg in a water bath set at 30°C for 1 h, added with 28 ml of deionized water and autoclaved for 1 h 121°C. After this time, the samples were vacuum filtered through a crucible and washed with boiling purified water. Klason lignin was determined by the mass residue after drying at 100 °C. Also, soluble lignin was determined on the combined filtrates by measuring the absorbance at 206 nm (Pujol et al., 2013) using a UV-vis spectrophotometer (Shimadzu-UV mini 1240). The values of Klason and soluble lignin were summed to obtain the total lignin content. Glucose, xylose and arabinose were measured by an HPLC system that consisted of a Beckman Coulter (CA, EUA) unit equipped with Karat32 software coupled to detectors: Beckman Diode Array (Wavelenght 220 nm) and a refractive index detector (RI Detector K-2301, Knauer, Germany). Ion exchange Aminex HPX-87H Column (300 × 7.8 mm) (Bio-Rad) was maintained at 55 ºC (CH-150 Column Oven; Eldex, US) to analyze sugars and organic acids.

The mobile phase used was 13 mM sulphuric acid at a flow rate of 0.5 mL/min. Running time was 30 min, and the injection volume was 50 µL. Each sample was injected in duplicate.
A standard curve was done with different concentrations for all sugars (0.05- 2 mg/ml) and inhibitors (0.3125- 5 g/L). Sugars and lignin concentrations were calculated according to equation 7,8,9 provided by the method (NREL, 2012) and inhibitors were calculated according to equation 10:

$$Sugar\ ext\ free\ \% = \frac{C \times V \times \frac{1g}{1000mg}}{Weight_{initial\ sample}} \times 100 \qquad (7)$$

Where, C is the concentration in mg/ml of sugars as determined by HPLC, V is the volume of filtrate 86.73 ml, weight $_{initial\ sample}$ is the mass of the initial samples (mg).
*calculate the percentage of each sugar on an extractive's free basis.

$$Insoluble\ lignin\ \% = \frac{Weight_{crucible\ plus\ insoluble\ lignin} - Weight_{crucible}}{Weight_{initial\ sample}} \times 100 \qquad (8)$$

Where, weight $_{crucible\ plus\ insoluble\ lignin}$ is the insoluble residue of lignin (g), weight $_{crucible}$ is the weight of empty crucible (g), weight $_{initial\ sample}$ is the mass of the initial samples in grams (g).

$$Acid\ soluble\ lignin\ \% = \frac{UV_{abs} * Volume_{filtrate} * Dilution}{\varepsilon * Weight_{initial\ sample} * Pathlength} \times 100 \qquad (9)$$

Where, UV $_{abs}$ is the average UV-Vis absorbance for sample at appropriate wavelength, volume $_{filtrate}$ is the volume of filtrate (86.73 ml), dilution is the dilution factor (if it is performed), Ɛ is the absorptivity of biomass at specific wavelength, weight $_{initial\ sample}$ is the mass of the initial samples in milligram, pathlength is the pathlength of UV-Vis cell in cm.

$$y = mx + c \qquad (10)$$

Where, y is the peak area, m is the slope of regression line, c is the intercept of the regression line with the y-axis. Dilution factors (D) and multipliers (M) may be used to calculate the final analyte concentration, if required.

2.4.2. Ash content determination

Ash content was determined according to NREL (2008). Porcelain markers were placed in the muffle furnace at 575 ± 25 °C for a minimum of four hours. Crucibles were removed from the furnace directly into a desiccator to cool down. The crucibles were weighed to the nearest 0.1 mg and weight was recorded. Weighed 2.0 g, to the nearest 0.1 mg of a test specimen into the tared crucible and placed in the muffle furnace at 575 ± 25 °C for 24 ± 6 h. Samples weight were recorded when they cool. Dry samples are used, and each sample were analyzed in duplicate. Ash was calculated according to following equation 11:

$$Ash\ \% = \frac{Weight_{crucible\ plus\ ash} - Weight_{crucible}}{Weight_{initial\ sample}} \times 100 \qquad (11)$$

Where, weight crucible plus ash is the sample and crucible weight after treatment(g), weight crucible is the weight of empty crucible(g), weight $_{initial\ sample}$ is the mass of the initial samples in grams (g).

2.4.3. Fourier transform infrared ray (FTIR) analysis

FTIR analysis was used to identify the functional groups of GS, GSWE and GSWEE samples. Spectra were obtained using KBr pellets and were recorded on an IRPrestige-21 infrared spectrophotometer (Shimadzu, Japan). Samples were grinded and mixed with KBr (sample/KBr ratio = 1/100) to prepare discs. The experiments were carried out using the wavenumber range of 500-4000 cm^{-1}, with a resolution of 4 cm^{-1} and a total of 32 scans for each sample.

2.5. Proof of concept: fermentation procedures using the bio-sugars for production of bio-ethanol

2.5.1 Microorganisms

Saccharomyces cerevisiae DSMZ 70449 and *Pichia stipitis* DSMZ 3651 yeast strains were used. Yeasts were obtained as a freeze-dried powder and the activation was done accordingly the indications of the supplier DSMZ in Potato Dextrose Broth (PDB) (Conda, Madrid, Spain) during three days at 25 °C then passed to slants. Stock cultures of these strains were prepared in 30 % glycerol water and stored at -80 °C for further use.

2.5.2. Fermentation procedures using the biosugars obtained from the two hydrolysis processes

2.5.2.1. Preparation of the fermentation media

The supernatants obtained from both hydrolysis processes were tested as liquid media for fermentation processes using the two-yeast species described previously. These were supplemented with nutrients such as 3 g/l $(NH_4)_2SO_4$ (Sigma, St. Louis, USA), 3 g/l K_2HPO_4(Sigma, Steinheim, Germany), 1 g/l $MgSO_4$, 5g/l yeast extract, 3.5 g/l peptone. To obtain sterile media for fermentations they were first autoclaved at 121°C for 15 min in Erlenmeyers flasks sealed with cotton caps covered with aluminum paper. Control media was prepared with 10 g/l glucose, 10 g/l xylose, 3 g/l $(NH_4)_2SO_4$ (Sigma, St. Louis, USA), 3 g/l K_2HPO_4(Sigma, Steinheim, Germany), 1 g/l $MgSO_4$, 5g/l yeast extract, 3.5 g/l peptone.

2.5.2.2. Preparation of the yeast inoculums, fermentation process and sampling along incubation time

Overnight yeast cultures were grown in PDB at 25-30 ºC. These were inoculated in the previously prepared fermentation media order to standardize an initial cell concentration of $1x10^5$ CFU/ml. The inoculated Erlenmeyer's were incubated at 25-30ºC for 7 days. 3 ml of samples were taken at times 0, 1, 3 and 7 days, 1 ml for evaluation of the yeast growth and 2 ml for pH analyses and glucose, xylose and ethanol quantification by HPLC according the methods described previously in section 2.4.1. The yeast growth was evaluated by performing decimal dilution in peptone water 0.1 % (w/v) and plating in potato dextrose agar by spread pour plating technique. Plates were incubated during 24 h at 25-30 ºC and the log CFU/ml were estimated. Ethanol yield, fermentation efficiency and ethanol productivity were calculated according to following equations.

$$Ethanol\ yield = -\frac{\triangle P}{\triangle S} \qquad (12)$$

Where, △P/△S stands for product (g/L of ethanol) produced per amount of substrate (g/L of glucose) consumed.

$$\text{Fermentation efficiency}\ \% = \frac{Amount\ of\ ethanol\ produced}{Amount\ of\ sugar\ consumed \times 0.511} \times 100 \qquad (13)$$

Where, amount of ethanol produced is the amount of product (g/L of ethanol) produced, amount of sugar consumed is the amount of substrate (g/L of sugars) consumed and 0.511 is the conversion factor of glucose or xylose to ethanol.

2.6. Statistics

Each trial was carried out in three replicates and the values reported as means ± SD. IBM SPSS® 19.0 (SPSS, Chicago, IL, USA) software for Windows was used to perform statistical analysis of variance (ANOVA) followed by Tukey's post hoc test (for means discrimination) to assess the significance of dilute acid hydrolysis and T-test was used to perform statistical analysis to assess the significance of ex-red and ex-white. Variance homogeneity was confirmed according to Levene's test. All significance tests were conducted at $p \leq 0.05$.

3. Result and Discussion

3.1. Compositional analyses of the red and white grape stalks

For lignocellulosic compositional analysis, is very important to remove the extractives, since they can cross-react with the acid and condense to acid insoluble components that will be associated-with and classified as Klason lignin (KL). The NREL method to take the extractives advises to use water and then water plus ethanol in a double Soxhlet extraction procedure. To decrease the processing times and the to avoid using ethanol, the fractioning of extractives was tested with water and 6 h of process (GSWE) and then with water and ethanol and 16 h of total time (GSWEE). Table 3.1 presents the compositional analyses performed in red and white grape stalks subject to these two processes.

Table 3. 1. *Compositional analyses of the GS extractives content (ODW, mean ± SD) in samples subject to the two types of extractives processes tested, using water (GSWE) or using the advised method by the standard protocol water followed by ethanol (GSWEE).*

	Red grape stalk		White grape stalk	
Compounds	GSWE	GSWEE	GSWE	GSWEE
Extractives (%, w/w)	56.29 ±3.46	58.86±4.43	75.08 ±2.11	76.28±3.89
Cellulose (glucose)	19.43±0.32	10.50±0.63	5.19±0.51	8.55±0.20
Hemicellulose	8.36±0.73	8.01±0.68	7.76±0.57	8.22±0.34
Xylose	7.55±0.52	7.20±0.55	7.41±0.52	7.54±0.26
Arabinose	0.81±0.21	0.81±0.13	0.65±0.05	0.68±0.08
Total lignin	39.16±2.95	48.47±1.88	54.62±5.35	60.22±0.98
Klason lignin	37.81±2.91	45.39±1.81	53.70±5.23	56.12±0.98
Soluble lignin	1.35±0.04	3.08±0.07	0.92±0.12	4.10±0.005
Ash (%, w/w)	4.90±0.03	2.27±0.09	3.12±0.08	1.54±0.28

* All data are yields of components (g) per 100g of oven-dried grape stalk, GSWE: Grape stalk water extracted, GSWEE: Grape stalk water and ethanol extracted.

Statistical analysis has been performed and there were no significant differences between both extractive's methods ($p>0.05$). These two methods were able to extract similar quantities of extractives and the higher quantities were obtained in the white variety GS (Figure 3.1 and 3.2). Extractives are water-soluble material, such as non-structural sugar, terpenes, etc. and ethanol soluble material including chlorophyll, waxes, etc. These values are not in agreement with Pujol et al. (2013), which obtained ca. 21 % for red grape stalk water extractives and ca. 28 % of ethanol extractives. Also, for the water extractives of red grape stalk is ca. 36.5 % (Spigno et al., 2013). Hence, in order to take advantage of this, we considered the valorization of the extractive fraction for the development of an antioxidant extract.

The information available for the composition of grape stalks is scarce. The different methods generate different values of the polysaccharides and lignin in some cases. According to

statistical analysis, there is significant different between cellulose quantities of GS-red and white ($p<0.05$). Cellulose quantities are higher in red than white variety, which is accordingly to the found in literature (Spigno et al., 2013, 2014). In addition, the extractives are higher in GS-white, which indicates that some cellulose maybe is lost during the process with water and ethanol. For hemicellulose and arabinose quantities, there are no differences between both varieties and extractives separation methods.

