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Final project

SOLID-STATE ENZYMATIC HYDROLYSIS OF AGRO-INDUSTRIAL RESIDUES AS ALTERNATIVE LOW-COST PROCESS FOR OBTAINING FERMENTABLE SUGARS

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Bachelor's degree in Biotechnology

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Acknowledgments

Four years ago, I started one of the most important stages in my life, when I decided to study a Bachelor's degree in Biotechnology. During this period of time, I have not only had the opportunity to acquire a lot of academic knowledge but also to pursue personal growth and collect lots of beautiful memories that I will always cherish.

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Summary

Title: Solid-state enzymatic hydrolysis of agro-industrial residues as alternative low-cost process for obtaining fermentable sugars

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Keywords: Solid-state enzymatic hydrolysis, Olive-mill solid waste, Grape Pomace, *Aspergillus niger*, Reducing sugars, waste to product.

Fermentable sugars (FS) are valuable intermediates used as source for obtaining a wide range of final products with higher value-added. Although FS can be obtained from different plants containing them such as from sugar cane or sugar beet, the use of second-generation sources such lignocellulosic biomass is of current interest. With such approach a two-fold aim is achieved. Producing FS economically while valorizing a waste stream. Among the lignocellulosic materials, the agro-industrial residues have been extensively studied as potential sources of diverse biotechnological applications. Since their structure contains significant cellulose and hemicellulose contents, they are prone to be source of FS after a hydrolysis step. Typically, hydrolysis of such materials is conducted using chemical (acids or basis) or enzymatic systems in liquid cultures. However, the use of solid-phase hydrolysis is gaining relevance due to their advantages over their liquid counterpart, such as a lower energy and water demand, and the reduced waste generation. In particular, the solid-state enzymatic hydrolysis (SSEH) has been used with several lignocellulosic materials to produced FS that can be further processed in both solid and liquid phases.

In the Catalonian region different lignocellulosic waste are produced, but some of the main include those derived from the wine and olive oil industries. Although these wastes are disposed, composted or burnt to produced energy, their characteristics make them potential source of valuable bioproducts such as FS. Therefore, by using a suitable approach for using such local wastes would provide an alternative to the conventional disposal strategies, while gaining value from a strain considered waste.

Thus, the aim of this study was to evaluate the potential of two local agro-industrial residues namely grape pomace (GP) and olive-mill solid waste (OMW) as raw materials for obtaining FS by using SSEH. Throughout the study, OMW and GP were hydrolyzed at different conditions. First, they were processed by a self-produced enzyme extract from *Aspergillus niger* that was previously obtained through solid-state fermentation. Then, they were hydrolyzed with a commercial extract (Viscozyme L).

It was found that the highest FS contents were achieved using GP as a substrate. Besides, the commercial enzyme showed more effectiveness than the self-produced enzymes from *Aspergillus niger*. It was also demonstrated that the moisture content affected the hydrolysis process, but differently depending on the type of enzymes used. These results show that using SSEH of selected agro-industrial residues is a promising valorization strategy that follows the framework of the circular economy.

This work was developed in the framework of the national project “Valorización de residuos agroindustriales para la producción de bioplásticos-VALORA” founded by the Spanish Ministerio de Economía y Competitividad (CTM2016-81038-R).

Resum

Títol: Hidròlisi enzimàtica en estat sòlid de residus agro-industrials com a alternativa de baix cost per la obtenció de sucres fermentables

Autora: Cristina Ordeix i Barrios

Supervisor: Oscar Mauricio Martínez Avila

Paraules Clau: Hidròlisi enzimàtica en estat sòlid, Residu d'oliva, Brisa de raïm, *Aspergillus niger*, Sucres Reductors, Residus com a producte.

Els sucres fermentables (SF) són uns productes intermediaris de valor utilitzats com a font per l'obtenció d'un ampli ventall de productes finals amb un alt valor afegit. Encara que els SF es poden obtenir de diferents plantes que els contenen, com per exemple de canya de sucre o la remolatxa sucrera, la utilització de fonts de segona generació com la biomassa lignocel·lulòsica és d'actual interès. Amb aquest enfocament s'aconsegueix el doble objectiu. Produir SF de manera econòmicament viable alhora que es valoritza un flux de residus. Entre els materials lignocel·lulòsics, els residus agro-industrials s'han estudiat àmpliament com a font potencial d'aplicacions biotecnològiques diverses. Degut a que la seva estructura conté quantitats significatives de cel·lulosa i hemicel·lulosa, són adequats com a font de sucres fermentables després d'un procés d'hidròlisi. Típicament, la hidròlisi d'aquests materials és duta a terme mitjançant la utilització de químics (àcids o bàsics) o sistemes enzimàtics en cultius líquids. No obstant això, la utilització d'una fase de hidròlisi en estat sòlid està guanyant rellevància degut als seus avantatges per sobre del seu homòleg en estat líquid, ja que requereix una menor demanda d'energia, aigua i una reducció de generació de residus. En concret, la hidròlisi enzimàtica en estat sòlid (HEES) ha estat utilitzada amb diversos materials lignocel·lulòsics per obtenir SF que puguin ser processats posteriorment tant en fases sòlides com líquides.

A la regió de Catalunya es produeixen diferents residus lignocel·lulòsics, però alguns dels principals són els derivats de les indústries del vi i de l'oli d'oliva. Tot i que aquests residus són eliminats, compostats o cremats per produir energia, les seves característiques els converteixen en una font potencial de bioproductes valuosos com els SF. Per tant, un enfocament adequat per a l'ús d'aquests residus locals

proporcionaria una alternativa a les estratègies convencionals d'eliminació, alhora que es guanyaria valor d'un material considerat com a residu.

Així doncs, l'objectiu d'aquest estudi era avaluar el potencial de dos residus agro-industrials locals que són la brisa de raïm (BR) i el residu d'oliva (RO) com a matèries primeres per obtenir SF mitjançant HEES. Al llarg de l'estudi, BR i RO van ser hidrolitzats a diferents condicions. En primer lloc, van ser processats per un extracte d'enzim elaborat a partir de *Aspergillus niger*, prèviament obtingut per fermentació en estat sòlid. Després, va ser hidrolitzat amb un extracte comercial (Viscozyme L).

Es va trobar que els continguts de SF més alts es van aconseguir utilitzant la BR com a substrat. A més, l'extracte comercial va mostrar més efectivitat que l'extracte produït a partir de *Aspergillus niger*. També es va demostrar que la humitat afectava el procés el hidròlisi, però de manera diferent depenent de l'extracte enzimàtic utilitzat. Aquests resultats mostren que l'ús de HEES dels residus agro-industrials seleccionats és una prometedora estratègia de valorització que segueix el marc de l'economia circular.

Aquest projecte s'ha desenvolupat en el context del projecte nacional "Valorización de residuos agroindustriales para la producción de bioplásticos-VALORA" fundat per el Ministerio de Economía y Competitividad de España (CTM2016-81038-R).

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1. Introduction

1.1. Project context and justification

In the last seventy years, there has been a massive plastic production by the industry, and the shortcomings of sustainable waste management have caused that 8 millions tons end up in the ocean every year (1), affecting the environment and the food chain. According to Greenpeace (2), it is estimated that 500 million tons will be produced during 2020, 900% more than twenty years ago, from which only 10% will be recycled. In the case of Catalonia, 5.5 millions of bottles and cans are not recycled per day (3) so it empathizes the need for change. However, in the past decades, the rapid economic development in many countries has promoted an increased need for renewable energy resources due to the finite supply of fossil-derived energy and materials, and an increasing concern of global warming.

In this context, the national project VALORA was born, aiming to valorize some local agro-industrial residues-produced in large amounts in the Catalonia region through the bioproduction of biodegradable bioplastics known as polyhydroxyalkanoates (PHA). VALORA project focus on obtaining these value-added compounds from waste streams, specifically from lignocellulosic-derived wastes using these as potential raw materials of an alternative technology: the solid-state fermentation (SFF), a more sustainable and potentially economic technology. Moreover, along with this project, other process stages are evaluated, such as potential pretreatments, and the enzymatic hydrolysis of the residues (in liquid and solid phases) to obtain organic fractions readily available for the microorganisms producing these bioplastics.

Having that context in mind, this work was focused on testing the solid-state enzymatic hydrolysis (SSEH) of two lignocellulosic residues produced locally: olive-mill solid waste (OMW) and grape pomace (GP). Thus, the SSEH has served as an alternative process to increase the fermentable sugar content of the residues, intending to enhance their potential for producing PHA via SSF within the VALORA project.

1.2. Lignocellulosic biomass

Lignocellulosic biomass is the term used to refer all kinds of vegetable dry matter, and it is mainly constituted by lignin, hemicellulose and cellulose (Figure 1). It has many advantages as raw material like their natural abundance, renewability, recyclability, low-price and easy of accessibility along the year and around the globe, making it as an eco-attractive and petro-alternative candidate. It is a potential substrate for the production of a range of high-value products, including biofuels such as bioethanol, biogas, organic acids, enzymes or bioplastics (4). Nowadays, lignocellulosic-derived wastes are eventually left untreated in landfills, incinerated or composted. Hence, eventually leading to their putrefaction, giving rise to toxic gases or leachate into the environment. However, bio-refinery is currently emerging as a sustainable approach that is focused on revalorize lignocellulosic materials into value-added products. These include composite, fine chemicals, animal feed, pulp and paper, biofuels and novel enzymes that are competent in the market presently dominated by the petroleum-based products (5).

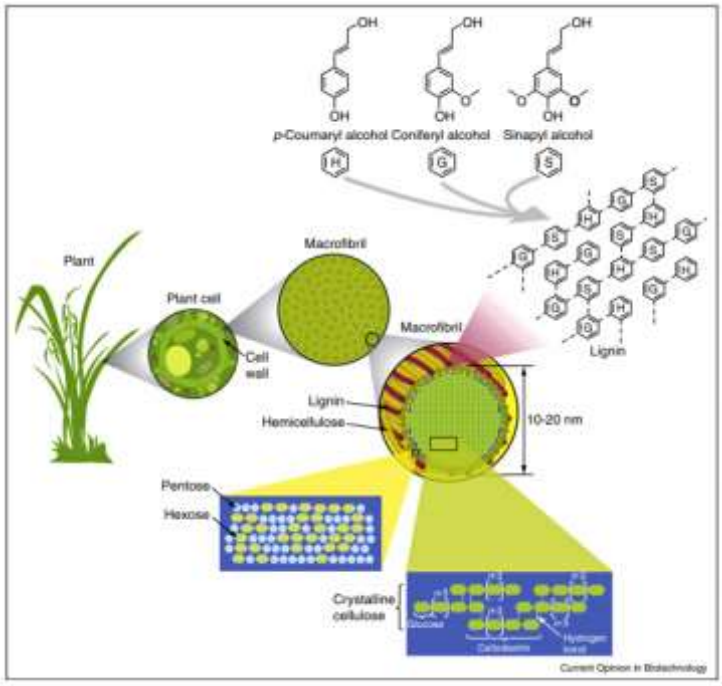

Figure 1. Typical structure of lignocellulosic material. Source (6)

One of the bioproducts obtained from lignocellulosic-derived biomass that has been deeply studied is the bioethanol. In general, this bioethanol production tends to be

more efficient in the use of resources such as the energy consumption, being up to 50.2% more efficient than other conventional technologies such as the thermochemical processes (5). Additionally, these bioprocesses are able to produce other valuable products simultaneously, such as PHA from the acid-rich effluent of fermentation (5).

