Lignocellulolytic enzymes production via Solid-State Fermentation of agroindustrial residues: process optimization and application

PhD Thesis

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Que el biotecnòleg i màster en enginyeria biològica i ambiental Jordi Llimós Turet ha realitzat sota la nostra direcció el treball amb títol " Lignocellulolytic enzymes production via Solid-State Fermentation of agroindustrial residues: process optimization and application", que es presenta en aquesta memòria i que constitueix la seva tesi per optar al Grau de Doctor per la Universitat de Vic-Universitat Central de Catalunya.

I perquè en prengueu coneixement i consti els efectes oportuns, es presenta a l'Escola de Doctorat de la Universitat de Vic-Universitat Central de Catalunya l'esmentada tesi, signant el present certificat.

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Overview of the thesis

This thesis is focused on the valorisation of agro-industrial waste through the use of solid-state fermentation (SSF) for enzyme production. This study was developed as part of the project *Valorización de residuos agroindustriales para la producción de Bioplásticos* (VALORA, CTM2016-81038-R) and it is the first study on SSF developed within the BETA Tech Center. The study has focused on the use of three different wastes from the food industry (brewer's spent grain, grape pomace and olive-mill solid waste) as potential substrates for the production of lignocellulolytic enzymes. These lignocellulosic-derived wastes are produced in large quantities in important local industries within Catalonia region, and their management still generates some environmental problems. However, these residues have properties that make them interesting as a source of lignocellulolytic enzymes, and therefore they can be potentially valorised by obtaining a high value-added product. In order to achieve this goal, three fungal strains have been evaluated as enzyme producers: *Aspergillus niger, Thermoascus aurantiacus* and *Trichoderma reesei.*

Chapter 4 sets out the selection of the substrate for enzyme production within the three selected residues. After the substrate was selected, a preliminary study was performed to determine the potential for producing lignocellulolytic enzymes from the three abovementioned fungal strains. In order to evaluate such a potential, the enzymatic activities served as the main performance parameter. Nevertheless, the efficiency of the obtained extracts to release sugars was also tested. Finally, a proof of concept was carried out, in line with the objective of the VALORA project to produce biodegradable bioplastics (polyhydroxyalkanoates) starting from the fermentable sugars produced with the enzymes. In a second stage, it was decided to continue working with two of the evaluated strains (*A. niger* and *T. aurantiacus*) to optimise the enzymatic production process at lab scale (Chapter 5). The *A. niger* SSF performed at the optimal conditions

found a maximum xylanase activity value of 309.0 ± 24.5 $U \cdot g^{-1}DM$ at 42 h and a maximum cellulase activity value of 7.9 ± 1.0 $FPU \cdot g^{-1}DM$ at 24 h. The *T. aurantiacus* SSF performed at the optimal conditions found a maximum xylanase activity value of 156.1 ± 15.8 $U \cdot g^{-1}DM$ at 48 h and a maximum cellulase activity value of 3.5 ± 0.5 $FPU \cdot g^{-1}DM$ at 48 h.

Once the process was optimised, a change of scale was made in order to study the process at bench scale and to evaluate different reactor configurations (Chapter 6). *The best results were obtained using A. niger with the PVC reactors with a maximum xylanase activity (245.5 ± 21.6 $U \cdot g^{-1}DM$, 5.1* ± 0.5 $U \cdot g^{-1}DM \cdot h^{-1}$ of productivity) at 48 h and a maximum cellulase activity (4.5 ± 0.2 $FPU \cdot g^{-1}DM$, 0.06 ± 0.00 $FPU \cdot g^{-1}DM \cdot h^{-1}$ of productivity) at 80 h.

Finally, the most promising extracts obtained during the study were subjected to different tests to assess their efficiency in the hydrolysis of diverse materials. In that step, it was also included the solid fraction of steam exploded materials as attempt to evaluate the enzymatic extracts in a wider range of materials (Chapter 7) and these were hydrolysed both in liquid and solid media.

In general, the results presented in this thesis constitute a first approach toward the use of SSF and pure fungal strains as a valid technology for the production of enzymes of industrial interest using low-cost raw materials.