Total lignin (Klason or insoluble lignin and soluble lignin) content is lower in red GS than in white GS. These values are according to the ones found by other authors such as Spigno et al. (2008) and Ping et al. (2011) which found in grape stalks, lignin contents between 40 and 47.3%. Nevertheless, low values around 17% can be also found in literature (Prozil et al., 2012). All these differences can be attributed first to the protocols used to quantify, to the interpretation of what is soluble or Klason lignin and finally to variations in the source variety and type of grape stalk.

The ash content values of grape stalks agree with the reported for grape stalks by Ping et al. (2011) ca. 3.9 %. As can be seen, there is a decrease on ashes content along the processing and red stalks present the higher content. This can be due to the significant amounts of potassium and other inorganic elements that are as well present (Pujol et al, 2013). Grape stalk has very low ash content which is potential benefit for the process of enzymatic hydrolysis as it is able to increase the efficiency of the hydrolysis (Bin et al., 2010).

The carbohydrate composition shows that glucose is the most abundant neutral monosaccharide and xylose is the second most abundant monomer followed by arabinose. Red GS has more sugar available than white GS ($p<0.05$).

Concerning the method used to separate the extractives, the water extraction during 6 h showed to be an alternative method to the advised by the standard (NREL, 2012), since it does not influence the content on cellulose and hemicellulose which will be the source of sugars for fermentation. The ethanol used in the advised method certainly extracts more dyes, waxes but in this specific case does not bring any extra function than using only water.

***Figure 3. 1**. Water extractives of white and red grape stalks, respectively.*

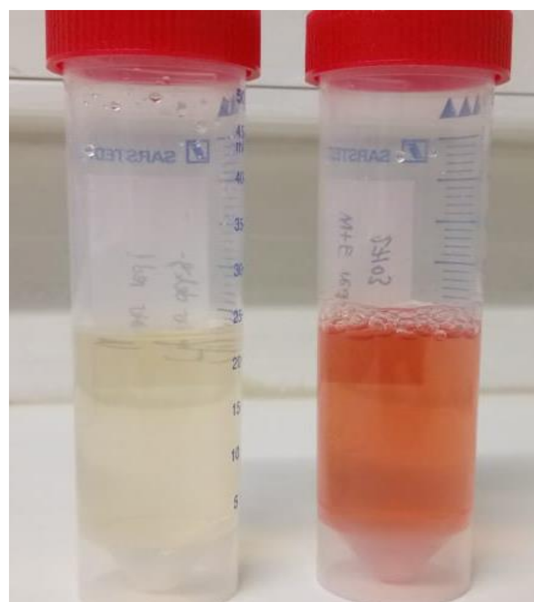


***Figure 3. 2.** Water and ethanol extractives of white and red grape stalks, respectively.*

3.1.1. Phenolic compounds

The fact that the white stalks present more extractives is attributed to the presence of phenolic compounds which was showed in Table 3.2. The performance of the successive extractions with water and ethanol only increases ca. 1 % of the extractive's removal. These extractives are rich in phenolic compounds as other compounds with bioactive properties, so this fraction can be tested for other properties, in the future, as an ingredient for food or cosmetic applications.

***Table 3. 2.** Crude extraction yield of phenolic compounds (mean ± SD).*

	% yield (%/d.w. stalks)
GSWEE-red	13.77±0.42
GSWEE-white	16.21±0.68

d.w: dry weight, GSWE: Grape stalk water extracted, GSWEE: Grape stalk water and ethanol extracted.

The results are not in agreement with reported for grape stalk by Spigno et al., (2007) (% yield 0.1-0.3). Because, generally the found methods are comparable for the used ratio raw material/solvent, but not for the other parameters time and temperature, solvent (which sometimes are even not specified) and analysis and expression of results.

3.2. Alkaline Pretreatment of Grape stalk

Figure 3. 3. *Black liquor after alkaline pretreatment*

This step is to remove lignin from the material. This step will facilitate the access to the polysaccharides to produce biosugars and will permit to obtain a fraction rich in lignin that can have value. The addition of NaOH to lignocellulosic biomass causes swelling of particles which increase enzymes and acid penetration into the cell-wall fine structure. Interaction of alkali and lignocellulosic biomass causes saponification of intermolecular ester bonds linkages within the biomass (Sun and Cheng, 2002; Feist et al., 1970). The removal of such linkages increases the porosity of lignocellulosic biomass and helps to separate the structural linkages between lignin and the complex carbohydrates and disrupts lignin structure (Fan et al., 1987). As it was mentioned, water extracted samples had more sugar than water and ethanol extracted samples, especially the red variety (Table 3.1). For this reason, water extracted GS materials were used to proceed for alkaline pretreatment. Table 3.3 shows the red and white GSWE that were pretreated with 1 % w/v NaOH solution at the ratio of 1: 10 (solid: liquid) for 15 and 60 min in autoclave. The delignification % (or % of solubility) of GSWE red and white samples were respectively 47.81 %, 42.04 %, when using 15 min and 50.61 % and 45.58 %, when using 60 min of processing time. These values are similar to the obtained by Pujol et al. (2013), (between 34.82%-49.19%). No significant differences were obtained between processing times and grape types ($p>0.05$), but we can say that the major proportion was obtained in GSWE red with 60 min. The samples with higher % of delignification were subject to sugars analyses to observe what remained after delignification. The higher quantity of sugars was detected in delignified samples from red variety. For this reason, red-DGS was chosen for the enzymatic and dilute acid hydrolysis process.

Table 3. 3. *Delignification % of the GSWE-red and GSWE-white samples (ODW, mean ± SD).and sugar analysis of GSWE treated with NaOH 1 % (w/v) using during the two testing times of reaction.*

Delignification %		
Time of process	GSWE-red	GSWE-white
15 min	47.81±2.61	42.04±2.02
60 min	50.61±1.62	45.58±0.86
Sugars % in DGS samples with 60 minutes		
Compounds (sugars)		
Cellulose	43.79±5.92	26.33±6.58
Hemicellulose	16.76±2.57	9.54±1.42
Xylose	16.38±2.5	9.54±1.42
Arabinose	0.38±0.07	N.D.
Total sugars	60.55±8.49	35.87±8

N.D.: Not detected; *Sugars data are yields of components (g) per 100g of oven-dried grape stalk. GSWE: Grape stalk water extracted, GSWEE: Grape stalk water and ethanol extracted.

3.3. Lignin recovery

3.3.1. Extraction of lignin derivatives from black liquors

Grape stalk the black liquors from delignification were also processed to extract lignin. The delignification was previously performed under an eco-friendly and cost-effective NaOH process, ether bonds break due to the function of NaOH and then lignin degrade gradually in the form of alkali lignin (Fan et al., 1987; Soccol et al., 2011). Black color comes from lignin compounds colored by alkali and dissolved to liquor. Afterwards, the lignin is precipitated or freeze dried from the black liquor to compare the different extraction conditions. Results are showed in Table 3.4, and it is possible to see that freeze-dried extracts from white GS generated higher lignin yields (9.47 %) comparing with precipitation process that had (1.98 %). Also, using the method of precipitation, the same type originated higher yield of lignin. Based on our investigation we can conclude that to have pure lignin precipitation process is the best recovery method of lignin. Nevertheless, higher yield and purity of lignin was obtained from water extracted materials which indicates that some lignin maybe is lost during the process with water and ethanol.

Table 3. 4. *Yield (%) and purity of lignin obtained from grape stalks without extractives using water or water plus ethanol procedures (mean ± SD).*

	Yield %		Purity %	
	Freeze dry	Precipitation	Freeze dry	Precipitation
GSWE-red	9.23±0.12	1.4±0.03	40.84±0.13	70.57±0.2
GSWEE-red	1.48±0.03	1.15±0.05	30.08±0.01	67.91±0.05
GSWE-white	9.47±0.08	1.98±0.01	56.02±0.06	73.73±0.04
GSWEE-white	1.42±0.15	4.27±0.04	39.50±0.09	67.01±0.01

GSWE: Grape stalk water extracted, GSWEE: Grape stalk water and ethanol extracted.

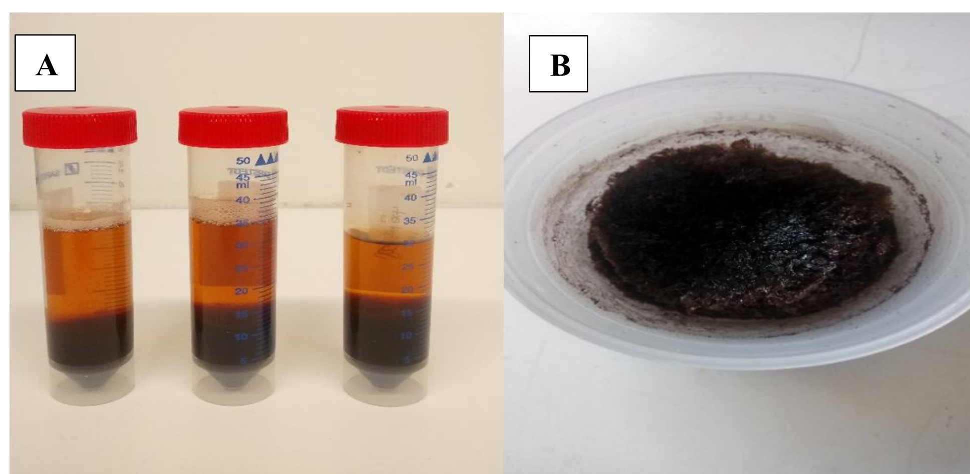


Figure 3. 4. *(A) Precipitated lignin from grape stalk; (B) freeze dried lignin from grape stalk.*

3.3.2. Lignin characterization by FTIR

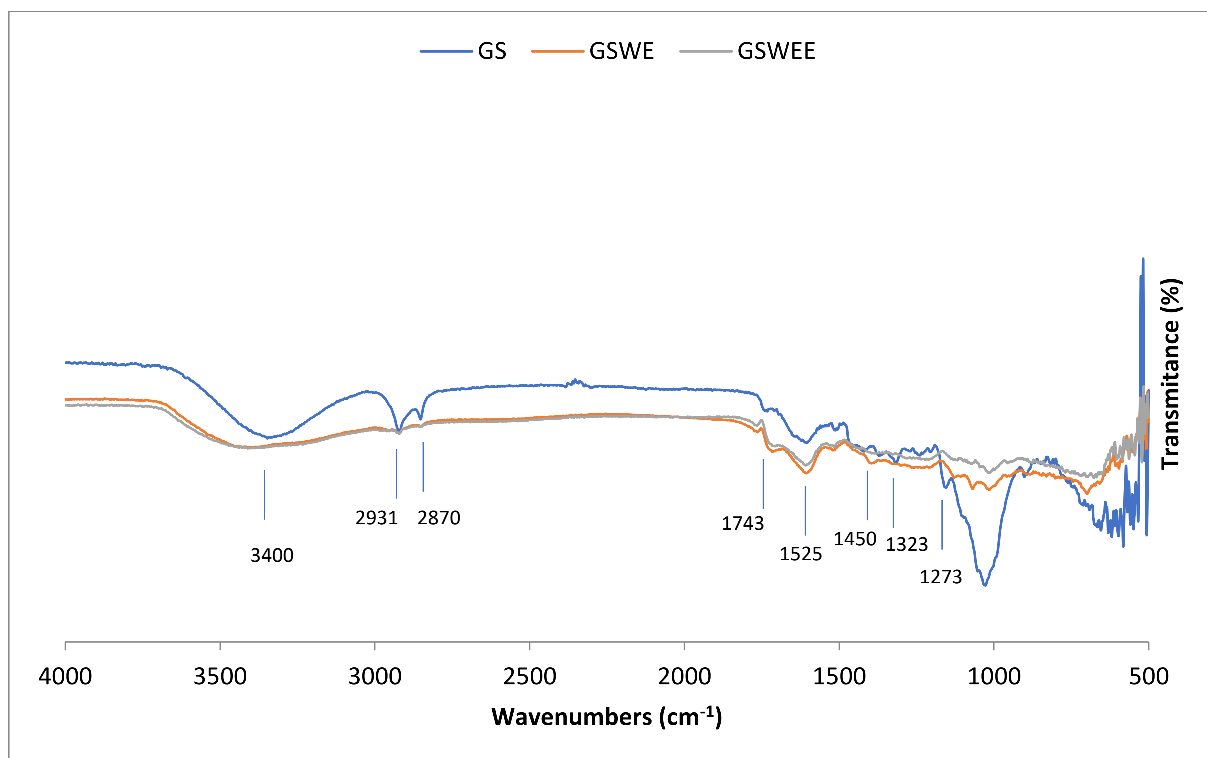


Figure 3. 5. *FTIR analysis of initial red grape stalk and extracted lignin of red grape stalk by precipitation.*