Lignocellulosic biomass can be considered a very heterogeneous material, but in general, its structure is composed of almost 75% of polysaccharides, making it a fiercely sought out raw material for value-added products. However, using these materials directly for such applications results difficult because of the complexity of the lignocellulosic structure, which is an entrapment of covalent bonding between lignin, hemicellulose and cellulose. Lignin acts as entrapment of the other two constituents, preventing the carbohydrate from being exposed, and therefore, limiting the availability of the polysaccharide (4). The degree of polymerization, acetyl groups, plant protein-enzyme interaction, the porosity of the material, the accessible surface area to enzymatic degradation and the residual surface area of biomass should be taken into account (7).

Thus, using lignocellulosic-derived biomass as raw materials for bioprocesses often requires the conditioning of cellulose and hemicellulose fraction to make more accessible the polysaccharides. Afterwards, obtaining fermentable sugars from the available polysaccharides is achieved through a hydrolysis step (4). Nevertheless, polysaccharides are not the only profitable fractions obtained from lignocellulosic materials. While hemicellulose and cellulose are subjected to hydrolysis to obtain sugar monomers like glucose, xylose, mannose, galactose, or other intermediate compounds such as acetic acid, ethylene, butadiene etc., the lignin fraction is mainly used for the production of aromatic compounds such as xylitol, phenols, glucaric acid, aspartic acid, glutamic acid, syringols, eugenol, toluene, xylene, styrene, 3-hydroxy propionic acid (3-HPA), among others (5).

Typically, before hydrolysis, different pretreatments are conducted over the lignocellulosic materials to reduce the negative effect of lignin on the hydrolysis step. These pretreatments cover a wide range of processes, including physical, chemical, and biological treatments (4). Figure 2 summarizes the most important pretreatments used with lignocellulosic materials. Selection of pretreatment depends on several aspects, but they are mostly focused on the type of material to be processed.

One of the main drawbacks of applying such pretreatments is the formation of inhibitor compounds as a result of enzyme activity, metabolism of the microorganisms, or chemicals released from cellulose, hemicellulose and lignin during the process. For instance, when a thermochemical treatment is applied, aliphatic acids (acetic, formic and levulinic), furaldehydes (furfural and 5-hydroxymethylfurfural), uronic acid, 4-hydroxybenzoic acid, vanillic acid, vanillin, phenol, cinnamaldehyde, formaldehyde

furan derivatives (2-furaldehyde, hydroxyl methyl furfural) could appear during the hydrolysis step (4). To overcome the formation of these toxic substances, a process should prevent the formation of inhibitor compounds during pretreatment and hydrolysis stages. One of the alternatives commonly used is the detoxification of hydrolysates prior fermentation step (4). Alternatively, using genetically modified microorganisms able to resist significant levels of the inhibitors found the culture, or to transform the toxic compounds into neutral products are also used (4). Ongoing examples of those technologies are the use of laccases and peroxidases enzymes to remove phenolic compounds, vacuum evaporation of most volatile fractions (acetic acid, furfural, vanillin, etc.), or the use of polyethylene glycol surfactant, activated charcoal and ethyl acetate for phenolic compounds (4).

Despite all the advantages, it is essential to consider some limitations of the bio-refinery approach. The main one is the uncertainly stable supply for continuous industrial production, the difficulties in reducing the high cost and pollution of pretreatments, and the challenge to reuse the enzymes maintaining the conversion rates.

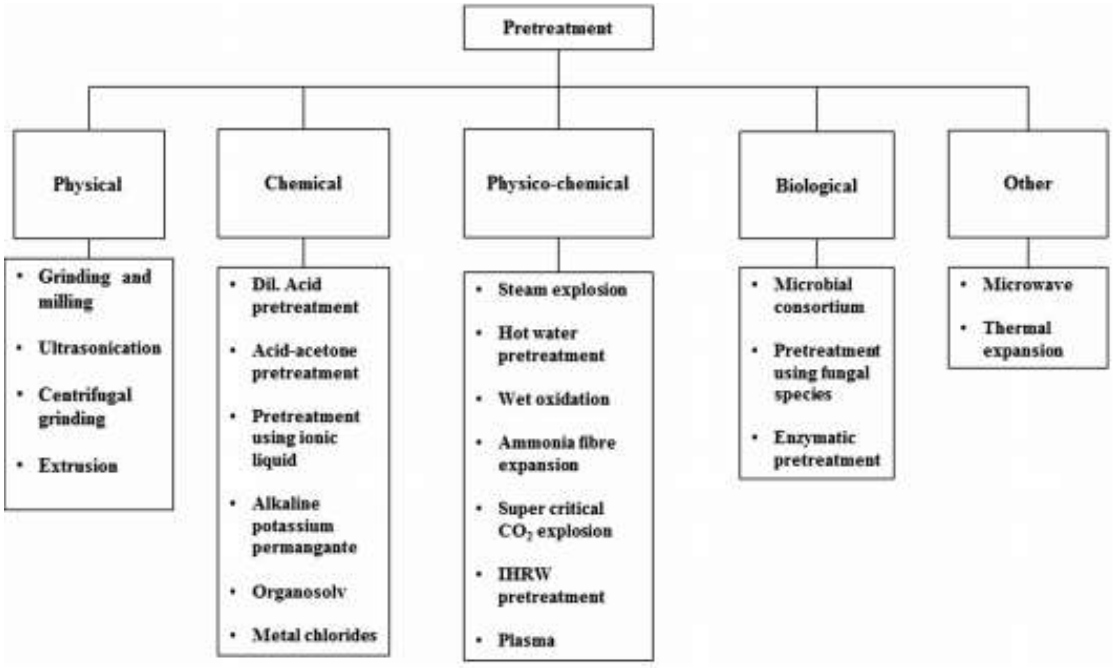

Figure 2. Pre-treatment strategies used with lignocellulosic biomass. Source (4)

1.2. 1. Lignin

Lignin can be considered the most abundant constituent in plant biomass (Figure 3), it has an aromatic and amorphous nature, and its monomers are basically phenylpropane units only differing in the substitution of methoxyl groups on the aromatic rings (4). The three main lignin monomers (monolignols) forming the lignin polymer are p-hydroxyphenyl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S)(4). In general, lignin is a structural polysaccharide imparting strength to the plant cell wall by covalently linking to hemicellulose and cellulose. Another property of lignin is its ability to adsorb enzymes irreversibly. However, some studies confirmed that higher temperatures, the addition of surfactants or specific pretreatments accelerate the cellulase adsorption process (4).

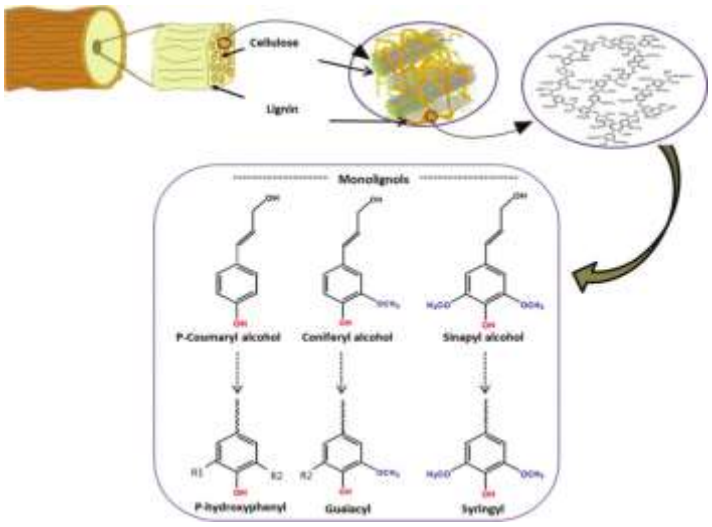

Figure 3. The three main monolignols that form the lignin polymer. Source (8)

1.2.2. Hemicellulose

Hemicellulose is a heterogeneous polymer made up of short chains of polysaccharide molecules, as shown in Figure 4. They approximately constitute 15–35% of the plant biomass and are composed of different sugar monomers such as D-xylose, L-arabinose (pentoses), D-galactose, D-mannose and D-glucose (hexoses) (4). Uronic acids, like α-D-glucuronic, α-D-4-O-methylgalacturonic and α-D-galacturonic acids have also been found in hemicellulose. Other sugars such as α-L-rhamnose and α-L-fucose may also exist in small quantities. Hemicelluloses are linked to cellulose by hydrogen bonds and lignin by covalent bonds (4).

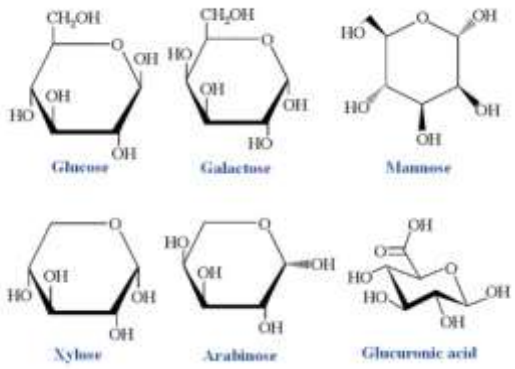

Figure 4. Different monomers presented in hemicellulose. Source (9)

1.2.3. Cellulose

Cellulose is a polysaccharide made up of D-glucose bonded to each by β(1→4) linkage forming linear chains (Figure 5). The plant cell wall is composed of microfibrils that are formed by cellulose chains bound together by hydrogen bonds, and each chain contains about 5000–14000 glucose molecules (4).

The bonds are intra- and inter-molecular between hydroxyl groups, and between hydroxyl groups and cyclic oxygen. At the supermolecular level, this capability to form hydrogen bonds consequences the elementary structural unit of cellulose fibers, a basic fibril, in which cellulose chains are prepared in corresponding over a hydrogen bond network (5). Basic fibrils are combined into fiber packages called micro-fibrils. Crystalline regions are caused by hydrogen bonds replacement with amorphous regions, in which cellulose chains display much less alignment with deference to each other. This makes that hydrolysis digestion of amorphous cellulose takes place before crystalline cellulose (4).

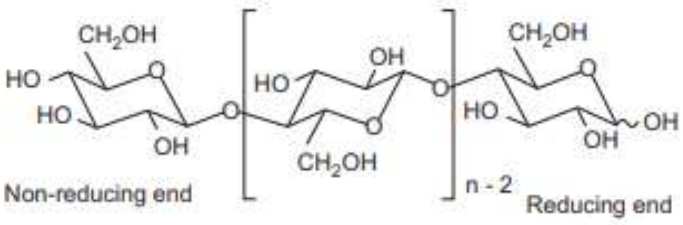

Figure 5. Cellulose structure. Source (10)

1.3. Enzymatic hydrolysis

Enzymatic hydrolysis is the process in which enzymes cleavage the bonds of a molecule generally in an aqueous solution to obtain smaller molecules (Figure 6). In the case of the hydrolysis of lignocellulosic biomass, the products of interest are fermentable sugars, the monomers of cellulose and hemicellulose that can be utilized as either a carbon source to produce ethanol, PHA or other value-added products employing different biotransformation processes. As stated before, lignin which acts as a protective physical barrier to cellulose and hemicellulose is commonly removed before enzymatic hydrolysis by a pretreatment as mentioned before. However, the need for pretreatment depends on the particular characteristics of each lignocellulosic material.