Resum

Aquesta tesis esta enfocada en la valorització de residus agroindustrials mitjançant la utilització de la fermentació en estat sòlid per a la producció enzimàtica. Aquest estudi es troba emmarcat dins el projecte *Valorització de residus agroindustrials per a la producció de bioplàstics* (VALORA, CTM2016-81038-R). Per a la producció enzimàtica, es van estudiar tres residus diferents provinents de la indústria alimentària: el bagàs de cervesa, la brisa de raïm i la pinyolada. Tots aquest són residus lignocel·lulòsics es produeixen en grans quantitats en indústries properes al centre d'investigació i la seva gestió pot comportar problemes mediambientals. Tot i això, aquests residus tenen unes propietats que els fan ser interessants com a font de enzims lignocel·lulòtics, i per tant, poden ser valoritzats obtenint un producte d'alt valor afegit. Per tal d'aconseguir aquest objectiu s'han avaluat 3 soques diferents de fongs com a productors enzimàtics: *Aspergillus niger, Thermoascus aurantiacus* and *Trichoderma reesei.*

En el primer capítol de resultats (Capítol 4) s'exposa la selecció del substrat per a la producció enzimàtica dins els tres residus estudiats. Un cop seleccionat el substrat, es va realitzar un estudi per avaluar el potencial productor d'enzims lignocel·lulòtics de les tres soques de fongs esmentades mitjançant la fermentació en estat sòlid a escala laboratori. Per tal d'avaluar el potencial de cada soca es va analitzar les activitats enzimàtiques obtingudes, però també l'aplicabilitat dels extractes enzimàtics obtingut per obtenir sucres fermentables de residus lignocel·lulòsics. Finalment, es realitza una prova de concepte, en consonància amb l'objectiu del projecte VALORA per tal d'obtenir bioplàstics a partir dels sucres fermentables obtinguts. Un cop obtinguts i avaluats els resultats de les diferents soques de fongs, es va decidir seguir treballant amb dues d'elles (*A. niger* i *T. aurantiacus*) i optimitzar el procés de producció enzimàtica mitjançant la fermentació en estat sòlid (Capítol 5). La SSF d'*A. niger* realitzada en les condicions

òptimes va trobar un valor màxim d'activitat xilanasa de 309,0 ± 24,5 U · g^{-1}DM a les 42 h i un valor màxim d'activitat cel·lulasa de 7,9 ± 1,0 FPU · g-1DM a les 24 h. La SSF de *T. aurantiacus* realitzada en les condicions òptimes va trobar un valor màxim d'activitat xilanasa de 156,1 ± 15,8 U · g^{-1}DM a les 48 h i un valor màxim d'activitat cel·lulasa de 3,5 ± 0,5 FPU · g^{-1}DM a les 48 h.

Un cop optimitzat el procés, es va fer un canvi d'escala per tal d'estudiar el procés a escala pilot i avaluar diferents configuracions del reactor (Capítol 6). Els millors resultats es van obtenir utilitzant *A. niger* amb els reactors de PVC amb una activitat xilanasa màxima (245,5 ± 21,6 U · g^{-1}DM, 5,1 ± 0,5 U · g^{-1}DM · h^{-1} de productivitat) a 48 h i una cel·lulasa màxima activitat (4,5 ± 0,2 FPU · g^{-1}DM, 0,06 ± 0,00 FPU · g^{-1}DM · h^{-1} de productivitat) a 80 h.

Finalment, els extractes més prometedors obtinguts durant l'estudi van ser sotmesos a diferents proves per avaluar-ne l'eficiència en la hidròlisi de diversos materials. En aquest pas, també es va incloure la fracció sòlida dels materials explotats amb vapor com un intent d'avaluar els extractes enzimàtics en una gamma més àmplia de materials (Capítol 7) i aquests es van hidrolitzar tant en medi líquid com sòlid.

En general, els resultats presentats en aquesta tesi constitueixen una primera aproximació cap a l'ús de soques de fongs purs i SSF com a tecnologia vàlida per a la producció d'enzims d'interès industrial utilitzant matèries primeres de baix cost.