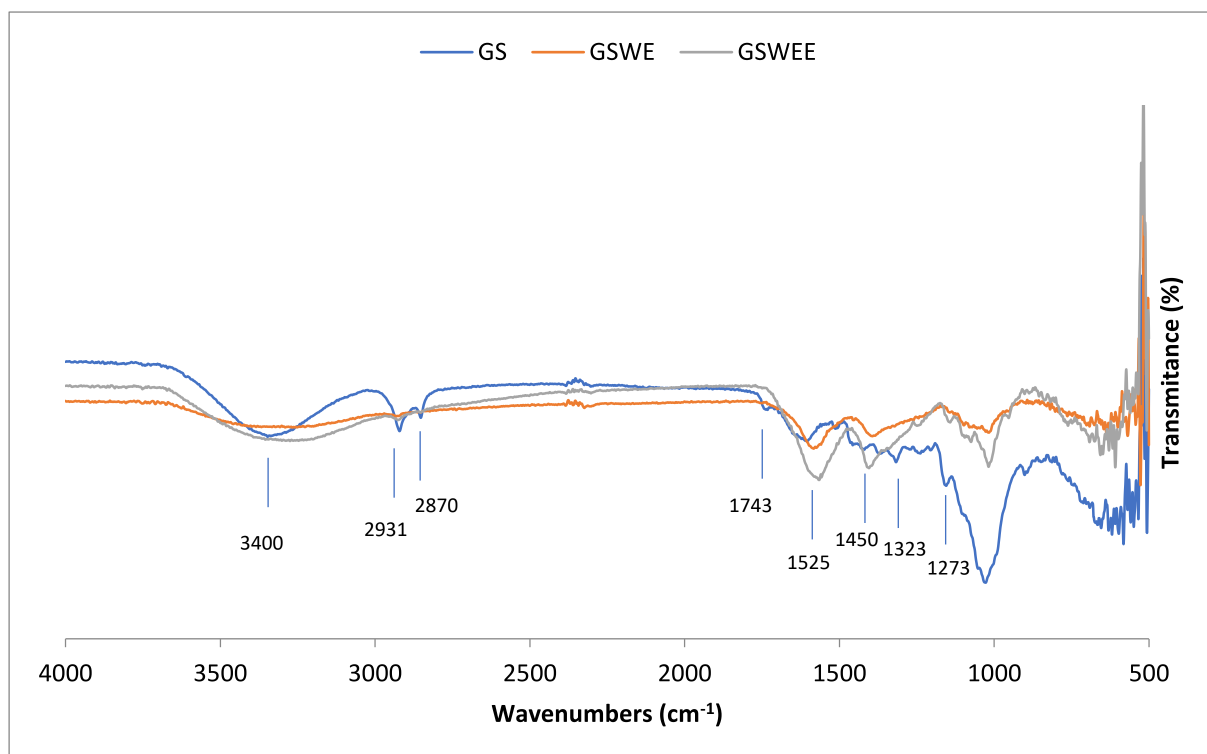


Figure 3. 6. *FTIR analysis of initial red grape stalk and extracted lignin of red grape stalk by freeze dry.*

Figure 3. 7. *FTIR analysis of initial white grape stalk and extracted lignin of white grape stalk by precipitation.*

Figure 3. 8. *FTIR analysis of initial white grape stalk and extracted lignin of white grape stalk by freeze dry.*

The isolated lignin was characterized with FTIR spectroscopic analysis. FTIR spectroscopy is a versatile, rapid, and reliable technique for lignin characterization. Using this technique, the phydroxyphenyl, guaiacyl, and syringyl units, methoxyl groups, carbonyl groups, and the ratio of phenolic hydroxyl to aliphatic hydroxyl groups can be determined.

The FTIR spectra of GS, GSWE, GSWEE (red and white) samples are presented in Figure 3.5, 3.6, 3.7 and 3.8. Spectra display a number of adsorption peaks indicating the complex nature of this material. The broad peak at around 3400 cm^{-1} is indicative of OH vibration modes. The two sharp peaks at 2931 $cm^{-1}$and 2870 cm^{-1}correspond to the asymmetric and symmetric vibration, respectively, of C-H in the olephinic chains, and the peak at 1743 $cm^{-1}$is attributed to the carbonyl C=O in ester groups. The presence of lignin is confirmed by the typical lignin bands at 1323 cm^{-1}, 1273 $cm^{-1}$and 1525 cm^{-1}, being the first two bands attributed to skeletal vibrations of syringil and guayacil aromatic rings with CO stretching, respectively, and the last one to aromatic skeletal vibrations (Hergert, 1971). The band at 1450 cm^{-1} associated to deformation vibration of C-H in aromatic ring of lignin moieties is less intense. The presence of polyphenols is confirmed by the characteristic band at 2931 $cm^{-1}$and the typical peaks of polysaccharides appear at 1075, 1118 and 996 cm^{-1}(Kacuráková et al., 2000). Comparison among the different FTIR spectrum of GS, GSWE and GSWEE samples show that the above-mentioned identified peaks are present in all fractions. Slight differences in the band's intensity can be observed in the white grape stalk compared to the red grape stalk. For the initial grape stalk, the most relevant differences are observed in the bands attributed to polysaccharides (1075, 1118 and 996 cm^{-1}) with higher peak intensities and to lignin (1525 and 1450 cm^{-1}) lower peak intensities compared to the extracted lignin of grape stalks.

3.4. Biosugars production from the delignified materials

3.4.1. Dilute acid hydrolysis

Acid hydrolysis is a common process to obtain sugar monomers that can be used e.g. in fermentation. Here, acid hydrolysis will involve breaking down the polysaccharide structure. In this study, sulphuric and acetic acids were chosen as suitable for weak/dilute acid hydrolysis. The use of these acids is related with the fact that sulphuric acid is the most commonly used catalysts for hydrolysis of lignocellulosic biomass (Lenihan et al., 2010) and acetic acid is already present in lignocellulosic biomass in the form of acetyl groups on the hemicellulose. Moreover, acetic acid can work as a co-solvent (Huber et al., 2006; Sun et al., 2009). On the other hand, cost of high concentrated acid treatment on biomass and need for recovery limit the process of released sugars through concentrated acid hydrolysis. Another drawback is effect of high acid concentration and time of processing that may lead to hydroxymethyl furfural (HMF) and furfural formation due to degradation of complex polysaccharides (Taherzadeh et al., 2000). Hence, the search for the content of sugars that released from red DGS samples using these two types of acid at different conditions and processing times was done and the results are exposed in Figure 3.9.

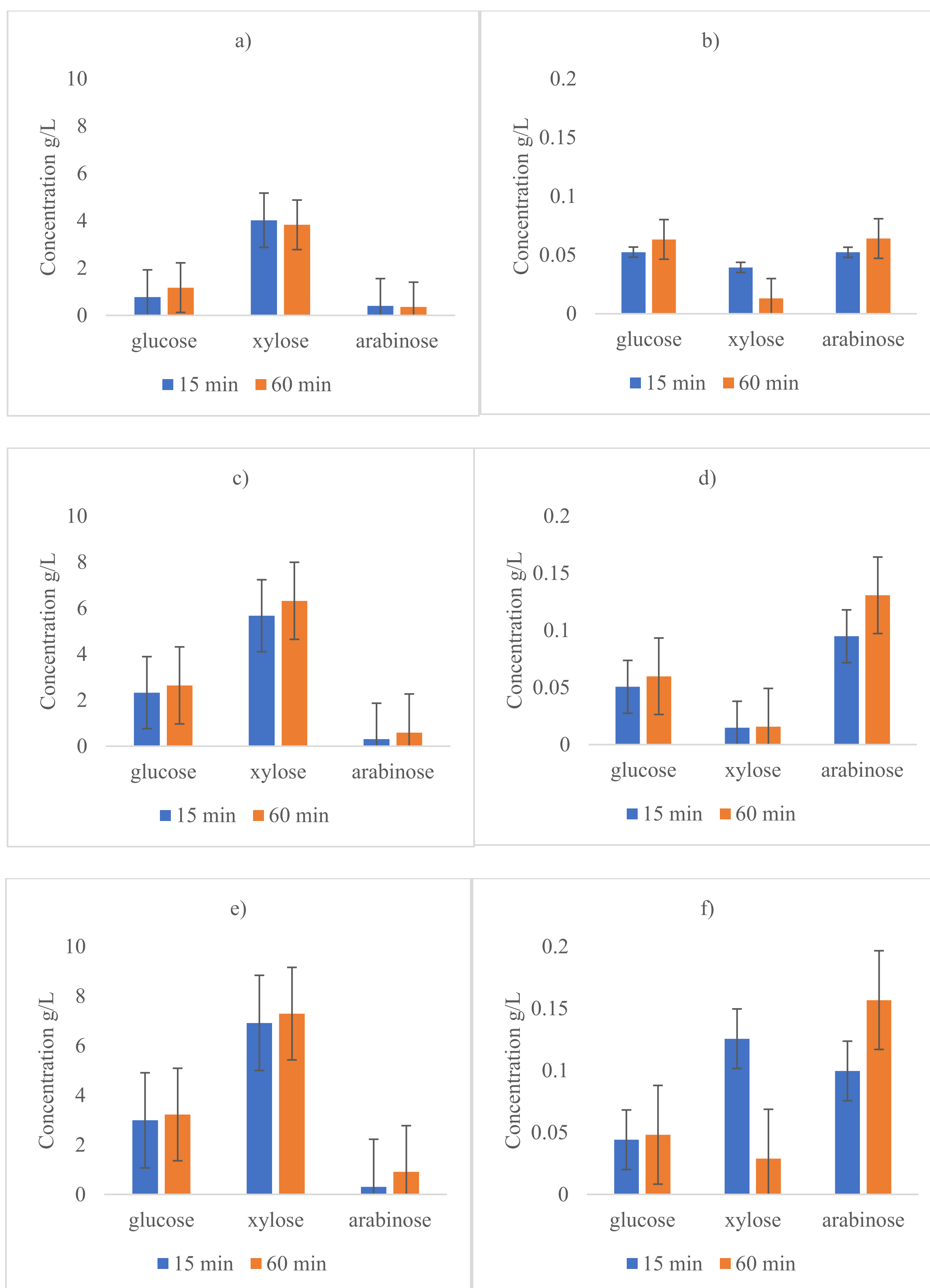


Figure 3. 9. *Monosaccharides composition and concentrations (means ± SD) in red DGS samples after dilute acid hydrolysis using two times of processing and 0.5 % (a and b), 2 % (c and d) and 3.5 % (e and f) of sulphuric acid and acetic acid, respectively.*

__Table 3. 5.__ Saccharification rates % of cellulose and hemicellulose in glucose, xylose and arabinose after acid hydrolysis procedures (means ± SD).