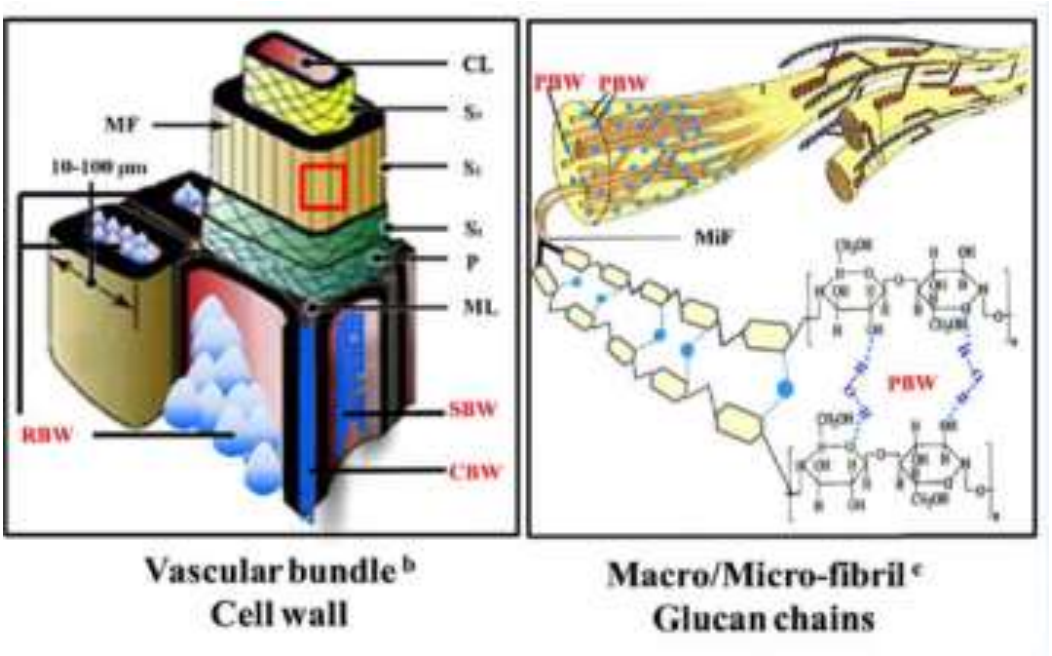

Figure 6. Place where the hydrolysis is produced. Five different water pools are existing in LCB suspensions due to the different interaction forces of biomass and water, including RBW,Restricted bulk water; CBW,Capillary bound water; SBW, Secondary bound water; PBW, Primary bound water; MF, Macro-fibril; MiF, Micro-fibril;CL:Cell-lumen; S,Secondary cell wall(S1, S2, S3);P,Primary cell wall; ML,Middle lamella. Source (11).

Hence, the global rate of this process is affected by the structural features of lignocellulosic biomass which make it a complex process. Many factors influence this process, and they can be broadly categorized into structural features of lignocellulose and the mechanisms and interactions related to enzyme kinetics such as the synergistic influence of other protein components on hydrolysis, and the release of hydrolyzed products into the bulk liquid (4).

Nonetheless, addressing the factors related to the structure of lignocellulose holds the key to efficient hydrolysis of the substrate. Among the aspects affecting the degradation of biomass residues in hydrolysis in can be mentioned: the lignin content, hemicellulose content, available amorphous cellulose as compared to its crystalline counterpart, the degree of polymerization, acetyl groups, plant protein-enzyme interaction, the association of cellulose with hemicellulose and lignin, the accessible surface area to enzymatic degradation, the porosity of the material and the residual surface area of biomass (4).

In the biorefinery approach, hydrolysis is mainly used for producing bioethanol (Figure 7) as an alternative fuel with attractive characteristics. For instance, its high heat of vaporization, high octane number and compatibility with vehicles (12).

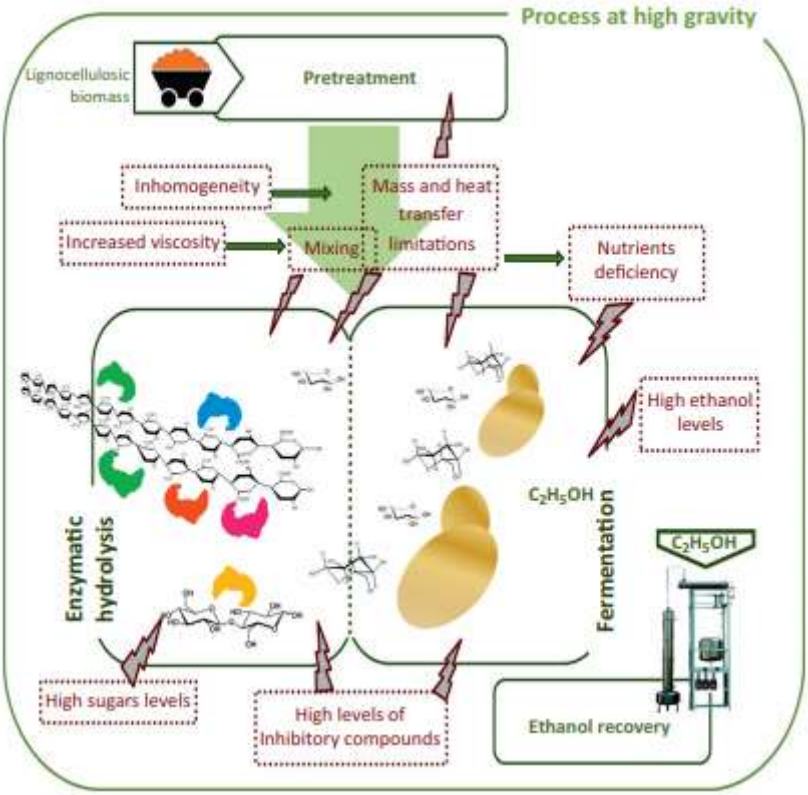

Figure 7. Different steps of the bioethanol production process from lignocellulosic materials.

Source (13)

In general, the enzymatic hydrolysis is run at temperatures between 30-50 ºC and pH 4-5 to increase the catalytic activity of the enzymes, and considering that elevated temperatures could inactivate the enzymes owing to the denaturation of protein structure causing a low reducing sugar yield.

Enzymes loading must be adjusted to the optimal saccharification performance since some studies have suggested that enzymes like cellulases can overcrowd accessible cellulose sites. Thus, not reaching the full hydrolytic potential for the given enzyme loading (14). Also, shear rates have been shown to disrupt the adsorption of cellulase on to biomass or even to cause the denaturation of cellulase.

Furthermore, it is known that the enzymatic hydrolysis involves a coproduction of inhibitor compounds that could affect the release of sugars, or the subsequent processes using the obtained hydrolysates. For example, cellobiose inhibits cellulase. To solve that aspect, typically cellulase is supplemented with β-glucosidase to reduce the inhibition by cellobiose. In addition, other factors, such as types of substrate, particle size, enzyme ratios, enzyme loadings, solids loadings, ab-/adsorption, and surfactants, also affect the synergistic system of multiple enzymes (11).

1.3.1. Lignocellulolytic enzymes

Lignocellulolytic enzymes are the enzymes capable of hydrolyzing the lignocellulosic biomass. These can be classified based on the fraction they can degrade. Thus, these can be divided into cellulases, hemicellulases and ligninases and the subtypes are related to the kind of polysaccharides they hydrolyze (cellulases for cellulase, xylanases for xylan, laccases for polyphenols of lignin, etc.). Here are presented the main enzymes used to hydrolyze lignocellulosic biomass.

1.3.1.1 Cellulases

Cellulases are the enzymes that hydrolyze β-1,4-glucoside linkages of cellulose chain and related cellooligosaccharides. There are three main types of cellulases (Figure 8): endo1-4-β-glucanase (EG, EC 3.2.1.4), cellobiohydrolase (CBH, EC 3.2.1.91) and β-glucosidase (BGL, EC 3.2.1.21). The difference between these lies in the cleavage. While endoglucanases cut the amorphous region of cellulose chain randomly, in the case of the exoglucanase is in the chain ends, and β-glucosidases hydrolyze the product of the exoglucanases releasing glucose monomers. So the synergistic action of these three types is vital for complete enzymatic hydrolysis (15).

Cellulases are inducible enzymes synthesized by a large diversity of microorganisms, including both fungi and bacteria during their growth on cellulosic materials. These microorganisms can be aerobic, anaerobic, mesophilic, or thermophilic. Among them, the genera of *Clostridium, Cellulomonas, Thermomonospora, Trichoderma,* and

Aspergillus are the most extensively studied cellulase producers (16). Recently they have also been produced by solid-state fermentation (SSF) (15), (17).

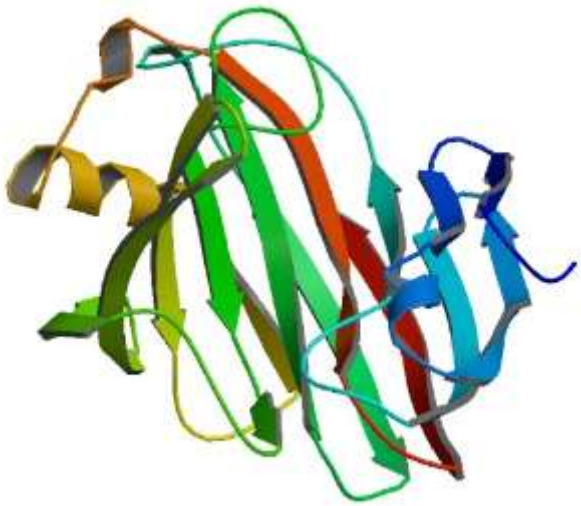

Figure 8. Structure of *Aspergillus niger* endoglucanase. Source (18)

1.3.1.2 Xylanases

Xylanases are a group of hemicellulases that degrade the linear polysaccharide xylan (the second most abundant polysaccharide) into xylose by catalyzing the hydrolysis of the glycosidic linkage (β-1,4) of xylosides (Figure 9). Xylanases have potential applications that include bioconversion of lignocellulosic material and agro-wastes into fermentative products together with other hydrolytic enzymes (19). They have been found to be widespread among fungi, actinomycetes, and bacteria, and some of the essential xylanolytic enzyme producers include *Aspergillus, Trichoderma, Streptomyces, Phanerochaetes, Chytridiomycetes, Ruminococcus, Fibrobacteres, Clostridia* and *Bacillus* (19).

Filamentous fungi are particularly interesting producers of xylanases because their enzyme levels are much higher than yeast and bacteria, they excrete the enzymes into the medium directly and produce several auxiliary enzymes required for the degradation of substituted xylan (19). Although most xylanase manufacturers use submerged fermentation techniques, the enzyme productivity via solid-state fermentation usually is much higher than that of submerged fermentation (19).