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CHAPTER 1. INTRODUCTION

1.1 Agro-industrial solid waste: feedstock for a circular economy

In the last years, the production and the consequent need for safe disposal of agro-industrial solid waste have become a concern due to the large number of tons generated. Nowadays, 147.2 million tons of agro-industrial solid waste are produced worldwide (Sadh et al., 2018). Typically, these solid wastes are disposed of in landfills, incinerated, or, in the best scenario, destined for animal feed or composting. The cost for the management of such wastes is significant, and it is mainly derived from the transportation of the waste to the landfills, the energy consumed in the incineration, the treatment of the solid waste, and in some cases, their conditioning to be suitable for animal feed (Sadh et al., 2018). In addition, some of the management strategies required for the proper treatment of such wastes entail major environmental issues that should be solved to develop more sustainable management of these waste (De Bhowmick et al., 2018).

Accordingly, to attain a more sustainable future it is necessary to not only solve these environmental issues but also implement a circular economy perspective through the valorisation of this solid waste. Therefore, a sustainable management of the waste is achieved as a product of interest is obtained, and consequently, obtaining an economic profit from waste. National efforts have been done in this line, for instance, the Bio-based industry consortium (BIC) is a European group of industrial companies, universities, research groups and technology organisations that are guided by the Strategic Innovation and Research Agenda (SIRA) (The Bio-based Industries Consortium, 2021). The main goals of the BIC are to promote bio-based products and to run towards a European sustainable circular bioeconomy. This vision searches for the valorisation of the domestic renewable raw materials, minimise the environmental impact of the production of food, bio-based products and energy, and promote sustainability through efficient use of feedstock with zero-waste objectives. To do so, the biorefinery concept arises as a tool to

integrate valorisation processes converting biomass into attractive bio-products and energy (Arevalo-Gallegos et al., 2017). In Spain political efforts have resulted in the current Spanish circular economy strategy (EEEC) and the 2012 bioeconomy strategy, aligned with the European legislation towards a sustainable EU economy (European Green Deal). Particularly the paragraph 2.1.6 "From farm to fork: designing a fair, healthy and environmentally-friendly food system", and consistent with the sustainable development goal (SDG) 12 "Responsible consumption and production" of the UN's 2030 Agenda.

Under this context, this research has been developed as part of VALORA project (*Valorización de residuos agroindustriales para la producción de bioplásticos*), financed and supported by the Spanish Ministerio de Economía, Industria y Competitividad. VALORA project aimed to evaluate the production of biodegradable bioplastics from local solid agro-industrial wastes integrating environmental and economically feasible technologies from a circular economy perspective.

1.1.1 Lignocellulosic wastes

Among industrial wastes, lignocellulosic wastes are a renewable resource obtained as a by-product in different industries, with a great potential to produce bioproducts and bioenergy. Lignocellulosic wastes are abundant carbon sources that are produced in many agroindustrial processes, reaching up to 10^{11} tons of biomass leftovers per year (De Bhowmick et al., 2018). These wastes comprise an extensive range of solids, from agro-industrial and forestry residues or subproducts to domestic solid wastes. Although the handling and disposal of these materials have been considered a problem for decades, their characteristics make them potential raw materials for the development of different applications. In general, lignocellulosic materials are attractive due to their renewable

nature. Furthermore, they are a widely distributed feedstock because of their worldwide production in large quantities, which make them a potential low-cost material. In fact, they have gathered most of the recent advances in the biorefinery sector for producing value-added products (Arevalo-Gallegos et al., 2017; Bilal et al., 2017).

Lignocellulosic waste consists of three different fibres: cellulose, hemicellulose and lignin. These fibres contribute to the structure of the plant and the resistance of the solid to mechanical forces. In order to process lignocellulosic materials, a hydrolysis step is often required to transform the cellulose and hemicellulose fractions entrapped into the lignin matrix into fermentable sugars (Arevalo-Gallegos et al., 2017). However, these fermentable sugars are not always accessible by enzymes and a pre-treatment is usually applied before the hydrolysis step (Malherbe and Cloete, 2002; Ravindran et al., 2018).