		Sulphuric acid		Acetic acid	
Saccharification rate (%)	**Concentration (%)**	**15 min**	**60 min**	**15 min**	**60 min**
	0.5	1.6±0.03	2.4±0.04	0.1±0.01	0.12±0.08
Glucose	**2**	4.79±0.01	5.43±0.02	0.1±0.05	0.12±0.05
	3.5	6.14±0.03	6.62±0.09	0.08±0.03	0.1±0.09
Xylose	**0.5**	22.08±0.6	21.04±0.06	0.22±0.01	0.05±0.01
	2	31.15±0.02	34.73±0.03	0.05±0.07	0.11±0.05
	3.5	38.02±0.09	40.05±0.01	0.71±0.03	0.16±0.07
Arabinose	**0.5**	12.51±0.05	32.17±0.04	4.47±0.01	5.36±0.03
	2	26.81±0.01	52.73±0.07	8.04±0.04	14.29±0.05
	3.5	27.70±0.06	81.33±0.01	8.04±0.03	11.61±0.08

It is possible to observe that there are differences in the sugar concentrations obtained for the two types of acids ($p<0.05$), but no differences between the used concentrations ($p>0.05$) (Figure 3.9). Maximum glucose, xylose and arabinose concentrations were obtained in hydrolysis using sulphuric acid at the higher concentration of 3.5 % and is according with the saccharification rates as observed in Table 3.5. Since cellulose is the major contributor of glucose and is present in higher concentrations, the % of saccharification is always lower than the others, which are present in lower quantities such as xylose and arabinose which are almost all released. With these concentrations it was possible to obtain ca. 7 g/L of xylose and ca. 3 g/L of glucose. The time of hydrolysis was also important, and it was possible to observe that with 60 min, the higher content of sugars was obtained with values of xylose with 7.29 g/L, glucose with 3.22 g/L of and arabinose with 0.91 g/L. The results were compared with some literature works reporting sugars concentrations of grape stalk obtained for sulphuric acid. The results are not in agreement with reported by Egüés et al. (2013) (glucose and xylose ca. 12-9 g/L respectively.). These differences can be due to the hydrolysis time in autoclave which in the case of Egüés et al. (2013) was 90 min. The increase in time of processing showed that promotes the saccharification in all cases as observed in Figure 3.9 and Table 3.5.

Moreover, inhibitor compounds started to be formed by hydrolysis of hemicellulose into xylose and further dehydration into acetic acid, furfural and 5-hydroxymethylfurfural (HMF) (Lu et al., 2009; Klinke et al., 2004). The concentrations of inhibitors produced are presented in Table 3.6. Furfural was not detected and as it was expected, the inhibitors increased with increasing

sulphuric and acetic acid concentration (Spronsen et al., 2011). According to this, the results showed that the inhibitors (acetic acid and 5-HMF) concentrations were not significantly different ($p > 0.05$) when using 15 and 60 min as processing times (Table 3.6). The concentration of acetic acid and HMF was increased with the 60 min of reaction time and 3.5% acid concentration. Acetic acid, HMF and furfural inhibit the growth of yeast cells and subsequent fermentation in a dose-dependent manner which is 4 g/L, 2 g/L, 1.86 g/L, respectively (Fosso-Kankeu et al., 2014; Delgenes et al.,1996; Favaro et al., 2013). And the values obtained are at lower concentrations than these ones, so the media can be used as fermentation media.

Concluding the use of 3.5 % of sulfuric acid and 60 min of hydrolysis time were the best conditions to obtain a high saccharification and low concentration of fermentation inhibitors production, and so these conditions were chosen to produce the media broths for fermentation used in the next section.

Table 3. 6. *Inhibitors concentration (means ± SD) present in solution after dilute acid hydrolysis.*

		Sulphuric acid		Acetic acid	
Compounds (g/L)	**Concentration (%)**	**15 min**	**60 min**	**15 min**	**60 min**
	0.5	0.083±0.97	0.077±1.46	0.086±1.23	0.088±0.29
HMF	**2**	0.084±0.11	0.078±1.94	0.087±0.17	0.090±0.55
	3.5	0.087±1.77	0.097±1.95	0.089±0.56	0.094±0.75
Acetic acid	**0.5**	1.1±97.75	1.2±138.50	0.3±193.08	0.4±177.69
	2	1.3±43.76	1.6±4.61	1.5±216.08	1.8±176.04
	3.5	1.5±7.46	2.5±196.45	2.7±166.78	2.9±153.96

HMF**:** 5-hydroxymethylfurfural.

3.4.2. Enzymatic Hydrolysis

In this experiment, polysaccharides of DGS were hydrolyzed to monosaccharides with commercial enzymes i.e. celluclast and β-glucosidase. Celluclast and β-glucosidase were chosen because to obtain high cellulose conversion and prevent cellobiose accumulation. Cellulase breaks the branched cellulose chains while the cellobiose formed is broken down with the action of ß-glucosidases producing glucose. The combined action of both enzymes considerably reduces the time required for hydrolysis (Garcia-Cubero et al., 2010). The main carbohydrates after enzymatic hydrolysis were glucose and xylose. Higher concentrations of sugars were obtained by enzymatic hydrolysis than by dilute acid hydrolysis, with values of

glucose with 6.06 g/L and xylose with 8.08 g/L (Table 3.7). Also, saccharification % was calculated and the higher values were obtained for xylose than glucose, 82.73 % and 25.97 %, respectively. The results show that xylose conversion was higher than glucose conversion in the DGS-red. Xylose recovery with these enzymes has not been mentioned before for grape stalk. Nevertheless, cellulose-to-glucose conversion agrees with the reported for grape stalk by Ping et al. (2011) which was ca. 25 %. Hemicellulose conversion to five basic monosaccharides (xylose, mannose, glucose, arabinose and galactose) is much higher than cellulose conversion, this can be because of the chain length in hemicellulose is much shorter (Andersen, 2007; Horn et al., 2012). According to literature, increasing of reaction time can be recover more glucose (Li et al., 2012; Han et al., 2012). Considering this, a second enzymatic hydrolysis process was carried out with more time. Anyway, the amount of sugars seems at the moment to be too low to make stalks economically exploitable for industrial fermentation, albeit this deserves further investigations and a certain amount of sugars can be also recovered.

Table 3. 7. *Results obtained for enzymatic hydrolysis (means ± SD). Concentration of glucose and xylose in samples after hydrolysis processes and saccharification rate % of glucose and xylose. Inhibitors concentration present in solution after enzyme hydrolysis.*

	Concentration of Glucose (g/L)	Concentration of Xylose (g/L)
DGS-red/ First saccharification	6.06±0.06	8.08±0.06
DGS-red/Second saccharification	2.15±0.39	0.004±0.15
	Glucose	Xylose
First saccharification rate %	25.97	82.73
Second saccharification rate %	12.9	3.27
Total (some of two processes)	38.87	86.00
	Inhibitors concentration after enzyme hydrolysis (g/L)	
HMF	0.018±0.4	
Acetic acid	0.2±0.1	
Furfural	0.008±0.05	

HMF**:** 5-hydroxymethylfurfural.

The concentration of inhibitors was lower than as mentioned before. According to this, the result revealed that the inhibitors do not effect of inhibitory on biomass production, substrate utilization and ethanol production during fermentation, so the media can be used as fermentation media.

3.5. Fermentation procedures using the bio-sugars obtained from the two hydrolysis processes

3.5.1. Fermentation

Separate hydrolysis and fermentation were carried out, so the hydrolysis by acid and enzymes were initially performed using the optimized conditions in the previous sections. The performance of saccharification and fermentation was done in separate because there is the intention of study the fermentative performance of two different yeast strains using the different media obtained. These microorganisms have their optimum conditions growth at 25-30 ºC and not 50 ºC, as celluclast enzymes need to have their optimum activity. Hence, the best conditions for each stage and the best yeast strain to perform fermentation in the two types of media were obtained. And so, fermentations were performed at lab scale in batch for 7 days with *S. cerevisiae* and *P. stipitis* as the fermentative microorganisms. *S. cerevisiae* species use glucose as the carbon source for ethanol production, while *P. stipitis* can consume both hexoses and pentoses (Cortez et al., 2010; Olofsson et al., 2008).

Figure 3. 10. *Flasks with resulting hydrolyzed sugars solutions used for fermentation purposes as well solutions produced as controls. Both were added with nutrients at the same concentrations. Sugars from dilute acid hydrolysis and enzyme hydrolysis were tested.*

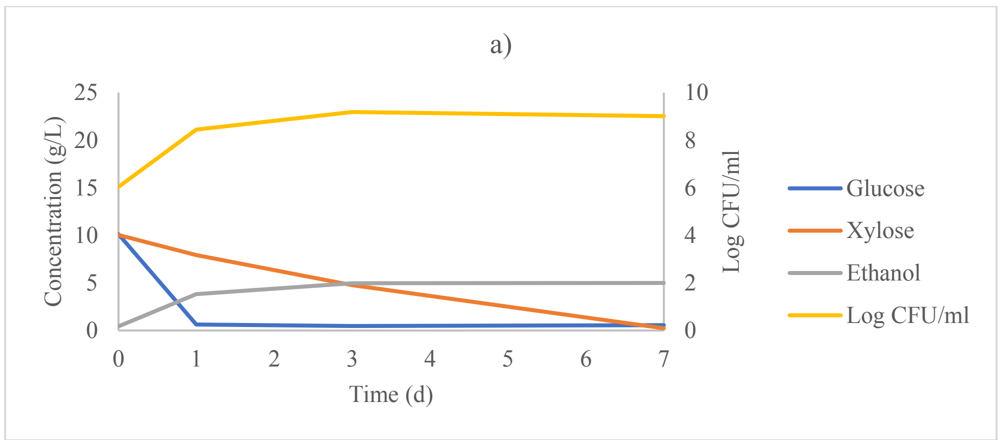
These fermentations were done with the goal of production of ethanol using grape stalks sugars as carbon sources by the yeasts *S. cerevisiae* and *P. stipitis*. Growth curves of *P. stipitis* and *S. cerevisiae* have been obtained for the different media. For both media, lower Log CFU/mL

were obtained for *S. cerevisiae* (log 7.43 CFU/ml, log 7.65 CFU/ml) than *P. stipitis* (log 8.80 CFU/ml, log 8.83 CFU/ml). In addition to that, the optimal pH range for *S. cerevisiae* and *P. stipitis* growth can vary from pH 4-6 and pH 4-5.5, respectively (Narendranath et al., 2005; Van Zyl et al., 1988). In the present study, the pH was accordingly the optimum (Table 3.8) with an expected decrease along fermentation time, and *S. cerevisiae* showed to be in general a more acidifying strain than *P. stipitis*.