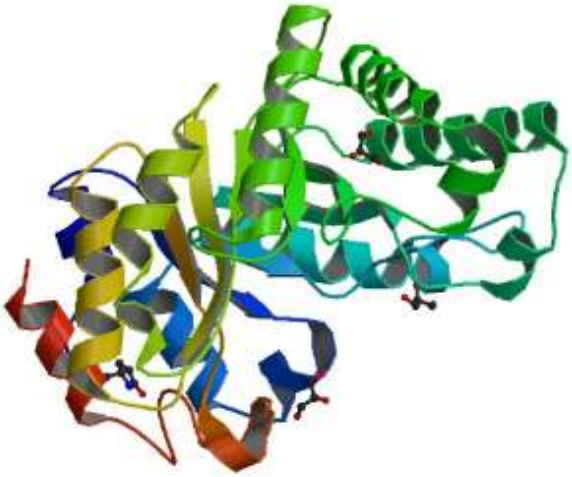

Figure 9. Crystal structure of an endo-beta-1,4-xylanase enzyme from *Aspergillus niger*. Source (20)

1.3.1.3 β-galactosidase

β-galactosidase is an exoglycosidase hydrolase enzyme that breaks lactose bonding to galactose and glucose to obtain a carbon source and energy production (Figure 10). It may also cleave fucosides and arabinosides but with much lower efficiency (21). These enzymes are widely used for industrial applications and are traditionally obtained from *Aspergillus spp*. and *Kluyveromyces spp* (22). They have also been successfully obtained through SSF. (23)

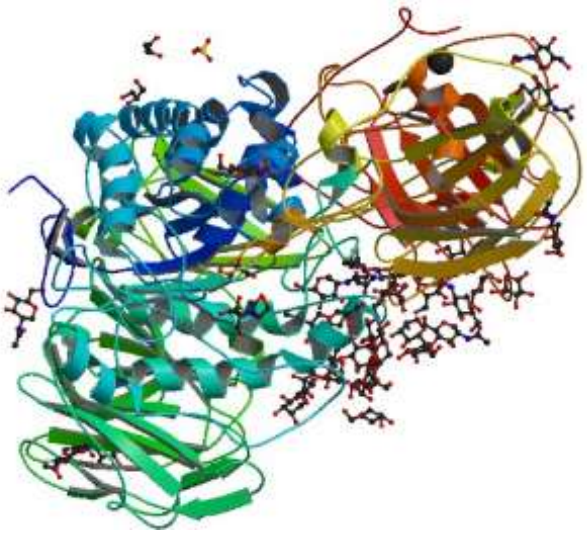

Figure 10. Structure of beta-galactosidase from *Aspergillus niger* in complex with different oligosaccharides.

Source (24)

1.3.2. Fermentable sugars

Fermentable sugars are carbohydrates including oligosaccharides, disaccharides, monosaccharides and polyols composed of short chains of sugar molecules, making them easy to break down, such that they can be easily used as a carbon source by a microorganism. Although the lignocellulosic materials contain some fermentable sugars, their content is typically limited. However, after hydrolysis part of the higher fractions (cellulose and hemicellulose) are converted into fermentable sugars (25). The most common fermentable sugars obtained from lignocellulose are glucose and xylose, which are also reducing sugars like the most of fermentable sugars (25). Reducing sugars are characterized by containing an open-chain form with an aldehyde group or a free hemiacetal group (Figure 11, Figure 12) (26).

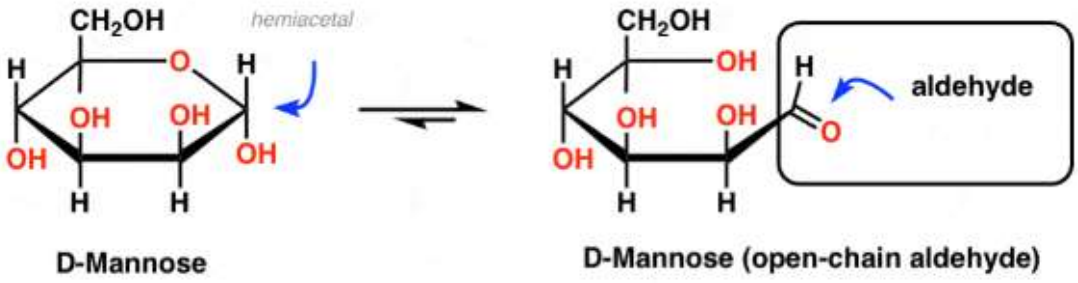

Figure 11. Mannose contains a hemiacetal group, so it is a reducing sugar since their open-chain form contains an aldehyde. Source (25)

Both aldoses, which have an aldehyde group, and the ketoses that have a ketone group can act as reducing sugars. However, ketoses must first be tautomerized (commonly resulting in a relocation of a proton) to aldoses before. This is the case of fructose (Figure 13, Figure 14) (26).

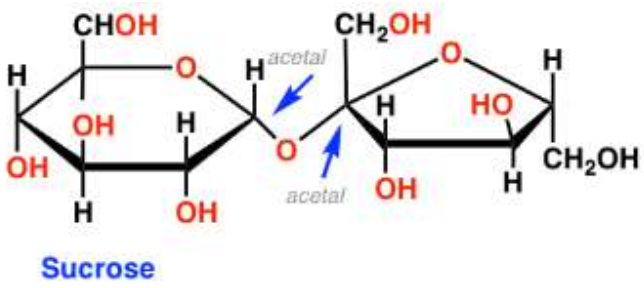

Figure 12. Sucrose is a non-reducing sugar. Acetals are locked and not in equilibrium with a ring-opened form with an aldehyde. Source (26)

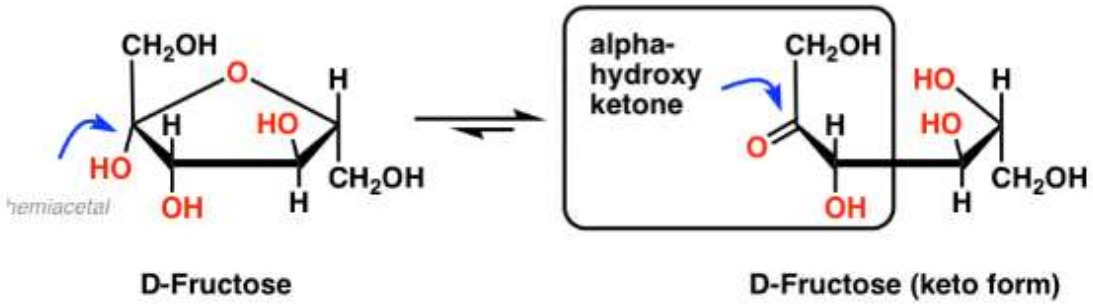

Figure 13. Fructose is a reducing sugar since it has a ketone group that can be tautomerized to aldose. Source (26)

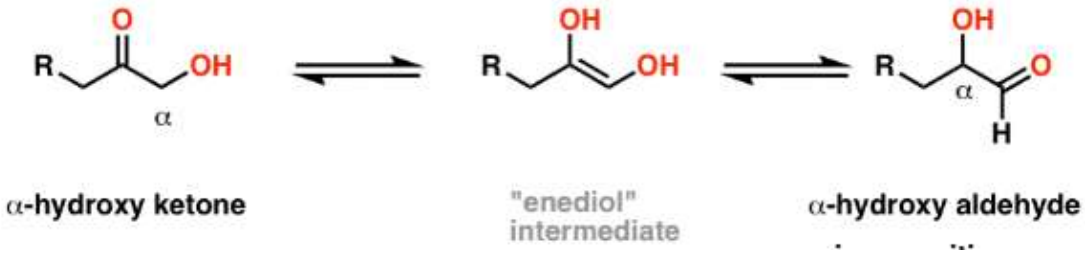

Figure 14. Tautomerization of ketose to aldose. Source (26)

They reduce another compound and being themselves oxidized, the carbonyl carbon of the sugar is oxidized to a carboxyl group (Figure 15). Many reagents can be used to oxidize aldehydes to carboxylic acids, and they are commonly used to determine their presence in a solution (26). One example is the 3,5-dinitrosalicylic acid, that is further explained in 3.4.3 section.

Figure 15. The aldehyde is oxidized to a carboxylic acid (break C-H, from C-O). The aldehyde reduces the oxidant. Therefore, the aldehyde is the reducing agent here. Source (26)

Among the many lignocellulose hydrolysis methods in order to get the fermentable sugars, enzymatic hydrolysis present unique advantages, such as mild reaction conditions, few by-products and more specificity compared to other chemical treatments (27).

1.4. Solid-state enzymatic hydrolysis

As it was mentioned before, hydrolysis is a process that has been traditionally run in liquid media. However, in recent years solid-state enzymatic hydrolysis has received ever-increasing interest all over the world as a result of the potentially high saccharification efficiency (28). SSEH is a process that occurs at high-solid loading concentration (≥ 15% solids w/w) (13) where there are no significant amounts of free water present (Figure 16).

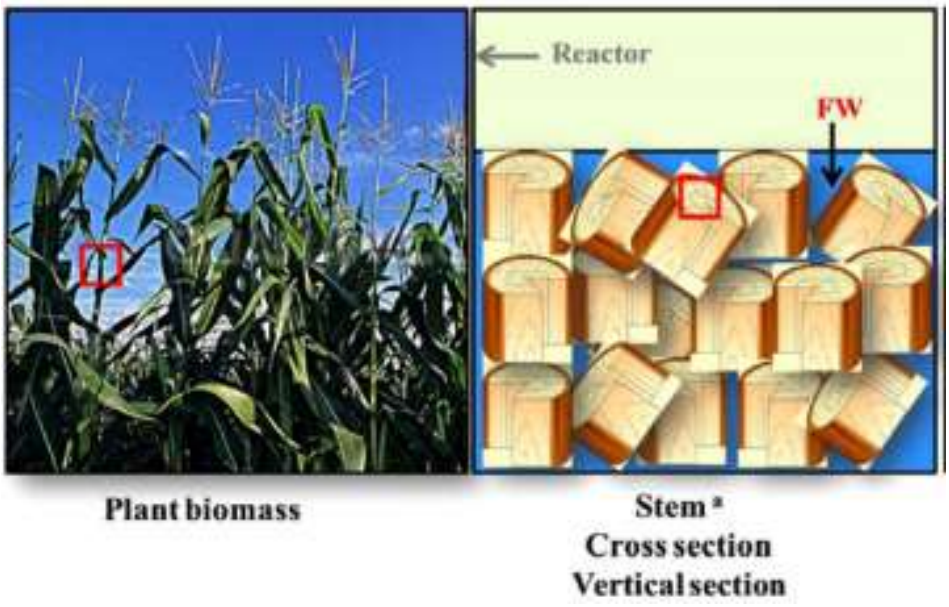

Figure 16. Reactor with a high loading concentration of lignocellulosic biomass. FW, Free water.

Source (11)

As it is known, water is essential to the enzymatic hydrolysis to assure a mass transfer by the solubilization of products and lubricity of the particles (14). Tsuchida et al. (29) studied water accessibility in cellulose of pretreated sugarcane bagasse and found that the stronger interaction of water with pretreated bagasse is consistent with better enzymes accessibility to cellulose and higher efficiency of the enzymatic hydrolysis. However, some other authors (30) found that the total solubilization of sugars does not depend on the liquid phase, and low water contents are also favorable by increasing the stability of enzymes. For example, moisture ratios can be optimized to provide enough water to an effective hydrolysis process, while facilitating the contamination control (15) (31).

Although research in this area is increasing, so far, the results are inconclusive because there is not any clear evidence about the factors that influence the water constraint and how they are correlated to the efficiency of SSEH (11). However, a comparison between solid-state and submerged fermentation has been analyzed (Table 1).