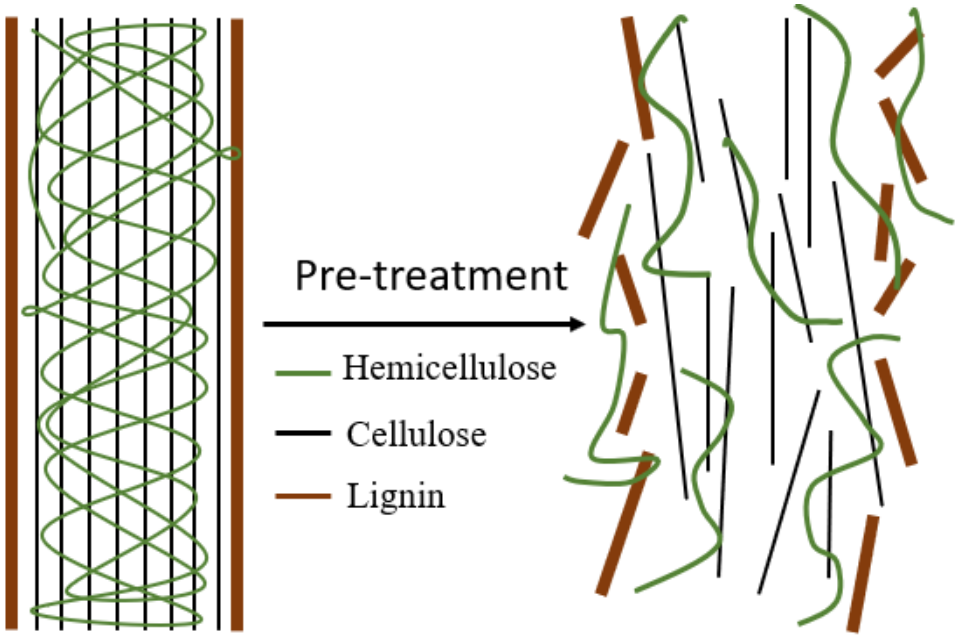

Figure 1.1 Fibres structure of the lignocellulosic materials and action of the pre-treatment on the structure.

Although hydrolysis step can be conducted using chemical hydrolysis (Binder and Raines, 2010), the current trend is to use enzymatic hydrolysis employing hydrolytic enzymes (H. K. Sharma et al., 2019), like cellulases and xylanases (Uçkun Kiran et al., 2014; Verma and Kumar, 2020). In general, enzymes act as catalysts, but in contrast to

their chemical counterparts, enzymatic processes work under milder conditions making them more attractive from techno-economical view (Singh et al., 2016).

- **Cellulose**

Cellulose ($C_6H_{10}O_5$) is a polymeric chain of D-glucose molecules with β-1,4-glycosidic bonds. It is the major component in lignocellulose (35-50%) and constitutes the skeleton of the cell wall of the plants. Also, it is the most abundant organic polymer on Earth. The cellulose fibres comprise 7.000-15.000 glucose molecules (Klemm et al., 2005).

- **Hemicellulose**

Hemicellulose is a polysaccharide chain composed of different sugars monomers, pentoses and hexoses, and uronic acids connected to the chain. It is the second most abundant organic polymer on Earth. The classification of hemicellulose depends on the type of constituent monomer: xylan, glucoxylan, arabinoxylan, glucuronoxylan, among others. It hinders the accessibility to the cellulose to allow the biotransformation of both fibres to products of interest. The hemicellulose chains are shorter than the cellulose, between 300-5.000 sugar molecules (Scheller and Ulvskov, 2010).

- **Lignin**

Lignin is an organic polymer made by cross-linking phenolic precursors. It forms the structural part of the tissues of most plants, and its main purpose is to provide strength and protection from pathogens. This cause that lignin does not allow the action of the microorganism and consequently the action of the produced enzymes on cellulose and hemicellulose, interfering with enzymatic hydrolysis of the lignocellulosic materials. (Vanholme et al., 2010)

1.1.2 Lignocellulosic waste from the food industry

The food industry is one of the primary producers of lignocellulosic waste materials, generating different kinds of lignocellulosic-derived materials such as seed, peels, husks, and other residues obtained during the processing of raw materials. These subproducts are generally produced in large quantities, but in some cases, they are produced only once or twice a year due to the seasonal behaviour of the production (Arevalo-Gallegos et al., 2017). This is essential information to know before selecting the lignocellulosic substrate. In this sense, a multi-feedstock biorefinery is an interesting concept to redesign the biorefineries to the seasonal production. Some of the commonly produced lignocellulosic wastes from the food industry that were available nearby are presented below.