In Figure 3.11 are showed the growth curves and sugar consumption of the strains tested in a control, acid dilute and enzymatic sugars media. Through all series of fermentations, *P. stipitis* and *S. cerevisiae* were also evaluated on their ability to produce ethanol and consequent consumption of sugars. In control media, both yeasts behave differently, especially in what concerns the consumption of xylose, which was higher for *P. stipitis* (Figure 3.11a). Also, this strain produced slightly more ethanol than *S. cerevisiae* (Figure 3.11b) with profiles of consumption of glucose like *P. stipitis*. In the enzymatic hydrolysis media, the fermentations were similar to the observed in control media, in what concerns glucose consumption and production of ethanol for both species of strains (Figure 3.11 c and d) but in acid hydrolysis media, strains showed to have a slower metabolic activity, maybe because of the presence of lower quantities of sugars, with low ethanol quantities production (Figure 3.11 d and e).

The same happened, with *P. stipitis* which showed to be successfully adapted to enzyme media than dilute acid hydrolysis media and agrees with the reported elsewhere (Groves, 2009; Gonçalves et al., 2016). To produce ethanol, the fermentation efficiency was 42.41% of *P. stipitis* compared to the *S. cerevisiae* 26.71% in the enzymatic hydrolysis media. The ethanol productivity and yields in the enzymatic media was doubled, when compared to acid hydrolysis media. The results show that fermentations of grape stalk can be optimized based on the enzymatic media with *P. stipitis* to have good ethanol yields, since this strain is able of using xylose and glucose at the same time for production of ethanol.

In addition to that, the optimal pH range for *S. cerevisiae* and *P. stipitis* growth can vary from pH 4 to 6 and pH 4-5.5, respectively (Narendranath et al., 2005; Van Zyl et al., 1988). In our experiment, pH range was ca. 5-4 for both yeasts. However, pH was decreased during the fermentation. This can be depending on acetic acid concentration, temperature, the presence of oxygen and the strain of yeast in fermentation (Narendranath et al., 2005; Casey et al., 2010; Codato et al., 2018).


a)
Concentration (g/L)
25
20
15
10
5
0
10
8
6
4
2
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Log CFU/ml
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3
4
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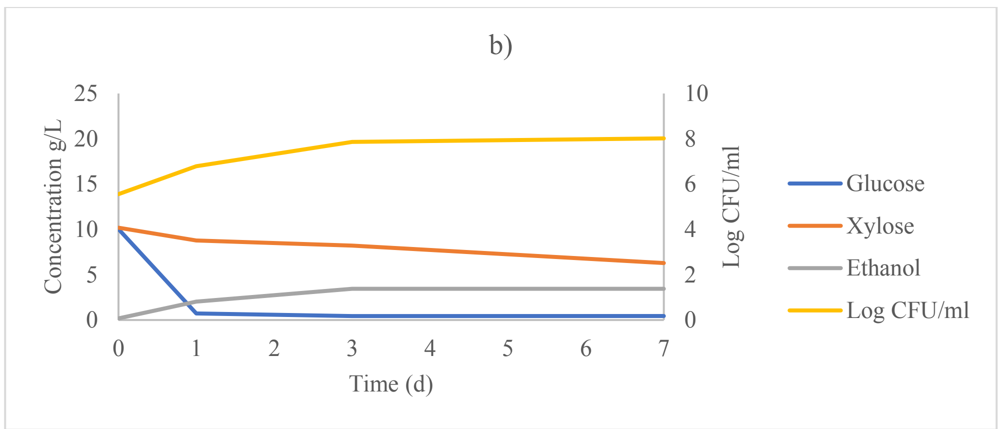
6
7
Time (d)
Glucose
Xylose
Ethanol
Log CFU/ml


b)
Concentration g/L
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Time (d)
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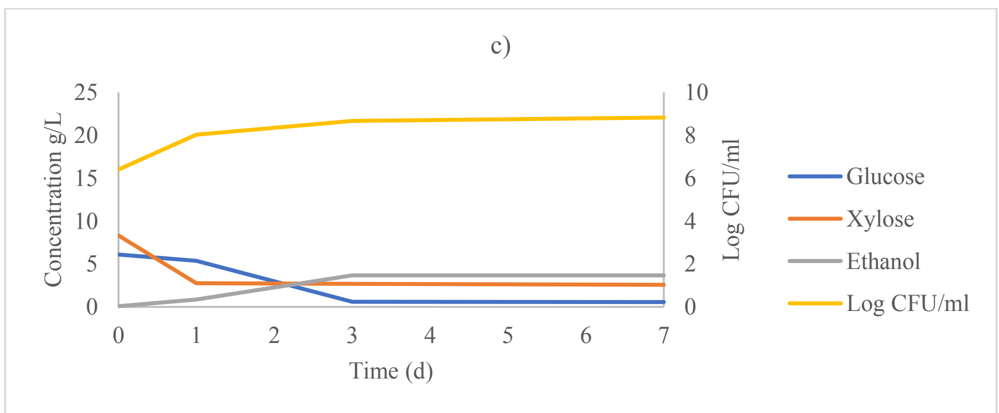
Log CFU/ml


c)
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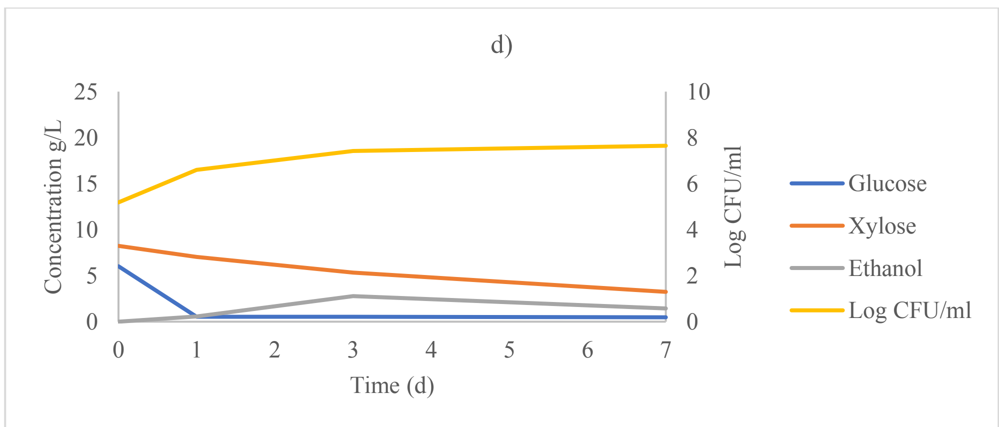
d)
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Time (d)
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Log CFU/ml

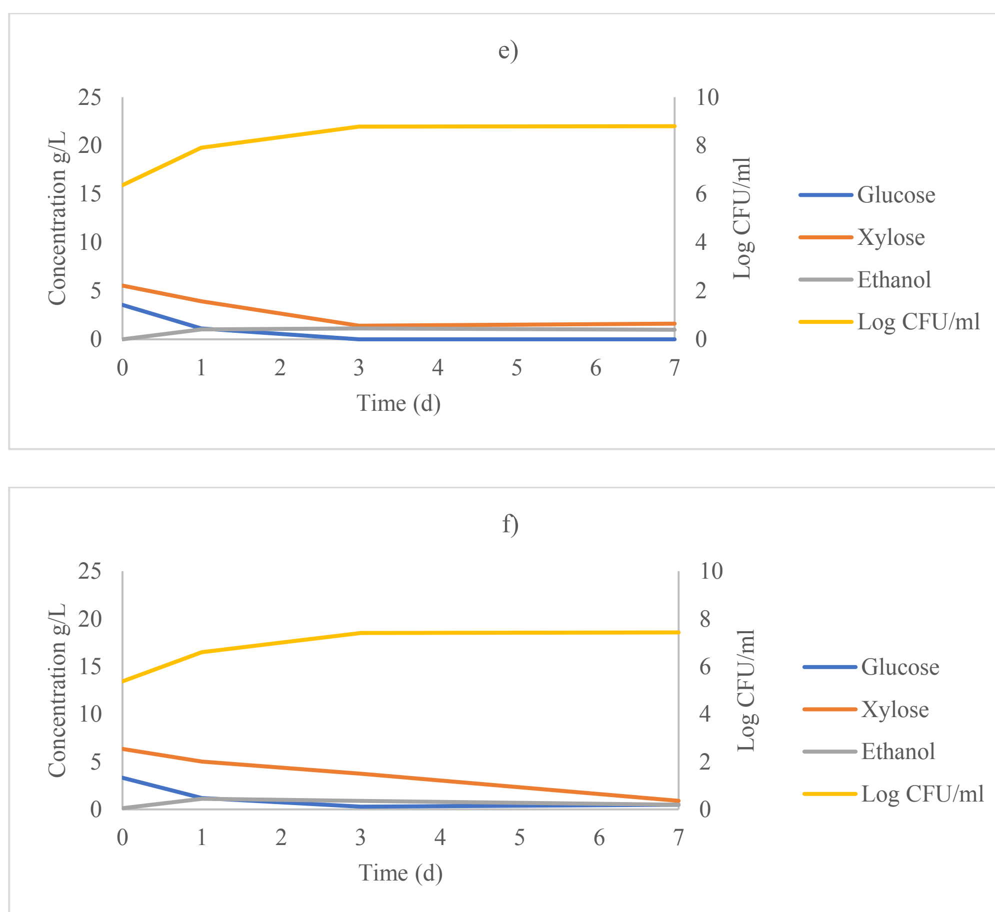

Figure 3. 11. *Evolution of the consumption of sugars along fermentation time and production of ethanol by P. stipitis and S. cerevisiae in control fermentation media (a and b), in rich-sugar media produced from enzymatic hydrolysis (c and d) and in rich-sugar media produced from dilute acid hydrolysis (e and f), respectively.*

Table 3. 8. *pH values during fermentation process of both species tested in the two different growth media produced with sugars from the dilute acid and enzymatic hydrolysis.*

	pH of Enzymatic media		pH of Acid hydrolysis media		pH of Control media	
	P. stipitis	*S. cerevisiae*	*P. stipitis*	*S. cerevisiae*	*P. stipitis*	*S. cerevisiae*
T0	5.10±0.2	5.08±0.7	5.55±0.4	5.52±0.8	4.79±0.5	4.74±0.2
T1	5.02±0.6	5.05±0.3	5.46±0.6	5.16±0.3	4.16±0.1	3.67±0.8
T3	4.85±0.1	4.82±0.8	5.15±0.3	4.82±0.9	4.03±0.4	3.60±0.3
T7	4.14±0.3	4.40±0.2	4.69±0.6	4.70±0.4	3.92±0.9	3.50±0.5

The initial sugar concentration, final ethanol concentration and yield values are given in Table 3. 9. The total sugars concentration was determined as the sum of monosaccharide (glucose and xylose) found in the dilute acid and enzymatic hydrolysis media. A maximum total sugars concentration ca.14 g/L was achieved with enzymatic medias. This followed with ca. 9 g/L with

dilute acid medias. The ethanol yields of both media were lower than the corresponding theoretical yields for glucose fermentations (0.51 g/g), nevertheless the enzymatic media results were always higher than in acid media. This can be the effect of having low sugar concentration or fermentation time (Agbogbo et al., 2007; Groves 2009). However, considering the different media and fermentation results, the *P. stipitis* enzyme media was determined to be the best media as substrate (14.42 g/L) owing to the results on the ethanol fermentation yield (0.22 g/L).