As detailed, SSEH has many advantages that make this option attractive for the industrial application of lignocellulosic materials. The most remarkable is its higher sugar concentration associated with higher solid loading. For most of the lignocellulosic materials, the initial solids loading should be above 20% w/w that is more than the double required to bring an efficient economic benefit in case of the ethanol industry as an example (14). Also, as the amount of water can be adjusted to a minimum during the extraction, the resulting solution is more concentrated (32). For example, López et al. (33) compared liquid and solid hydrolysis in chestnut by three different conditions: one prove to obtain a more concentrated hydrolysate than in submerged hydrolysis, and other two (despite having a 15% less of yield), resulted adequate for simultaneous solid-state hydrolysis and fermentation processes.

Table 1. Advantages and disadvantages of SSEH compared to submerged hydrolysis

Advantages	Disadvantages
Higher sugar concentration	Inhibition effects
Low capital and operating costs	Water constraint
Less energy input	Rheology change
High economic feasibility	Low efficiency

Another relevant aspect of SSEH is related to the size reduction of equipment and lower water consumption. It is demonstrated that smaller equipment and/or fewer reactors can be utilized to produce an equivalent output (34). Taking into account the abundance and cost-competitive lignocellulosic biofuel production with minimal effects on the environment can be envisioned by operating at high loading levels with high economic feasibility. Moreover, the cost of lignocellulosic enzymes has been considered one of the main bottlenecks for commercialization. Still, a recent focus on the development of new enzymes with improved properties and the low-cost production of efficient enzyme cocktails using high solid loadings, would, in turn, decrease the overall cost of the process (13).

However, there are also several technical challenges hindering the large-scale implementation of SSEH. As explained before, high solid loadings entail a high sugar concentration, which could also inhibit the activity of the enzymes. Simultaneously, degradation products generated from cellulose, hemicellulose, and other polymer compositions such as lignin during pretreatment and hydrolysis could also inhibit the process (11). As said before, water constraint could complicate the process due to the high viscosity and rheology problems resulting in the reduced efficiency of mass transfer concomitantly. By the moment, most scientific attempts on high solids hydrolysis reported reduced efficiency of enzymatic hydrolysis as the cellulose content increased, but all of these limitations can be overcome as explained below.

In general, it could be stated that conversion in SSEH depends on (but not limited to) many other factors apart from water. These include lignocellulosic material, type of pretreatment, synergistic system of multiple enzymes and their source, conditions of enzymatic hydrolysis, intensification methods, fed system and design of the reactor. Then, the challenge is to find the best conditions that maximize the release of fermentable sugars, at the same time that, being able to take it on a large-scale being environmentally-friendly and economically feasible (11).

In order to succeed on it, some elements have to take into account: best adsorption between the enzyme and the substrate, reduction of the inhibition product concentrations as well as the energy consumption.

Some studies have demonstrated that these conditions can be enhanced. To achieve the most efficient adsorption between the enzyme and the substrate (adsorption between cellulose and cellulase as an example) the specific area of the substrate can be increased modifying supplied water, temperature, particle size and enzyme loadings (28). However, some others (14) found that less-than-ideal condition in one property exacerbates the negative effects of another property. For example, the supplementation of additives to improve the aspects associated with rheology (decreasing the particle size, increasing the porosity, etc.) could inhibit the hydrolytic enzymes (14).

As occurs in the liquid phase, in the solid media, the presence of inhibitor compounds is also an issue. In fact, the degradation of the lignocellulosic material into these by-products is increased when working at high-solid loadings (especially weak acids, furan derivatives, and phenolic compounds) inhibiting the activity of the enzymes (11). Nevertheless, several methods to reduce their concentration have been carried out. For instance, the washing of the acid pretreated biomass with water before enzymatic hydrolysis. It was demonstrated that 87% of formic acid and 64% of acetic acid was removed in a wheat straw experiment (35), or the evidence that a post-washing could remove the inhibition effect of phenolics (36). However, it would require a significant water input and some fermentable sugars could be removed, which reduces the utilization efficiency of sugars.

Another alternative is to increase the enzymes load. Liu et al. (37) proved that by increasing a 25% the enzyme loading in a steam-exploded corn stover improved the glucan conversion reducing the inhibition effects. But it accounted for a higher cost of the process. Other methods proved to be adequate to limit the adverse effects of inhibitor compounds include using fungus to delignify, or the selective removal of these by-products using activated carbon or $Ca(OH)_2$ treatments (38), (39).

With the increase of solid concentration, sugars concentration also tends to increase. However, this induces the so-called sugars feedback inhibition effect (40). To avoid

that, the synergism of different enzymes has been a strategy. Hu et al. (41) gave evidence that accessory enzymes added to cellulases improves the SSEH performance and a higher proportion of xylanase is required when the substrate contains relatively high xylan content at high solids loadings. Furthermore, Garcia-Aparicio et al. (42) found that xylanase has a positive effect, especially in the early stages of enzymatic hydrolysis.

To further study that, several experiments have been run with enzyme supplementation supporting the idea that overall enzyme loadings could be reduced if a better conversion is achieved by incorporating an array of different enzymes. For example, it was demonstrated that supplementing cellulase with β-glucosidase has long been used to minimize end-product inhibition of the cellulase and achieve higher conversions and improves with xylanase addition (14).

On the other hand, an improvement could be achieved by studying other microorganisms. While the cellulase system of *T. reesei* is one of the most commonly studied enzyme systems, other microorganisms also produce cellulolytic enzymes that could potentially impart superior activity under certain conditions. This is the case of the report of Ingram et al. (43) that compares the conversion efficiencies of enzymes from two different organisms, *T. reesei* and a genetically-modified strain of *Penicillium janthinellum* (for increasing cellulase production). Both enzyme extracts contained cellulases, β-glucosidases and xylanases but despite higher enzyme loadings of *P. janthinellum*, cellulases were needed to achieve the same conversion levels.

Other challenges specific to high-solids enzymatic hydrolysis include long hydrolysis times since the reaction time needed for most enzymes to convert lignocellulose into sufficient glucose concentrations for fermentation is three days on average (14). This can reduce the increasing enzyme loading, which has a high price and even can saturate the accessible cellulose sites. Also, smaller particle size may reduce the time required for enzymatic hydrolysis, and the corresponding reduction in viscosity may allow higher solids loading and the reduction of reactor sizes during large-scale processing (44).

Regarding biomass feeding, batch approaches are the most common. However, fed-batch strategies are used to reduce the high loading of biomass. In this regard, some experiments have proved to be inconclusive (45) since they obtain approximately 66% and 90% efficiency level for steam pretreated corn stover at different enzyme loadings, respectively. Still, when the solids were fed at 24 h intervals, the respective yields resulted lower (approximately 55% and 80%) and the hydrolysis rates slower, probably caused by, non-productive binding of the enzyme to xylan or lignin fractions of the substrate or the inability of the enzyme to desorb from partially hydrolyzed substrate and find accessible cellulose sites in the fresh substrate.

On the contrary, other studies found that such an approach is an effective strategy to improve the efficiency of SSEH. For instance, the fed-batch process reduces the initial viscosity of the enzymatic hydrolysis system providing time for the solids to liquefy (14). The fed-batch process also maintains the free water level high in the initial stage of enzymatic hydrolysis, allowing to minimize, or even avoiding the diffusion and mixing limitations (46). Additionally, the fed-batch process should be helpful for the recycle of enzymes and the reduction of inhibition effect (47). Even though the prolongation of time and the increase in cost due to significant complexity should be evaluated.

Nonetheless, the fed system is not the only factor affecting hydrolysis behaviour Zhang et al. (48) developed a novel helical impeller to enhance the SSEH efficiency of steam explosion pretreated corn stover compared with a Rushton impeller that demonstrates to have better performances in the saccharification yield, ethanol titer, and energy cost than those of the Rushton impeller stirring (37). Hence, showing that a suitable mixing of the solid phase contributes to the development of the process.

Another aspect that definitively contributes to the improvement of enzymatic hydrolysis at high solids load is the reactor design. Due to the high viscosity and lack of homogeneity, the horizontal orientation of the reactor could provide many advantages over typical vertical stirred tanks. In that case, such configuration ensures better enzyme distribution, minimizes particle settling and local accumulation of reaction products within the reactor, and the feed requirements are lower with the same level of adequate mixing. Moreover, they are easily scalable from bench-scale to pilot-scale and industrial scale. Some studies (14) demonstrate a 10% more production of glucose in the horizontal reactor compared to shake flasks, achieving the maximum at 20% initial solids loading. The paddles also provide a scraping action that removes lignocellulosic material from the reactor walls, improving heat transfer between the reactor and the biomass.

In general, SSEH has been applied at lab and bench scales. However, several plants are operating at a pilot-scale, something that can encourage the industry to improve upon the challenges and limitations that are not recognized at the laboratory scale. One example of an SSEH pilot plant is located in Denmark, and it is capable of producing 5.3 million liters of ethanol each year, performing at 25-30% (w/w) solids content (49). Additionally, pretreatment (20-40%) and fermentation (18% dry matter) are performed at high-solids loadings, and the remaining lignin-rich material (40-95% dry matter) is burned to produce heat and electricity that can be cycled back into the conversion operation (49).

Also, the National Renewable Energy Laboratory (Golden, CO, USA) is capable of processing about 0.5-1 ton dry into ethanol each day operating in a semi-continuous process at solids loading ≥ 20% (w/w) with pretreatment occurring in horizontal

reactors with paddles, and after liquefaction, the slurry is subjected to stirred tank reactors to complete the enzymatic hydrolysis of the material (14).

Another pilot-scale platform Chen et al. (50) developed an industrial-level of simultaneous saccharification and co-fermentation system (SSCF). Glucose produced from enzymatic hydrolysis is timely consumed by yeast, and the ethanol is then separated online through gas stripping followed by activated carbon adsorption. By using reactors of 400 m^3, it was achieved more than 4% w/w ethanol concentration (corresponding to 72.3% of the ethanol's theoretical yield) with the added value of the coproduction of lignin plastic composite material and compress natural gas.

2. Objectives

The general objective of this project is to assess the solid-state enzymatic hydrolysis (SSEH) as an economical and eco-friendly tool to increase the fermentation potential of two lignocellulosic wastes produced in the Catalonian region. The proposed hypothesis is that olive-mill solid waste and grape pomace could be hydrolyzed through SSEH to obtain fermentable sugars using a self-produced enzymatic extract without needing a pretreatment stage.

The project has been split into the following specific objectives:

- To characterize the selected lignocellulosic wastes to identify their potential as substrate for SSEH.
- To evaluate the performance SSEH of the selected waste materials using commercial enzymes.
- To evaluate the performance SSEH of the selected waste materials using self-produced enzymes from *Aspergillus niger*.
- To determine the effect of the initial moisture content on the efficiency of the SSEH of the selected wastes.

3. Material and Methods

3.1. Substrates used in the study

The residues used to evaluate the SSEH were grape pomace (GP) and olive-mill solid waste (OSW). GP is the solid organic waste containing grape skin, stem, pulp, and seed, which are deliberately discarded in various grape processing industries, such as wine or juice manufacturing (Figure 17). Its high content in polysaccharides has made it an attractive bioactive compound to its phenolic extraction in recent years (51). This residue was collected from the vineyard of Celler Cooperatiu d'Espolla (Catalunya, Spain).