- **Brewer's spent grain**

The brewery industry is one of the most productive industries of beverages in the world (Aliyu and Bala, 2011), and it generates large quantities of brewer's spent grain (BSG), a lignocellulosic material that is commonly considered a leftover and is used as animal feed (Ibarruri et al., 2019). BSG has significant amounts of protein and fibres (Mussatto et al., 2006) that can be used as carbon and nitrogen source for different microorganisms to produce value-added products. Although BSG has been previously used to obtain different hydrolytic enzymes (de Castro and Sato, 2015), just a few reports show BSG as raw material to obtain fermentable sugars (Leite et al., 2019; Outeiriño et al., 2019; Paz et al., 2019). Furthermore, many of the reports just focus on the enzyme production, but not on their applicability to obtain other added-value products, which opens an opportunity to study the complete process from raw material to final products, covering the entire production chain and using the full potential of the residue (Xiros and Christakopoulos, 2012).

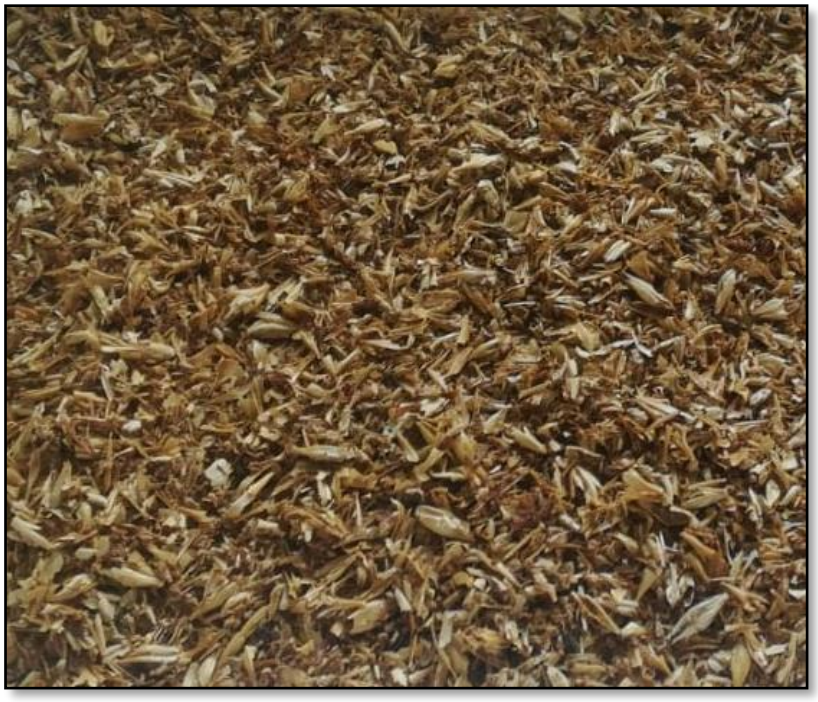

Figure 1.2 Brewer's spent grain appearance

- **Grape pomace**

The grape pomace is the solid that remains of grapes after the production of wine, the peel of the grape, seeds and the stem. It constitutes from 20% to 25% of the weight of the grape. The peel and the seed are rich in anthocyanins and phenolic antioxidants, respectively. Grape pomace also contains lipids, proteins, minerals and fibres. Grape pomace has an important potential to be a source of high nutritional food, antioxidants gluconic acid and enzymes (Dulf et al., 2020; Papadaki et al., 2020; Singh and Singh, 2006; Teles et al., 2019; Yu and Ahmedna, 2013).

Figure 1.3 Grape pomace appearance

- **Olive mill solid waste**

The olive mill solid waste is the waste generated from olive oil production. It generates environmental issues due to their large-scale production and in a short period of time during the year. Furthermore, Spain is the first producer worldwide, followed by Greece and Italy. This material contains a large amount of organic matter and different nutrients that could be used to produce value-added products (Abu Tayeh et al., 2020). The valorisation of this waste has been studied, and different technologies have been proposed, such as solid-state fermentation (SSF), anaerobic digestion or composting (Roig et al., 2006). Olive mill solid waste has an important potential to be a source of antioxidants, enzymes and ethanol (Abu Tayeh et al., 2020; Filipe et al., 2020; Zaier et al., 2021).