Table 3. 9. *Ethanol yields obtained by P. stipitis and S. cerevisiae using as culture media added with sugars obtained from dilute acid and enzymatic hydrolysis as fermentation media.*

Species and type of media	Initial total concentration of sugars (g/L)*	Final total concentration of sugars (g/L)	Ethanol yield (g/L)	Fermentation efficiency (%)	Ethanol productivity (g/L/h)
P. stipitis control	20.17	0.81	0.24	46.08	0.65
P. stipitis enzyme media	14.42	3.15	0.22	42.41	0.39
P. stipitis dilute acid media	9.10	1.63	0.13	26.20	0.14
S. cerevisiae control	20.22	6.74	0.21	40.94	0.40
S. cerevisiae enzyme media	14.26	3.71	0.14	26.71	0.21
S. cerevisiae dilute acid media	9.65	1.38	0.005	8.99	0.05

*All sugars included (glucose, xylose and arabinose)

3.6. Nanocellulose

The grape stalks were used to extract the nanocellulose. HGS was processed without bleach process to produce CNC. The yields of CNC obtained were 5.79 % and 1.20 % from enzymatic process derived CNC and acid, respectively. At the end of second enzyme hydrolysis, samples were bleached to remove color because samples were not able to be analyzed by DLS. After the bleaching process, samples were without color, but they lost their whiteness using of 64 % sulfuric acid. After dialysis of samples, ultrasonication is a process for the defibrillation of cellulose fiber with the hydrodynamic forces of the ultrasound. Samples were freeze dried and powder was obtained as shown in Figure 3.12.

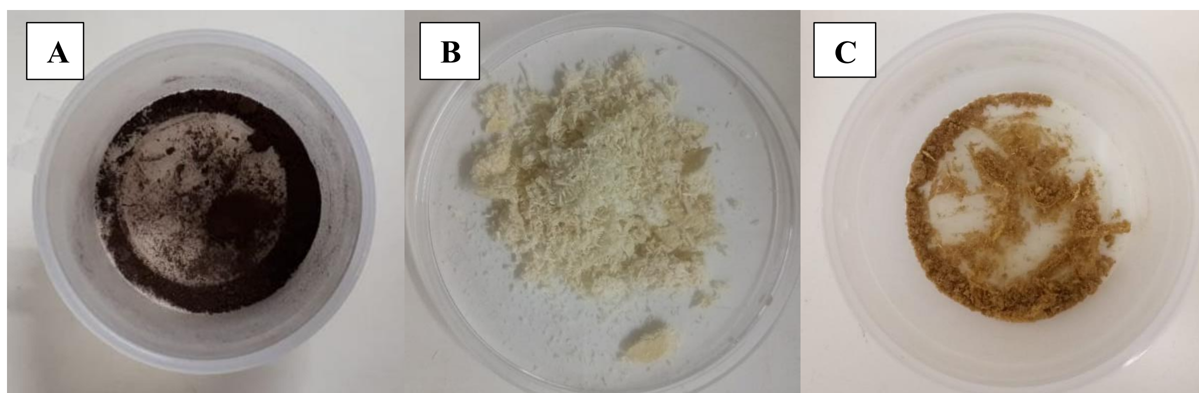

Figure 3. 12. *(A) HGS without bleach process; (B) HGS with bleach process; (C) HGS with bleaching process after dialysis.*

Table 3. 10. *CNC % (w/w) (means ± SD) of the products obtained during samples processing.*

	Acid hydrolysis (without bleach)	enzyme hydrolysis (without bleach)	second enzyme hydrolysis (with bleach)
CNC % (w/w)	1.20±0.25%	5.79±0.63%	0.56±0.48%

Table 3. 11. *Chemical characterization in % (w/w) (means ± SD) of the products obtained during samples processing.*

	Particle size (nm)	Polydispersity index	Zeta potential (mv)
Bleached grape stalk-red	294.6±19.01	0.321±0.080	-36.7±0.751

Samples were analyzed by DLS to determine the CNC particle size in suspension and charge (Table 3.11). The sizes obtained were high since crystals length is also measured using a DLS scatter that is dynamic, and agglomeration may occur in solution. Nevertheless, smaller CNCs were obtained. The polydispersity index (PI) indicates the variation in the distribution of the particle size. A high polydispersity shows the existence of particle families with different sizes, which may mean the occurrence of aggregation (Hanaor et al, 2012). In general, all samples showed PI values much higher than 0.3, which indicates a polydisperse distribution of CNCs. On the other hand, zeta potential (ZP) can give us an indication of whether repulsion between adjacent, similarly charged particles in dispersion will occur or not. When ZP is high (whether they are positive or negative values) means stability between the particles, whereas when the potential is low, particles tend to coagulate/flocculate as attraction exceeds repulsion in the dispersion. The ZP values where ca. 30 mV in the CNCs extracted from grape stalk, which

means a moderate stability. The values are negative, which is a result of the acid hydrolysis, in which sulphuric acid removed the amorphous regions in the cellulose fibres, leaving only the highly ordered crystalline regions intact, resulting in negatively charged, sulphonated nanoparticles.

Several researchers investigated shapes and size distributions of nanocellulose obtained in hydrolysis of different type of fiber using acid or enzymes (Tsukamoto et al., 2013; Mandal et al., 2011; Oksman et al., 2011; Abraham et al., 2011; Filson et al., 2009). However, this study is reporting the first time that biomass from grape stalk is a source of nanocellulose, potentially adding value to grape stalk. Therefore, grape stalks should be explored further as raw material for producing nanocellulose.

4. Conclusions

A complete chemical characterization of different variation of grape stalks was performed after milling of the biomaterial. Results confirm that two methods were able to extract similar quantities of extractives and the higher quantities were obtained in white variety GS. Nevertheless, the samples subject to water extraction have higher content on sugars. The alkaline pretreatment of grape stalks performed for 60 min showed to be the best method for delignification. After delignification Red GS was selected for progressive stages, because of the higher sugar quantities present the composition.

The process was followed by a dilute acid or enzyme hydrolysis for the recovery of structural reducing sugars from red GS. The best condition with acid in terms of yield of sugars and production of inhibitors could be obtained using 3.5 % sulphuric acid for 60 min. Nevertheless, the best process was hydrolysis with enzyme seemed to be a good way to recover monosaccharides from grape stalks.

Ethanol was successfully produced using via *S. cerevisiae* and *P. stipitis* fermentation. It is very important to find and present new raw materials for the use of renewable energy producing sectors, to guarantee a sustainable future. The work performed showed the potential use of GS for ethanol production via *S. cerevisiae* and *P. stipitis* fermentation using biosugars in the growth medium. Two media were tested to determine the optimum for ethanol production by *P. stipitis* and *S. cerevisiae.* Nevertheless, *P. stipitis* produced higher ethanol yields than the *S. cerevisiae* in enzymatic media. The grape stalks also were used to extract the nanocellulose. Grape stalks should be explored further as raw material for producing nanocellulose. The results show that grape stalks can be a promising source of natural valuable products.

5. Future Work

The present work studied the valorization of grape stalks for production of value-added compounds and the production of sugars for second generation ethanol. To increase profitability of grape stalk biomass biofuel production, it becomes increasing important to characterize and utilize the by product. Considering of extractives, the concentration of phenolic compounds should be obtained, and the phenolic compounds profile should be described by analytical methods. Also, the antioxidant and antimicrobial activities should be determined to evaluate the potential application of these extracts e.g. as preservatives for food products. Lignin obtained as a by-product from the black liquor, it would be also interesting to tested for antioxidant and antimicrobial properties.

Grape stalk biomass possesses high potential as a renewable substance for producing important nanobiomaterials such as nanocellulose. Another interesting approach would be to find possible applications for nanocellulose utilization of renewable grape stalk biomass for the preparation of nanomaterials which could be useful in diverse applications.

6. Reference

Abraham, E., Deepa, B., Pothan, L. A., Jacob, M., Thomas, S., Cvelbar, U., Anandjiwala, R. 2011. Extraction of nanocellulose fibrils from lignocellulosic fibres: a novel approach. *Carbohydr. Polym.* **86**: 1468.

Agbogbo, F. K., Coward-Kelly, G., Torry-Smith, M., Wenger, K., Jeffries, T. W. 2007. The effect of initial cell concentration on xylose fermentation by *Pichia stipitis. Applied Biochemistry and Biotechnology* **137**: 653–662

Alonso, A. M, Guillén, D. A., Barroso, C. G., Puertas, B., García, A. 2002. Determination of antioxidant activity of wine byproducts and its correlation with polyphenolic content. *Journal of Agricultural and Food Chemistry* **50**: 5832–5836.

Amendola, D., De Faveri, D.M., Egües, I., Serrano, L., Labidi, J., Spigno, G., 2012. Autohydrolysis and organosolv process for recovery of hemicelluloses phenolic compounds and lignin from grape stalks. *Bioresour. Technol.* **107**: 267–274.

Andersen, N. 2007. Enzymatic Hydrolysis of Cellulose: Experimental and Modeling Studies. PhD Thesis. BioCentrum. Lyngby, Denmark: Technical university of Denmark.

Arvanitoyannis, I. S., Ladas, D., Mavromatis, A. 2006. Potential uses and applications of treated wine wastes: a review. *International Journal of Food Science & Technology* **41**: 475-487.

Bali, G., Meng, X., Deneff, J.I., Sun, Q., Ragauskas, A.J. 2015.The effect of alkaline pretreatment methods on cellulose structure and accessibility. *ChemSusChem.* **8**: 275-9.

Banerjee, G., Scott-C, J. S., Walton, J. D. 2010. Improving Enzymes for Biomass Conversion: A Basic Research Perspective. *Bioenerg. Res.* **3**: 82–92.

Bansal, P., Hall, M., Realff, M. J., Lee, J. H., Bommarius, A. S. 2009. Modeling cellulase kinetics on lignocellulosic substrates. *Biotechnol Adv.* **27**:833-48.

Bansal, P., Vowell, B. J., Hall, M., Realff, M. J., Lee, J. H., Bommarius, A. S. 2012. Elucidation of cellulose accessibility, hydrolysability and reactivity as the major limitations in the enzymatic hydrolysis of cellulose, *Bioresource Technology* **107**: 243–250.

Bayrak, E. 2013. Utilization of wine waste for fermentative processes. Izmir Institute of Technology. Thesis (Master of Science in Food Engineering)-The Graduate School of Engineering and Sciences of Izmir Institute of Technology, Izmir, Turkey. pp.73.

Bin, Y., Hongzhang, C. 2010. Effect of the ash on enzymatic hydrolysis of steam-exploded rice straw. *Bioresource Technology* **101**: 9114–9119.

Bon, E. P. S., Ferrara, M. A. 2007. Bioethanol Production via Enzymatic Hydrolysis of Cellulosic Biomass. 2007. Document prepared for "The Role of Agricultural Biotechnologies for Production of Bioenergy in Developing Countries", an FAO seminar held in Rome.

Brown, E.E., Hu, D., Lail, N.A., Zhang, X. 2013. Potential of nanocrystalline cellulose–fibrin nanocomposites for artificial vascular graft applications. *Biomacromolecules* **14**:1063–71.

Bustamante, M. A., Paredes, C., Morales, J., Mayoral, A. M., Moral, R. 2009. Study of the composting process of winery and distillery wastes using multivariate techniques. *Bioresource Technology* **100**: 4766–4772.