Figure 17. Grape pomace residue

On the other hand, OSW is an olive industry by-product that is mainly produced during the olive oil extraction (Figure 18). Although it is commonly discarded, it has demonstrated to be suitable for the conversion to energy by thermos-chemical and bio-chemical technologies in a laboratory scale (52). The lignocellulosic product was provided by a local olive oil producer in the Lleida region (Catalunya, Spain).

The physic-chemical characterization of the used residues is shown in Table 2.

Figure 18. Olive-mill solid waste.

Table 2. Characterization of the evaluated lignocellulosic residues.

Parameter	Grape pomace	Olive-mill solid waste
Total Kjeldahl nitrogen (g kg^{-1})	8.1 ± 1.5	9.7 ± 2.0
Oxidizable carbon (g kg^{-1})	624 ± 23	437 ± 5
Total phosphorus (g kg^{-1})	169 ± 13	57.2 ± 10.1
C:N (mass basis)	77.0 ± 3.2	45.1 ± 1.5
Total solids as received (% dry basis)	54.6 ± 0.8	91.4 ± 0.3
Volatile solids (% dry basis)	81.6 ± 0.4	80.7 ± 0.2
Water holding capacity (g_{H20} $g^{-1}_{dry\ material}$)	0.62 ± 0.01	0.85 ± 0.05
pH (1:10)	3.6 ± 0.3	5.4 ± 0.2
Reducing sugars (g $100g^{-1}$ dry basis)	6.8 ± 0.4	0.38 ± 0.02
Cellulose (% dry basis)	11.0 ± 0.4	10.8 ± 0.5
Hemicellulose (% dry basis)	3.5 ± 0.1	37.7 ± 0.7
Lignin (% dry basis)	16.9 ± 0.6	45.8 ± 0.8

3.2. Enzymatic extracts used in this study

In this study, two enzymatic extracts were used to hydrolyze the two lignocellulosic materials. The first was a self-produced extract from *A. niger,* and it was compared with commercial enzymes that have demonstrated efficiency in the lignocellulosic degradation.

3.2.1. Enzymatic extract from *A.niger*

The self-produce enzyme extracts were obtained by solid-state fermentation with 10% inoculum of *A. niger* following the method developed in the BETA group by Llimós et al. (53). The substrate was Brewer's Spent Grain (BSG) with 78% moisture content and fermentation process lasted 48 h at 37ºC with a flow rate of 25 mL/min (Figure 19). The enzymes produced in the solid phase were extracted using a citrate buffer 0.05M (pH 4.6) using a 1:10 solid-liquid ratio. These extracts were characterized in terms of the cellulase and xylanase activity following standardized procedures (54), (55). Xylanase activity of the extract was 230 ± 23 units (U) mL^{-1}, and the cellulase activity was 2.5 ± 1.0 filter paper units (FPU) mL^{-1}. These activities were measured following the methods proposed by Ang et al. (55) and Ghose et al. (54).

Figure 19. Bioreactors, where the solid-state fermentation to produce the enzymes, took place. They are connected to the respirometer to control the oxygen source and consumption.

3.2.2.Viscozyme ® L

Viscozyme® L was used as a commercial multi-enzyme complex. It was provided by Sigma-Aldrich, and it contains a wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase, and it has been used in the extraction of biomolecules from lignocellulosic material. Despite its high efficiency, it has to be diluted to maintain the proper pH for the microorganism in the post-fermentation process. In this study, the concentration was 2%, diluted with buffer solution (25% acetic acid, 25% sodium acetate). This dilution implied a xylanase activity of 260 ± 21 U mL^{-1}, and a cellulase activity of 2.9 ± 1.1 FPU mL^{-1}.

3.3. Substrate preparation and hydrolysis experiments

In order to prevent the degradation of the lignocellulosic materials, they were dried overnight at 60°C in an air oven and stored at -20°C until used. Before performing the hydrolysis process, the samples were sterilized at 121ºC for 15 min. Preparation of the substrates consisted of adding the enzymatic extracts (section 3.2.) to 80 ± 2 g of dried substrates placed in a cylindrical reactor (0.6 L) such that the final MC coincided with the requirement of each experiment (Table 3). Since the enzymatic extracts were diluted in a buffer solution, this assured the solid-media pH was always in the range 4.6-5.0, ideal for the type of enzymes used. After addition of the enzymatic extracts, the reactors were placed in an incubator at 40°C, and the hydrolysis was monitored for 48 h by taking samples at predefined intervals.

3.4. Analytical methods

The moisture content (MC), water holding capacity (WHC), pH, total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN), total phosphorus and oxidizable carbon (OXC) have been determined following standard procedures (56).

3.4.1. Moisture content

The moisture content is the amount of liquid water that is contained in the solid matrix. In other words, it is the complementary measure of total solids. This variable has a considerable impact in the physical proprieties of the material such as rheology, the reactivity of chemical compounds, binding properties of bulk materials (30), and

consequently in the solid-state enzymatic hydrolysis process and that is the reason for its monitoring.

Usually, the water content is difficult to measure because of the complex intermolecular bonding properties within the substance matrix so for that reason it was determined following the thermogravimetric method drying process that is universally recognized as an efficient reliable and cost-effective method and can be utilized in any environment. It is based on the weight loss of mass that occurs as the material is heated. The sample weight is taken prior to heating and again after reaching a steady-state mass subsequent to drying. Accordingly, samples were weighed before and after heated at 105ºC during a minimum of 24 h and calculated as follows:

$$Moisture\ content = \frac{Wet\ weight(g) - Dry\ weight(g)}{Wet\ weigth(g)} \quad (1)$$

Where wet weigh corresponds to the taken sample before being heated, and Dry weight corresponds to the weight of the sample after 24 h of drying.

3.4.2. Water-Holding Capacity

The water holding capacity (WHC) is the ability of the residue to bind and hold water within the matrix and depends on the chemical structure of the residue. It is relevant because the moisture content cannot exceed this quantity of water, which was useful to monitor the moisture content during the hydrolysis.

In order to know the maximum volume of water the residue was capable of retaining, the residue was homogenized and poured into a funnel with a filter paper as seen in Figure 20. Then, water was added and collected in a beaker. The residues were weight before and after the addition of the water, so the WHC was calculated as:

$$WHC = \frac{Wet\ weight(g) - Dry\ weight(g)}{Dry\ weigth(g)} \quad (2)$$

Where wet weigh stands for the residue after water absorbance and the Dry weight corresponds to the weight of the sample before that.

Figure 20. WHC estimation setup

3.4.3. Reducing sugars content

Reducing sugars contained in the solid residues were determined following the DNS method (57) from the supernatant obtained after a solid-liquid extraction using water (Figure 21). First of all, distilled water was added in a ratio of 1:10 and put in the shaker at 30ºC and 160 rpm during 35 min. Then the tubes were centrifuged at 4200 rpm during 10 min, and the supernatant was recovered and stored at 4°C until used.

Figure 21. Sample extraction for DNS analysis.

Dinitro Salicylic Acid method is a colorimetric technique that consists of a redox reaction between the 3,5-dinitrosalicyclic acid and the reducing sugars present in the sample. The reducing power of these sugars comes from their carbonyl group, which can be oxidized to the carboxyl group by mild oxidizing agents. At the same time, the DNS (yellow) is reduced to 3-amino-5-nitrosalicylic acid (red-brown) which can be

quantified by spectrophotometry at 540 nm, wavelength of maximum absorbance. The intensity of the color is proportional to the concentration of sugars (Figure 22). The reaction is carried out in an alkaline medium. Figure 23 shows the oxidation-reduction reaction.

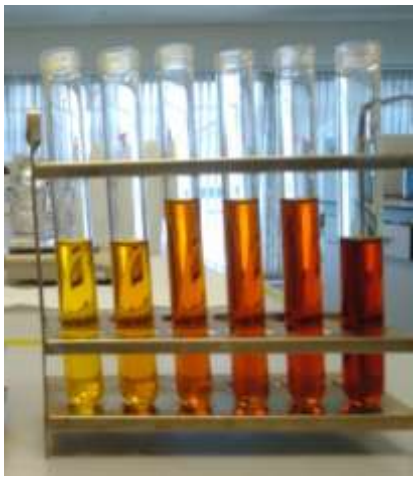

Figure 22. Bank of dilutions of glucose concentration used to DNS calibration curb. The intensity of the colour is proportional to the concentration of sugars.

The appropriate dilution of each sample was done and 3 mL of DNS were added afterwards. Samples were boiled for six minutes to let the reaction take place and later on the tubes were cooled. Finally, 20 mL of distilled water, and after shaking, the absorbance of the samples were measured at 540nm. Reducing sugars content was computed by using a calibration curve using glucose as standard in the range from 0.2 and 2.0 g L^{-1}.

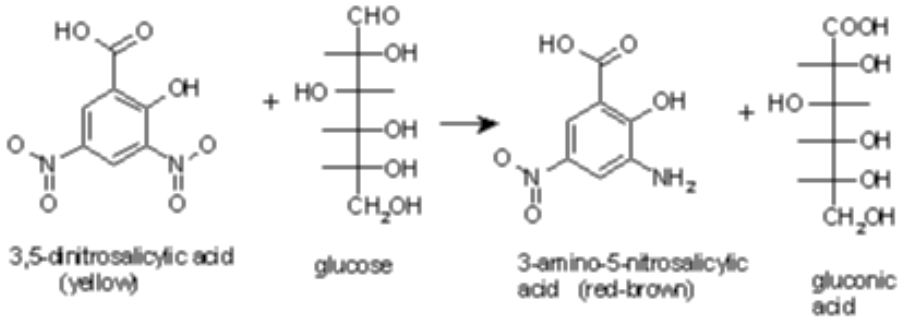

Figure 23. Conversion of reducing sugars by DNS. Source (58)

3.5. Study design

Experiments consisted of testing the effect of two variables on the release of fermentable sugars measured as reducing sugars when using the SSEH in the evaluated residues. In order to achieve that goal, a 2^k factorial design has been run in both residues, being k the number of factors. In this case, the factors or variables studied were the initial moisture content (MC_0) of the residues and the type of enzymatic complex used. Development of this design of experiments resulted in the execution of four experiments for each residue. Table 3 summarizes the levels used for each of the factors in the 2^2 design. Furthermore, to identify the point of maximum release of reducing sugars, the hydrolysis process was followed for 48 h, taking samples in predefined intervals to create a kinetic of the process.

Table 3. Summary of the study design

Residue	Factor	Low level	High level
GP	MC (%)	15	30
	Enzyme type	Self-produced *A. niger*	Viscozyme L
OMW	MC (%)	25	36
	Enzyme type	Self-produced *A. niger*	Viscozyme L

GP: grape-pomace; OMW: Olive-mill solid waste; MC: moisture content.

3.6. Statistical analysis

A one-way ANOVA and Tuckey's multiple comparison tests were performed using Minitab 18 with a p-value of 0.05. One-way ANOVA was performed to test the equality of the eight means, and the Tuckey's multiple comparison tests were run to identify differences among the evaluated groups. For this one-way ANOVA, reducing sugar release was used as the response variable, and the residue with specific moisture and specific enzyme were the factors.