Figure 1.4 Olive mill solid waste appearance

1.2 Solid-state fermentation

Solid-state fermentation (SSF) is a promising technology commonly used to valorise lignocellulosic materials due to its economic and sustainable potential and the versatility to process an extensive range of waste materials to produce a vast set of bioproducts (Sadh et al., 2018). SSF is defined as a fermentation process conducted in the absence or near absence of free water, with natural or inert support as a solid material (Thomas et al., 2013). SSF has been known for long ago since the bread-making by Egyptians (2600 B.C) and the cheese making and the koji process in Asia (B.C) (Rodríguez Couto and Sanromán, 2005). During the 18th century, SSF processes became more relevant, and from 1900 onwards, the production of enzymes by SSF started to be a trend. However, the submerged fermentation (SmF) processes had become a model technology to produce compounds through fermentation due to the development of this technology to produce penicillin. Consequently, more profound knowledge of the technology has been acquired (Rodríguez Couto and Sanromán, 2005).

Among the main traits defining SSF, it can be mentioned the selection of the solid support for the process. Its selection tends to be a critical aspect determining the success of the process. Different factors are important when selecting the support for a SSF process, such as porosity, size, chemical composition, cost and availability (Ooijkaas et al., 2000). The SSF solid support can be divided into two types: inert supports and non-inert supports. Inert supports only act as an attachment for the microorganism. Otherwise, non-inert support also acts as a supply of nutrients. Non-inert supports are usually named as substrates and normally are lignocellulosic materials, a fact that helps to solve the economic and environmental problems related to the disposal of this kind of materials (Ooijkaas et al., 2000; Webb, 2017). The substrate composition is also important as it will determine the bioproduct produced, depending on the fibres composition or substances that it contains. Non-inert supports are more commonly used than inert supports, but working with inert supports helps in the product recovery, and the purification step tend to be more accessible than with non-inert supports. However, media and production costs are higher with non-inert supports (Rodríguez Couto and Sanromán, 2005).

Nevertheless, some substrates normally need a bulking agent to give structure and porosity to the solid matrix. In this sense, at high solid loads can also avoid compaction problems, to ensure a correct aerobic environment and allowing the correct fungal growth. The commonly bulking agent used are straw, sawdust or polyurethane foam (Jiménez-Peñalver et al., 2018; Kumar et al., 2021; Martínez-Avila et al., 2021b; Rodríguez et al., 2021).

1.2.1 Challenges for a commercially competitive SSF

Despite their advantages, it could be stated that SSF is not a consolidated technology, and most studies are laboratory-scale where a small amount of substrate is used, limiting its application at an industrial scale. Currently, the scale-up of the SSF process still faces

some challenges, such as problems related to mass and heat transfer when handling large amounts of solid waste and difficulties in avoiding biological contamination. Also, the lack of homogeneity in the majority of the solid matrix of the SSF difficult the monitorization of the different parameters during the fermentation and, therefore, the use of control systems (Bellon-Maurel et al., 2003; Sturm et al., 2008; Webb, 2017).

One of the main difficulties in the scale-up of the SSF and their applications at a commercial scale is the limited knowledge related to the design and operation of large-scale reactors. The bioreactor constitutes the environment where the biological reaction occurs, so the parameters as pH, humidity, temperature and aeration, among others, play an important role in the fermentation and must be taken into account for each different fermentation process (Singhania et al., 2009).

Different reactor configurations have been proposed to perform SSF processes. Most of the common configurations include packed-bed reactor, tray-type reactor or rotating drum reactor (Webb, 2017).

A packed-bed reactor consists of a column, normally made by plastic, steel or glass, filled with the substrate and/or the support and the specific microorganism. The reactor has a forced aeration with continuous humidified air supply, that improve the moisture gradients and the temperature control inside the reactor. Also, the solid bed could be mixed periodically to avoid compaction problems (Wang and Yang, 2007; Webb, 2017). Figure 1.5 shows a general schematic packed-bed bioreactor.