Cárcel, J.A., García-Pérez, J.V., Mulet, A., Rodríguez, L., Riera, E. 2010. Ultrasonically assisted antioxidant extraction from grape stalks and olive leaves. *Phys. Procedia* **3**: 147–152.

Cardona, C. A., Quintero, J. A., Paz, I. C. 2010. Production of bioethanol from sugarcane bagasse: Status and perspectives. *Bioresource Technology* **101**: 4754–4766.

Casey, E., Sedlak, M., Ho, W.Y. N., Mosier, N. S. 2010. Effect of acetic acid and pH on the cofermentation of glucose and xylose to ethanol by a genetically engineered strain of *Saccharomyces cerevisiae. FEMS Yeast Research* **10**: 385–393.

Cheng, Yu-S., Zheng, Y., Yu, C. W., Dooley, T. M., Jenkins, B. M., VanderGheynst J. S. 2010. Evaluation of High Solids Alkaline Pretreatment of Rice Straw. *Appl Biochem Biotechnol*. **162**: 1768–1784.

Codato, C. B., Martini, C., Ceccato-Antonini, S. R., Bastos, R.G. 2018. Ethanol production from Dekkera bruxellensis in synthetic media with pentose. *Braz. J. Chem. Eng.* **35**: 11-17.

Cortez, L. A. B., 2010. Sugarcane BioEthanol R&D for productivity and sustainability. Editor Edgard Blucher, Sao Paulo,Brazil.

Delgenes, J. P., R. Moletta, and J. M. Navarro. 1996. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehatae*. *Enzyme Microb. Technol.* **19:**220-225.

Devesa-Rey, R., Vecino, X., Varela-Alende, J.L., Barral, M.T., Cruz, J.M., Moldes, A.B. 2011. Valorization of winery waste vs. the costs of not recycling. *Waste Manag.* **31**:2327-35.

Dogaris, I., Vakontios, G., Kalogeris, E., Mamma, D., Kekos, D. 2009. Induction of cellulases and hemicellulases from Neurospora crassa under solid-state cultivation for bioconversion of sorghum bagasse into ethanol. *Ind Crops Prod* **29**:404–411

Domínguez-Robles, J., Sánchez, R., Espinosa, E., Savy, D., Mazzei, P., Piccolo, A., Rodríguez, A. 2017. Isolation and Characterization of Gramineae and Fabaceae Soda Lignins. *Int. J. Mol. Sci.* **18**: 327.

Edwards, J.V., Prevost, N.T., Condon, B., French, A., Wu, Q. 2012. Immobilization of lysozyme-cellulose amide linked conjugates on cellulose I and II cotton nanocrystalline preparations. *Cellulose* **19**:495–506.

Egüés, I., Serrano, L., Amendola, D., De Faveri, D.M., Spigno, G., Labidi, J. 2013. Fermentable sugars recovery from grape stalks for bioethanol production. *Renewable Energy* **60**: 553-558.

Eyholzer, C., De Couraça, A.B., Duc, F., Bourban, P.E., Tingaut, P., Zimmermann, T., Månson, J. A., Oksman, K. 2011. Biocomposite hydrogels with carboxymethylated, nanofibrillated cellulose powder for replacement of the nucleus pulposus. *Biomacromolecules* **12**: 1419–27.

Fan, L.T., Gharpuray, M.M., Lee, Y.H. 1987. Cellulose hydrolysis. 1st ed. New York: Springer-Verlag.

FAO-OIV (Food and Agriculture Organization of the United Nations; the International Organisation of Vine and Wine); Table and dried grapes, fao-oiv focus 2016. Non-alcoholic products of the vitivinicultural sector intended for human consumption. pp. 64.

Favaro, L., Basaglia, M., Trento, A., Rensburg, E. V., García-Aparicio, M., Zyl, W.H.V., Casella, S. 2013. Exploring grape marc as trove for new thermotolerant and inhibitor-tolerant Saccharomyces cerevisiae strains for second-generation bioethanol production. *Biotechnology for Biofuels* **6**:168.

Feist, W. C., Baker, A. J. and Tarkow. H. 1970. Alkali requirements for improving digestibility of hardwoods by rumen microorganisms. *J. Anim. Sci.* **30**: 832-835.

Ferrer, A., Pal, L., Hubbe, M. 2017. Nanocellulose in packaging: Advances in barrier layer technologies. *Industrial Crops and Products* **95**: 574–582.

Filson, P. B., Dawson-Andoh, B. E., Schwegler-Berry, D. 2009. Enzymatic-mediated production of cellulose nanocrystals from recycled pulp. *Green Chem.* **11**: 1808-1814.

Fortunati, E., Rinaldi, S., Peltzer, M., Bloise, N., Visai, L., Armentano, I., Jiménez, A., Latterini, L., Kenny, J.M. 2014. Nano-biocomposite films with modified cellulose nanocrystals and synthesized silver nanoparticles. *Carbohydr Polym* **101**:1122–33.

Fosso-Kankeu, E., Marx, S., Meyer, A. 2014. Susceptibility of Saccharomyces Cerevisiae to Inhibitors and Impact on Bioethanol Production Yield. *Renewable Energy & Environmental Engg*. **7**: 27-28.

Frederick, W.J., Lien, S.J., Courchene, C.E., DeMartini, N.A., Ragauskas, A.J., Lisa, K. 2008. Co-production of Ethanol and Cellulose Fiber from Southern Pine: A Technical and Economic Assessment. *Biomass and Bioenergy* **32**:1293-1302

Galbe, M., Zacchi, G. 2002. A review of the production of ethanol from softwood. *Applied Microbiology and Biotechnology* **59**: 618-628.

Gama, R., Van Dyk, J. S., Pletschke, B.I.,2015. Optimisation of enzymatic hydrolysis of apple pomace for production of biofuel and biorefinery chemicals using commercial enzymes. *3 Biotech* **5**: 1075–1087.

Garcia-Cubero, M. T., Marcos, M., Bolado, S., Coca, M., Gonzalez-Benito, G. 2010. Optimization of the operating conditions in enzymatic hydrolysis of pretreated lignocellulosic materials. *Chemical Engineering Transactions* **21**: 1285-1290.

García-Pérez, J.V., García-Alvarado, M.A., Carcel, J.A., Mulet, A., 2010. Extraction kinetics modeling of antioxidants from grape stalk (Vitis vinifera var Bobal): influence of drying conditions. *J. Food Eng.* **101**: 49–58.

Gonçalves, F. A. , Ruiz, H. A. , Santos, E. S. D. , Teixeira, J. A., Macedo, G. R. D .2016. Bioethanol production by *Saccharomyces cerevisiae*, *Pichia stipitis* and *Zymomonas mobilis* from delignified coconut fibre mature and lignin extraction according to biorefinery concept. *Renewable Energy* **94**: 353-365.

González-Centeno, M.R. Rosselló,C. Simal, S. Garau, M.C. López,F. A.Femenia. 2010. Physico-chemical properties of cell wall materials obtained from ten grape varieties and their byproducts: grape pomaces and stems. *LWT- Food Science and Technology* **43:** 1580-1586.

Groves, S. L. 2009. Optimization of ethanol production by yeasts from lignocellulosic feedstocks. Master's Thesis, Michigan Technological University.

Gupta, A., Verma, J. P. 2015. Sustainable bio-ethanol production from agro-residues: A review. *Renewable and Sustainable Energy* **41**: 550-567.

Han, L., Feng, J., Zhang, S., Ma, Z., Wang, Y., Zhang, X. 2012. Alkali pretreated of wheat straw and its enzymatic hydrolysis. *Braz. J. Microbiol.* **43**: 53-61.

Hanaor, D., Michelazzi, M., Leonelli, C., & Sorrell, C. C. (2012). The effects of carboxylic acids on the aqueous dispersion and electrophoretic deposition of ZrO_2. *Journal of the European Ceramic Society* **32**: 235– 244.

Harmsen, P.F.H., Huijgen, W.J.J., Bermúdez López, L.M., Bakker, R.R.C. 2010. Literature Review of Physical and Chemical Pretreatment Processes for Lignocellulosic Biomass. Wageningen University & Research centre - Food & Biobased Research (WUR-FBR, NL), Energy Research Centre of the Netherlands (ECN, NL), Abengoa Bioenergía Nuevas Tecnologías (ABNT, ES). pp.49.

Hergert, H.L. 1971. Lignins: Occurrence, formation, structure and reactions. In: Sarkanen KV, Ludwig CH (eds) Wiley, New York, pp 267–297.

Holladay, J.E., White, J.F., et al., 2007. Top Value-added Chemicals from Biomass. In: Results of Screening for Potential Candidates from Biorefinery Lignin, Vol. 2. Pacific Northwest National Laboratory (PNNL) and the National Renewable Energy Laboratory (NREL). pp.87.

Horn, S. J., Vaaje-Kolstad, G., Westereng, B., Eijsink, V.G.H. 2012. Novel enzymes for the degradation of cellulose. *Biotechnology for Biofuels* **5**:45.

Huber, G.W. Iborra, S., Corma, A. 2006. Synthesis of Transportation Fuels from Biomass: Chemistry, Catalysts, and Engineering. *Chem. Rev.* **106** :4044-4098

Jackson R. S. 2008. Wine Science Principles and Applications. 3th edition. Academic Press. San Diego,CA.

Jackson, J.K., Letchford, K., Wasserman, B.Z., Ye, L., Hamad, W.Y., Burt, H.M. 2011. The use of nanocrystalline cellulose for the binding and controlled release of drugs. *Int J Nanomedicine.* **6**:321-30.

Kacuráková, M., Capek, P., Sasinková, V., Wellner, N., Ebringerová, A. 2000. FTIR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses. *Carbohydr Polym.* **43**: 195–203.

Khot, M., Kamat, S., Zinjarde, S., Pant, A., Chopade, B., Kumar, A., 2012. Single cell oil of oleaginous fungi from the tropical mangrove wetlands as a potential feedstock for biodiesel. *Microb. Cell Fact.* **11**: 71.

Klinke, H.B., Thomsen, A.B., Ahring, B.K. 2004. Inhibition of ethanol producing yeast and bacteria by degradation product produced during pretreatment of biomass. Appl. *Microbiol. Biotechnol.* **66**:10–26.

Kumar, P., Barrett, D. M., Delwiche, M. J., Stroeve, P., 2009. Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. *Ind. Eng. Chem. Res* **48**: 3713–3729.

Lafka, Theodora-I., Sinanoglou, V., Lazos, E. S. 2007. On the extraction and antioxidant activity of phenolic compounds from winery wastes. *Food Chemistry* **104**: 1206–1214.

Lee, H.V., Hamid, S.B.A., Zain, S.K. 2014. Conversion of Lignocellulosic Biomass to Nanocellulose: Structure and Chemical Process. *Scientific World Journal* **2014**: 20.

Lenihan, P.; Orozco, A.; O'Neil, E.; Ahmad,M.,N.,M.; Rooney, D.,W. & Walker G.,M. 2010. Dilute acid hydrolysis of lignocellulosic biomass. *Chemical Engineering Journal* **156**: 395-403.

Li, Y., Qin, C., Lei, Y. 2012. The Study of Enzyme Hydrolysis Saccharification Process of Stems and Leaves of Banana. *Energy Procedia*. **16**: 223 – 228.

Limayem, A, Ricke, S. C. 2012. Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. *Progress in Energy and Combustion Science* **38**: 449-467.