4. Results

4.1. SSEH using Viscozyme L

Viscozyme L has been used as a reference for the process considering its proven ability to hydrolyze lignocellulosic materials of similar nature to those evaluated in this study. As it can be seen in Figure 24, by applying this enzymatic agent on the studied residues produced different results. In the first place, it was found that there were significant differences among the maximum reducing sugar levels obtained with OMW and GP after the SSEH. While in OMW it was obtained a low sugars level (approximately 3% of reducing sugars (dry basis)), in the case of GP the maximum level reached up to 16.8%.

Furthermore, these tests allowed to identify that, by using the high MC levels in each of the residues, higher reducing sugars content were obtained compared to the low MC levels. Although this difference was not such marked when using OMW, it resulted significant in the case of GP. In that scenario, such difference implied 20% more of reducing sugars content in the hydrolyzed residue.

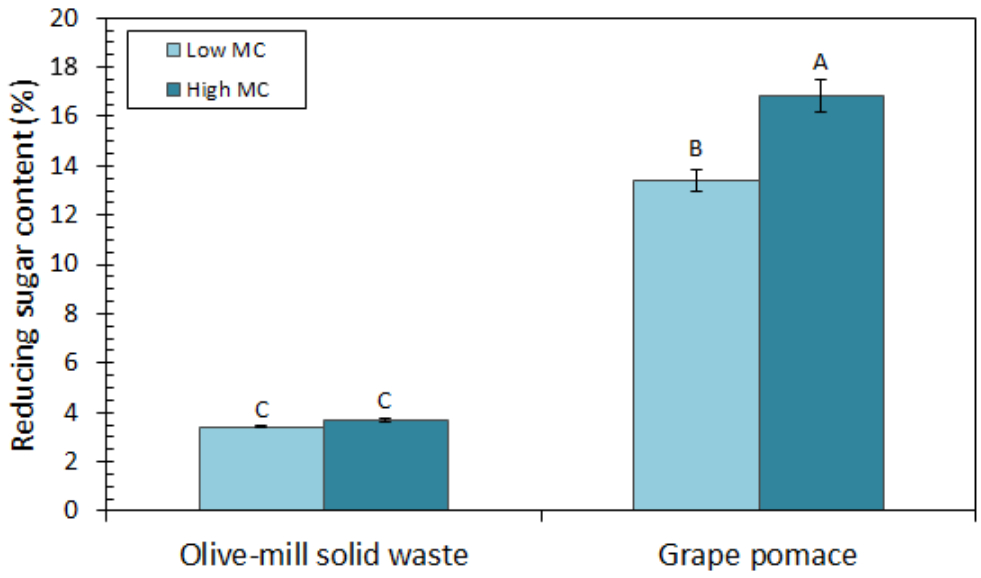

Figure 24. Final reducing sugars content of the evaluated residues using Viscozyme L. Results are expressed in % (g of reducing sugars/g of total solids). MC: Initial moisture content. Different capital letters mean significant differences among the evaluated groups according to the Tukey test ($p < 0.05$).

4.2. SSEH using the enzymatic extract from *A. niger*

Enzymatic extracts from *A. niger* were also tested to hydrolyze the lignocellulosic materials evaluated in this study. As Figure 25 shows, as occurred with the commercial extract, in this case, there were also significant differences between both residues. Thus, while in OMW it was obtained significant low levels (around 0.5% of reducing sugars (dry basis)), in the case of GP the maximum level reached up to 13%.

Moreover, it could be seen that MC had a different influence between both residues. Whereas in GP, higher reducing sugars content were obtained in low MC levels, in the case of OMW, the results were the opposite. It can also be seen that differences were not significant in OMW experiments, but for GP, they resulted in 20% more of reducing sugars content when using low MC.

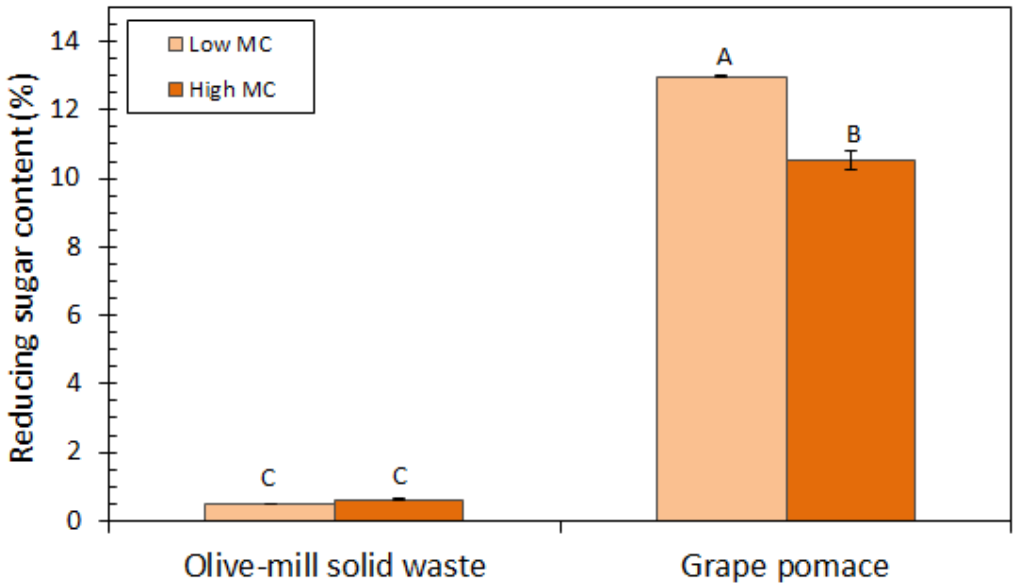

Figure 25. Final reducing sugars content of the evaluated residues using enzyme extract from *A. niger*. Results are expressed in % (g of reducing sugar/ g of total solids). MC: Initial moisture content. Different capital letters means significant differences among the evaluated groups according to the Tukey test ($p < 0.05$).

4.3. Efficiency of the evaluated enzymatic extracts

The results depicted in Figure 26 present the increase in reducing sugars after SSEH referred to the initial reducing sugars content of the residues. As seen, for OMW, it was found that there was a vast difference among the increase of reducing sugars when using Viscozyme L and enzyme extract from *A. niger*. Whereas with the

commercial extract it was gained an increase greater than 700%, using *A. niger* extract the maximum increase was just 50%.

Conversely, concerning GP, the obtained increases were not such remarked among the enzymatic extracts used. While with the utilization of self-produced enzyme, it reached 60%, in the case of Viscozyme L, more than 150% was obtained. However, there were no significant differences regarding GP 15%, since using both extracts it was obtained almost 100% of reducing sugars increase.

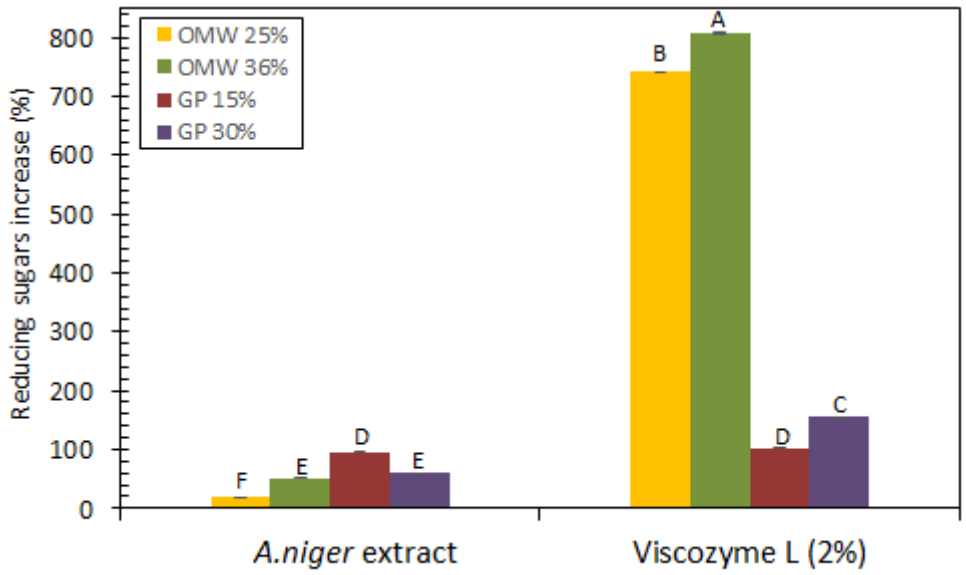

Figure 26. Increase in reducing sugars for the evaluated residues after the solid-state enzymatic hydrolysis with both enzymatic agents. Results are referred to the initial reducing sugars content of the residues. Different capital letters means significant differences among the evaluated groups according to the Tukey test ($p < 0.05$).

These results can also be seen from the point of view of the substrates yield. In that sense, it was found that for OMW these yields ranged between 0.08 gRS/gTS and 3.3 gRS/gTS using both enzymatic extracts. While the lower yields corresponded to the use of *A. niger* enzymatic extract (up to 0.21 gRS/gTS), the maximum yields were achieved by Viscozyme L.

On the other hand, for GP, the trend was significantly different. While the maximum yield was reached using the commercial extract at high MC (10.2 gRS/gTS), using the same MC level with *A. niger* enzymatic extract produced only 3.9 gRS/gTS. Despite these differences, it was also found that working at 15% significantly reduced the differences among enzymatic extracts, reaching 6.8 gRS/gTS and 6.4 gRS/gTS with Viscozyme L and the self-produced extract, respectively.

4.4. Kinetics of hydrolysis for the evaluated residues using Viscozyme L

Results compiled in sections 4.1 and 4.2 correspond to the maximum levels found during the monitoring of the SSEH. However, in order to obtain such data, it was needed to perform a kinetic study for each of the experiments. In such a way, it could be assured that the point of maximum reducing sugar content would be reported.

Thus, Figure 27 shows the time course of the SSEH of the residues when using Viscozyme L. As observed, in both tested residues there was an asymptotic tendency independently of the MC used, but with marked differences regarding the reducing sugars levels achieved. It can be seen that, in the SSEH of OMW, the maximum reducing sugars levels were reached after 7 h of hydrolysis (3.1%), and those levels remained almost constant up to the end of the monitoring. The trend also shows that for this residue, no significant changes occurred due to the low and high MC levels.

On the other hand, Figure 27 also shows that the SSEH of GP proceeded without significant differences during the first 7 h for both MC levels. However, at that point, the trend has changed and it was found that working at 30% MC resulted in higher reducing sugars contents. As observed, in that scenario, the maximum levels were reached after 18 h of hydrolysis (16.8%), followed by a consistent decrease that resulted in an average reducing sugar content of 14.5%. In the other scenario (GP MC 15%), the maximum level was obtained after 32 h of hydrolysis (13.4%). Then, as occurred at high MC, a decrease left the final reducing sugar content in 11.1%.