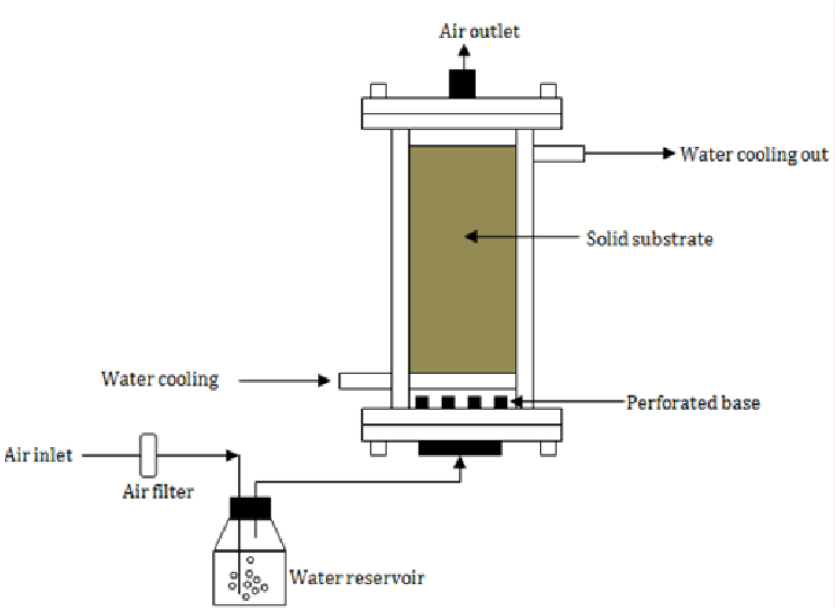

Figure 1.5 Scheme of a packed-bed bioreactor. Figure from: (Webb, 2017)

The tray-type bioreactor has been used widely in traditional SSF. Their simple design consists of a serial of trays, with the substrate and the microorganism placed inside, in a thick layer of a few centimetres. This type of reactor normally uses passive aeration and in some cases the substrate has to be mixed to enhance nutrient distribution and oxygenate the solid bed. The substrate has to be added in the trays in thin layers to avoid the overheating and to maintain the aerobic conditions (Leite et al., 2021; Webb, 2017). Figure 1.6 shows a general schematic tray-type bioreactor.

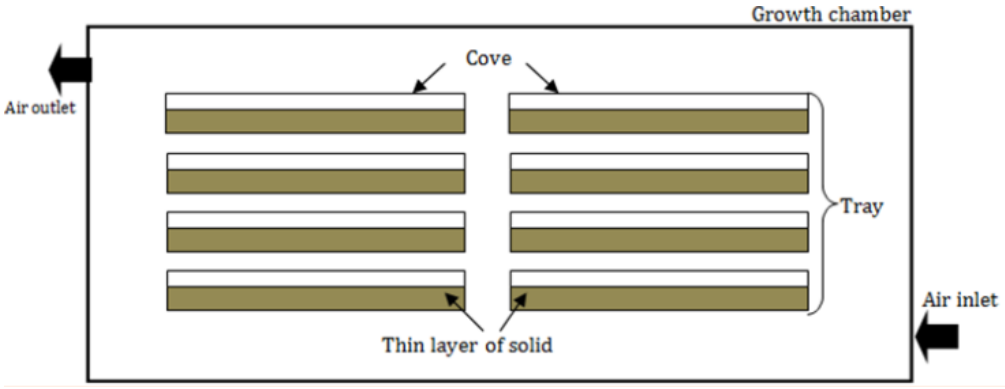

Figure 1.6 Scheme of a tray-type bioreactor. Figure from: (Webb, 2017)

The rotating drum bioreactor is formed with a cylinder with the support and the microorganism placed inside. The drum is semi-filled with the substrate and it has a slow rotation that mix the solid bed, favouring nutrient distribution and improving heterogeneity. This rotation also improves oxygen transfer because the contact with the air of the upper part of the reactor is enhanced (Rodríguez Couto and Sanromán, 2005; Webb, 2017). Figure 1.7 shows a general schematic rotating drum bioreactor.