Lorenzo, M., Moldes, D., Rodríguez Couto, S., Sanromán, A., 2002. Improving laccase production by employing different lignocellulosic wastes in submerged cultures of Trametes versicolor. *Bioresour. Technol.* **82**: 109–113.

Lu, X., Yamauchi, K., Phaiiboonsilpa, N., Saka S. 2009. Two-step hydrolysis of Japanese beech as treated by semi-flowhot-compressed water. *J.Wood Sci*. **55**:367–375.

Madureira, A. R., Atatoprak, T., Çabuk, D., Sousa, F., Pullar, R. C., Pintado, M. 2018. Extraction and Characterisation of Cellulose Nanocrystals from Pineapple Peel. *International Journal of Food Studies* **7**: 24–3.

Mandal, A.; Chakrabarty, D. 2011. Isolation of nanocellulose from waste sugarcane bagasse (SCB) and its characterization. *Carbohydr. Polym.* **86**: 1291.

Martínez, N.D., Venturini, R.B., Soé Silva, H., González, J.E., Rodríguez, A.M., 2009. Copper on activated carbon for catalytic wet air oxidation. *Mater. Res.* **12**: 45–50.

Mazzaferro, L.S., Monteiro Cu˜na, M., Breccia, J.D., 2011. Production of xylooligosaccharides by chemo-enzymatic treatment of agricultural by-products. *BioResources* **6**: 5050–5061.

Mondal, S. 2017. Preparation, properties and applications of nanocellulosic materials. *Carbohydr Polym.* **163**: 301-316.

Mood, S. H., Golfeshan, A. H., Tabatabaei, M., Jouzani, G. S., Najafi, G. H., Gholami, M., Ardjmand, M. 2013. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. *Renewable and Sustainable Energy Reviews* **27**: 77–93.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology* **96**: 673-686.

Mussatto, S.I., Roberto, I.C. 2004: Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: A review. *Bioresource Technology* **93**: 1- 10.

Nada, A., Ibrahem, A.A., Fahmy, Y., Abo-Yousef, H.E. 1999. Peroxyacetic acid pulping of bagasse and characterization of the lignin and pulp. *Journal of Scientific and Industrial Research* **58**:620, 628.

Narendranath, N.V., Power, R. 2005. Relationship between pH and Medium Dissolved Solids in Terms of Growth and Metabolism of Lactobacilli and *Saccharomyces cerevisiae* during Ethanol Production. *Appl Environ Microbiol*. **71**: 2239–2243.

Ng, Hon-M., Sin, L. T., Tee, Tiam-T., Bee, Soo-T., Hui, David., Low, Chong-Y., Rahmat, A.R. 2015. Extraction of cellulose nanocrystals from plant sources for application as reinforcing agent in polymers. *Composites Part B: Engineering* **75**: 176-200.

OIV (International Organisation of Vine and Wine); Global Economic Vitiviniculture Data. Paris, 20 October 2016. pp. 4.

Oksman, K., Etang, J. A., Mathew, A. P., Jonoobi, M. 2011. Cellulose nanowhiskers separated from a bio-residue from wood bioethanol production. *Biomass Bioenergy* ***35***: 146-52.

Oliveira, M., Duarte, E., 2016. Integrated approach to winery waste: waste generation and data consolidation. *Front. Environ. Sci. Eng.* **10**: 168–176.

Olofsson, K., Bertilsson, M., Lidén, G. 2008. A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnology for Biofuels* 1:7

Olsson, L., Jorgensen H., Krogh K., Roca C. 2005. Bioethanol Production from Lignocellulosic Material, in Polysaccharides: Structural Diversity and Functional Versatility, Second Edition. Marcel Dekker, New York, pp 957-993.

Pan, X.; N. Gilkes, J.F. Kadla, K. Pye, S. Saka, D. Gregg, K. Ehara, D. Xie, D. Lam & J.N. Saddler (2006): Bioconversion of hybrid poplar to ethanol and co-products using an organosolv fractionation process: optimization of process yields. *Biotechnology and Bioen-gineering* **94**: 851-861.

Pengilly, C. 2013. Study of Enzymatic Hydrolysis to Improve Sugar Production from Steam-Pretreated Sweet Sorghum Bagasse and Triticale Straw. Master thesis. Stellenbosch University. pp. 186.

Ping, L., Brosse, N. Sannigrahi, P. Ragauskas, A.2011. Evaluation of grape stalks as a bioresource. *Industrial Crops and Products* **33** :200–204.

Pocha, J., Villaescusa, I., 2010. A model to describe Cr (VI) kinetics biosorption. *J. Hazard. Mater.* **175**: 770–778.

Prozil, S. O., Evtuguin, D. V., Lopes, L. P. C. 2012. Chemical composition of grape stalks of Vitis vinifera L. from red grape pomaces. *Industrial Crops and Products* **35**: 178– 184.

Pujol, D., Liu, C., Fiol, N. M., Olivella, À., Gominho, J., Villaescusa, I., Pereira, H. 2013. Chemical characterization of different granulometric fractionsof grape stalks waste. *Industrial Crops and Products* **50**: 494– 500.

Sakata Y, Zhuang, H, Kwansa, H, Koehler, R C, Doré, S. 2010. Resveratrol protects against experimental stroke: putative neuroprotective role of heme oxygenase 1. *Experimental Neurology* **224**: 325– 329.

Santamaría, B, Salazar, G, Béltran, S, Cabezas, J. L. 2002. Membrane sequences for fractionation of polyphenolic extracts from defatted milled grape seeds. *Desalination* **148**: 103–109.

Sarkar, N., Ghosh, S. K., Bannerjee, S., Aikat, K. 2012. Bioethanol production from agricultural wastes: An overview. *Renewable Energy* **37**: 19-27.

Silva, C. F., Arcuri, S. L., Campos, C. R., Vilela, D. M., Alves, J. G. L. F., Schwan, R. F. 2011. Using the residue of spirit production and bio-ethanol for protein production by yeasts. *Waste Management (New York, N. Y.)* **31**: 108–114

Singhania, R. R., Patel, A. K., Sukumaran, R. K., Larroche, C., Pandey, A. 2013. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. *Bioresour Technol.* **127**: 500-507.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. 2012. Determination of Structural Carbohydrates and Lignin in Biomass. Laboratory Analytical Procedure (LAP) and National Renewable Energy Laboratory (NREL). The Alliance for Sustainable Energy, LLC (“ASE”) for the Department of Energy. pp. 18.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton. 2008. Determination of Ash in Biomass. Laboratory Analytical Procedure (LAP) and National Renewable Energy Laboratory (NREL). The Midwest Research Institute (“MRI”) for the Department of Energy. pp. 8.

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. 2008. Determination of Extractives in Biomass. Laboratory Analytical Procedure (LAP) and National Renewable Energy Laboratory (NREL). The Midwest Research Institute (“MRI”) for the Department Of Energy. pp. 12.

Soccol, CR., Faraco, V., Karp, S., Vandenberghe, LPS., Thomaz-Soccol, V., Woiciechowski, A., Pandey, A. Lignocellulosic bioethanol: current status and future perspectives. In: Pandey A, Larroche C, Ricke SC, Dussap CG, Gnansounou E, editors. Biofuels: Alternative Feedstocks and Conversion Processes. San Diego: Academic Press; 2011. p. 101-122.

Spigno, G., De Faveri, D.M., 2007. Antioxidants from grape stalks and marc: influence of extraction procedure on yield, purity and antioxidant power of the extracts. *J. Food Eng.* **78**: 793–801.

Spigno, G., Maggi, L., Amendola, D., Dragoni, M., De Faveri, D. M. 2013. Influence of cultivar on the lignocellulosic fractionation of grape stalks. *Industrial Crops and Products* **46**: 283-289.

Spigno, G., Pizzorno, T., De Faveri, D.M., 2008. Cellulose and hemicelluloses recovery from grape stalks. *Bioresour. Technol.* **99**: 4329–4337.

Spronsen, J. V., Cardoso, M. A. T., Witkamp, G. J., Jong, W., Kroon, M. C. 2011. Separation and recovery of the constituents from lignocellulosic biomass by using ionic liquids and acetic acid as co-solvents for mild hydrolysis. *Chemical Engineering and Processing* **50**: 196–199.

Sun, N., Rahman, M., Qin, Y., Maxim, M.L., Rodríguez, H., Rogers, R.D. 2009. Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3-methylimidazolium acetate. *Green Chemistry* **11**: 646-655.

Sun, Y. and Cheng, J. (2002) Hydrolysis of Lignocellulosic Materials for Ethanol Production: A Review. *Bioresource Technology* **83**: 1-11.

Taherzadeh, M. J., Gustafsson, L., Niklasson, C., Liden, G. 2000. Inhibition effects of furfural on aerobic batch cultivation of *Saccharomyces cerevisiae* growing on ethanol and/or acetic acid. *J.Biosci. Bioeng.* **90:**374-380.

Tsukamoto, J., Duran, N., Tasic, L. 2013. Nanocellulose and bioethanol production from orange waste using isolated microorganisms. *J. Braz. Chem. Soc.* **24**: 1385-1543.

Van, Zyl, C., Prior, B.A., Du Preez, J.C. 1988. Production of ethanol from sugar cane bagasse hemicellulose hydrolyzate by *Pichia stipitis*. *Applied Biochemistry and Biotechnology* **17**: 357–369.

Verardi, A., De Bari, I., Ricca, E., Calabrò, V. 2012. Hydrolysis of Lignocellulosic Biomass: Current Status of Processes and Technologies and Future Perspectives. In: Bioethanol (Marco Aurelio Pinheiro Lima), InTech. pp. 95-122.

Waldron K. W. 2010. Bioalcohol production: Biochemical conversion of lignocellulosic biomass. 1st edition. In: A volume in woodhead publishing series in energy.ISBN 978-1-84569-510-1. pp.496.

Watkins, D., Nuruddin, M., Hosur, M., Tcherbi-Narteh, A., Jeelani, S. 2015. Extraction and characterization of lignin from different biomass resources. *J Mater Res Technol* **4**: 26-32

Wilson, D., del Valle, M., Alegret, S., Valderrama, C., Florido, A., 2012. Potentiometric electronic tongue-flow injection analysis system for the monitoring of heavy metal biosorption processes. *Talanta* **93**: 285–292.

Yalcin, D., Ozcalik, O., Altiok, E., Bayraktar, O. 2008. Characterization and recovery of tartaric acid from wastes of wine and grape juice industries. *Journal of Thermal Analysis and Calorimetry* **94**: 767–771.

Zabed, H., Sahu, J.N., Suely, A., Boyce, A.N., Faruq, G. 2017. Bioethanol production from renewable sources: Current perspectives and technological progress. *Renewable and Sustainable Energy Reviews* **71**: 475-501.

Zacharof, Myrto-P. 2017. Grape Winery Waste as Feedstock for Bioconversions: Applying the Biorefinery Concept: *a review*. *Waste and Biomass Valorization* **8**: 1011–1025.

Zhang, B., Shahbazi, A. 2011. Recent Developments in Pretreatment Technologies for Production of Lignocellulosic Biofuels. *Journal of Petroleum & J Environmental Biotechnology* **2**: 111.

Zhang, F., Lin, J., Zhao, G. 2016. Preparation and Characterization of Modified Soda Lignin with Polyethylene Glycol. *Materials* 9: 822.

Zhu, F., Du, B., Zheng, L., Li, J. 2015. Advance on the bioactivity and potential applications of dietary fibre from grape pomace. *Food Chem* **186**: 207-12.