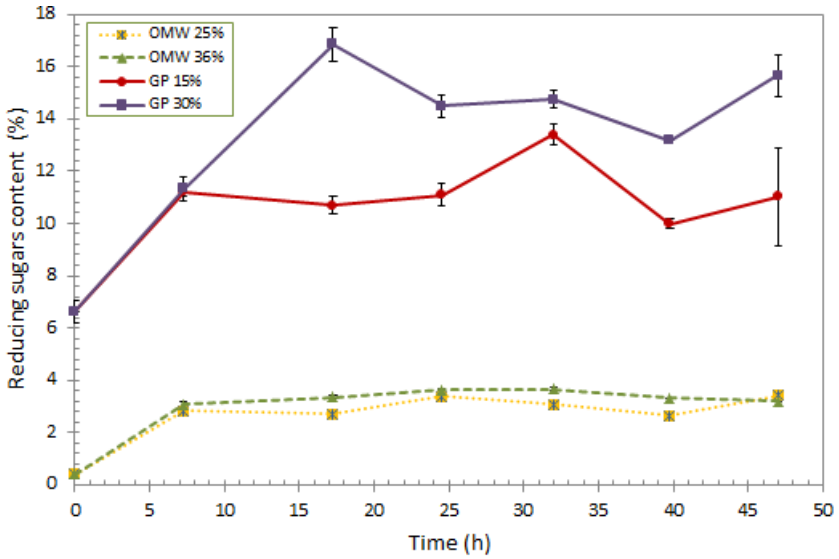

Figure 27. Time course of the solid-state enzymatic hydrolysis for the evaluated residues using Viscozyme L.

OMW: Olive-mill solid waste; GP: Grape pomace.

4.5. Kinetics of hydrolysis for the evaluated residues using enzyme extract from *A. niger*

In Figure 28, it is exposed the time course of the SSEH of the evaluated residues when using *A. niger* extract. It can be seen that there was an asymptotic tendency for GP while in OMW, the sugar content remained almost constant during all the process. While in OMW, MC had no influence in kinetics neither reducing sugar release, in the case of GP, it produced a remarkable effect. As it is shown, in the SSEH of OMW, the reducing sugars levels were around 0.04% all the time, demonstrating that no changes occurred if MC varied.

Also, as observed in Figure 28, SSEH of GP did not present relevant variations during the first 25 h independently of the MC level. From that point, the trend has changed and it was found that working at 15% MC resulted in higher reducing sugars contents. In that situation, the maximum reducing sugar levels were achieved after 32 h of hydrolysis (13%) proceeding by a steady decrease until 10.7% after 48 h.

On the other hand, GP with 30% MC obtained a first peak around after 15 h (9.4%) followed by a decrease until 32 h (8.2%), the moment where a second increase allows the SSEH to reach the maximum levels close to 48 h by reaching up to 10.5%.

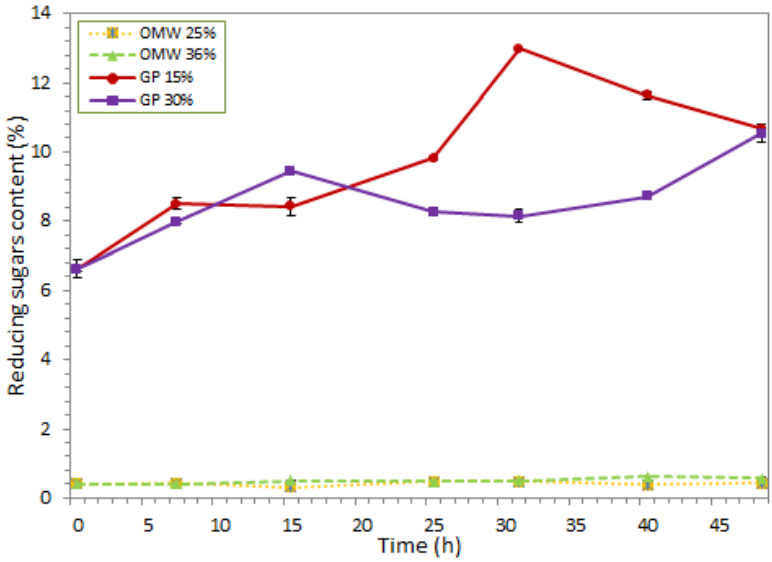

Figure 28. Time course of the solid-state enzymatic hydrolysis for the evaluated residues using enzyme extract from *A. niger*. OMW: Olive-mill solid waste; GP: Grape pomace.

5. Discussion

The primary purpose in a SSEH study is to found the best conditions of the process to achieve the optimization of fermentable sugar content to be consumed by a microorganism to produce value-added products such as bioplastics throw fermentation. Throughout this work, two lignocellulosic wastes have been investigated as a potential low-cost source using an economical and eco-friendly SSEH.

As it was stated in section 4, referring to the kind of lignocellulosic material, it was observed that the SSEH of GP was much better in all the evaluated conditions in comparison to OMW. Although OMW contains a higher polysaccharide fraction as cellulose and hemicellulose (Table 2) that is prone to be converted into fermentable sugars by the action of lignocellulolytic enzymes, its lignin content can also be considered very high (Table 2). In this sense, OMW could be negatively affected by this factor, limiting the efficiency of the used enzymes. Even though, by using Viscozyme L resulted significantly better than the self-produced enzymatic extract for hydrolyzing this material. However, the reducing sugars increase still limited compare to the levels achieved for the other residue.

On the other hand, the characterization of the residues (Table 2) also suggests that GP could be less prone to the negative effect of lignin. It can be seen that such content is close to 17%, almost three times lower than the content in OMW. Moreover, GP could be considered a material with higher porosity than OMW, which could help in the accessibility of enzymes, improving enzymatic degradation (7).

Although the negative effect of lignin could have been overcome by using a pretreatment stage, in this study, it was not considered based on the recent results of a study performed in the BETA group using the same residues. In the study of Corchado et al. (59), these residues have been tested in liquid culture as a source for PHA production. After comparing the effect in the hydrolysis step of diluted-acid pretreatment respect to the process without pretreatment, it was found that despite the higher increase in the sugar concentration, the pretreatment induced significant amounts of inhibitor compounds. These compounds, in turn, resulted deleterious for the fermentation stage, limiting the benefits of the hydrolysis step. Thus, in this study, it was expected an equivalent behavior.

Concerning the extract enzyme, the utilization of the commercial one resulted more effective in general terms (Figure 26), so it suggests that these enzymes were able to solubilize the polysaccharide fractions better as expected. It is important to take into account that Viscozyme L is an enzymatic cocktail where all enzymes contained were obtained from different microorganisms according to the maximum yield and obtained by a carefully optimized process. On the other hand, the enzyme mix resulting of the

SSF from a single microorganism (*A. niger*) was a basic extract that requires a refined process since the enzyme activity decreases through hydrolysis in a more pronounced way than the commercial extract (data not shown). In this sense, it could be stated that such differences in performance were expected due to the nature of each extract; consequently, the obtained results.

Nevertheless, it has to be considered that despite the good results using the commercial extract, by using a self-produced enzyme could induce a significant effect on the economy of the process. Such condition can be clearly seen analyzing that the price of concentrated Viscozyme L is beyond 1 euro per milliliter. On the contrary, producing the extract from *A. niger* could cost up to 50% less in optimized conditions (53). Besides, it should be considered that both the production and extraction of the self-produced enzymes are still prone to be optimized. Such a scenario would let to better results regarding the hydrolysis efficiency and the economy of the process.

Furthermore, in this study, it has been demonstrated that the self-produced extract from *A. niger* resulted as effective as the commercial enzyme in the GP 15% MC (Figure 26). In that case, achieving better hydrolysis kinetics (Figure 28) since the hydrolysis with commercial enzyme was more evident in the first hours while the other was increasing as the time drew on, may be caused by a less inhibitory effect. This supports the idea that SSEH is a promising valorization strategy (33).

Talking about the effect of MC on the SSEH performance, it is clear that there was no clear tendency in neither the residues nor the enzymatic extracts. In this sense, authors such as Gawande et al. (31) indicate that the initial moisture level at which free moisture occurs varies considerably between substrates, depending on their water-binding characteristics. Besides, the water presented in the SSEH system exists either in a complex form within the solid matrix, as a thin layer adsorbed to the surface of the particles or less tightly bound within the capillary region of the solid. Based on that context, Felipe et al. (30) demonstrated that the moisture content did not influence the amount of fermentable sugars obtained from sugarcane bagasse in the case of no pre-treatment application, attributing a deficit of mass-transfer by the low solvent and enzyme mobility. A similar effect could explain the non-affectation of OMW (Figure 24, Figure 25) since water could not properly bond to the substrate, independently of MC. It has to be remained that OMW is a non-porous material, and this condition reflects a drawback when using such residue in solid-state applications.

On the other hand, the same study (30) but using an ultrasound-assisted system confirms that the highest moisture content (95%) led to the lowest efficiency of hydrolysis, the highest efficiency was obtained using 75% of moisture content. In contrast, at 55%, the hydrolysis efficiency presented intermediary results. The best result was justified as being an equilibrium between enzyme stability in the presence of ultrasound irradiation (better absorption) and mass-transfer effects, which can be

equivalent applied in the kinetics of GP 15% using self-produced enzyme extract (Figure 28) compared to 30%. Another study run with drying rice (29) demonstrated that higher moisture contents could cause significant changes in particle size, density, and surface characteristics, and these, in turn, could significantly affect the degradation of the solid so that it could explain the case of GP using Viscozyme L (Figure 24).

The different results obtained with the enzymatic extracts regarding the MC effect could be also be understood considering the fact that *A. niger* works better in low MC contents probably because this extract contains some traces of the microorganism formed in SSF (*A. niger*), that in the SSEH process may have grown in the solid media. Such contamination could be consuming part of the reducing sugars contained in the solid media, and therefore, it could be limiting the extent of the hydrolysis process. Thus, when the MC is low, the ability of such traces to growth is limited (since there is a limitation of the water required to perform its metabolisms) (60), and the reducing sugars content is less affected by the contaminants. These results suggest that a suitable strategy should be implemented when using this kind of extracts, looking for an equilibrium between technical and economic aspects.

6. Conclusions

- Grape pomace is a potential substrate for SSEH since it contains an important part of polysaccharide fraction and a considerable fermentable sugars concentration can be obtained. So, it has the potential to be applied in the fermentation process to produce valuable products.
- Olive-mill solid waste did not show evidence to be a suitable substrate for SSEH. However, their delignification should be further studied since it also contains a high content of cellulose and hemicellulose that could be profitable.
- The commercial enzyme was more effective in almost all the evaluated scenarios for hydrolyzing the residues.
- A low initial moisture content (15%) in grape pomace can improve sugar yield using self-produced enzymes from *Aspergillus niger*. In comparison, a high moisture content (30%) increases the sugar releasement in case of using Viscozyme L.
- Olive-mill solid waste was not affected by moisture content in those conditions.
- The maximum sugar release was near 17% of reducing sugars (dry basis) obtained in the grape pomace 30% MC hydrolysis using Viscozyme L.

- A self-produced enzyme from *Aspergillus niger* has the potential to be used in SSEH to obtain a considerable concentration of fermentable sugars for a subsequent biofuels production through fermentation.

7. Future work

- Once the best results for SSEH are found, the next step would be to prove the hydrolyzed residue as a substrate for the SSF for producing PHA. It would be essential to prove that the microorganism is suitable for that MC since fungus normally work better at high MC but in bacteria is the opposite (61).
- The SSEH process could be further studied with GP but including other relevant factor affecting the process to identify potential improvements. For instance, the addition of nutrient solutions (nitrogen source and additional mineral salts) to promote the enzyme activity (62).
- The extraction process of the self-produced enzyme could be improved to enhance its performance in SSEH.
- It could be studied the effect of a suitable pretreatment on the tested residues, to identify if this induces significant improvements on the SSEH yields.

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