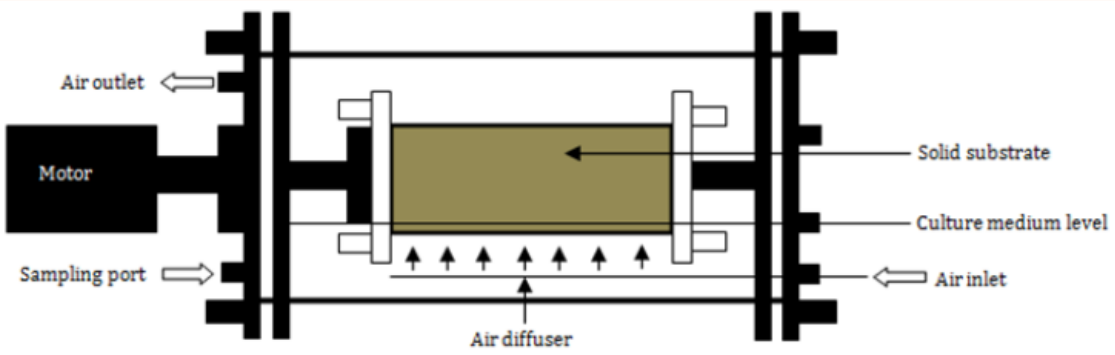

Figure 1.7 Scheme of a rotating drum bioreactor. Figure from: (Webb, 2017)

1.2.2 Products obtained through SSF

SSF has led to a new way of bioconversion of organic solid wastes into a wide variety of biomolecules. This has been possible thanks to the versatility of the technology using different types of microorganisms and a wide range of solid wastes with different components and properties. In this sense, the variety of bioproducts that could be obtained through the bioconversion of these organic solid wastes takes a wide range. Furthermore, the valorisation of these wastes into bioproducts generates a benefit (Yazid et al., 2017).

Table 1.1 shows different examples of value-added products obtained through SSF. As it can be seen, there is a large variety of microorganisms and substrates used to obtain different types of products. Although one of the most studied products obtained via SSF are enzymes, SSF has been successfully used in combination with solid organic wastes to produce other value-added products such as biosurfactants (Jiménez-Peñalver et al.,

2016), base chemicals (Martínez-Avila et al., 2019), antioxidants (Leite et al., 2019) or biopesticides (Sala et al., 2020), showing that this is a versatile and fruitful technology.

Table 1.1 Products obtained through SSF with different microorganisms and substrates

Microorganism	Substrate	Product	Reference
Aspergillus fumigatus	Oil palm trunk	Cellulase and xylanase	Ang et al., 2013
Native microbial populations	Soy fibre and coffee husk	Alkaline protease	Abraham et al., 2013
Starmerella bombicola	Winterization oil cake	Biosurfactants	Jiménez-Peñalver et al., 2016
Thermoascus aurantiacus	Wheat bran and soy bran	Amylases	De Oliveira et al., 2016
Aspergillus oryzae and A. japonicas	Oil cake	Lipases	Jain and Naik, 2018
Lichtheimia ramosa	Sugarcane bagasse	Cellulases and hemicellulases	Garcia et al., 2018
Kluyveromyces marxianus	Sugarcane bagasse	Aroma compounds	Martínez-Avila et al., 2019
Aspergillus niger and A. ibericus	Brewer's spent grain	Antioxidants	Leite et al., 2019
Beauveria bassiana and Trichoderma harzianum	Rice husk	Biopesticides	Sala et al., 2020
Bacillus thuringiensis	Digestate	Biopesticide	Mejias et al., 2020
Burkholderia cepacia	Brewer's spent grain and grape pomace	Polyhydroxyalkanoates and enzymes	Martínez-Avila et al., 2021

The selection of the proper microorganism it is crucial to obtain the desired product. Different microorganisms have been employed in SSF processes. For enzyme production mainly bacteria and fungi are commonly used, and among them, fungi have gained interest due to SSF conditions reproduce the natural living environment for fungal growth and have been shown that their synergy is an interesting relation to produce enzymes

(Gowthaman et al., 2001). Due to the fibre composition, lignocellulosic substrates are an interesting substrate because they induce the enzyme production. The fungi that growth on the lignocellulosic substrate, excretes the specific enzymes to break the fibres of the solid and then obtain nutrients for the biological growth.

Other interesting products obtained through SSF are briefly explained below:

- **Polyhydroxyalkanoates**

One of the most interesting processes needing sustainable and inexpensive substrates is the polyhydroxyalkanoates (PHA) production. These bioplastics have become of primary interest since they could be potential substitutes of their petroleum-based counterparts, which have a negligible biodegradability and negatively affect the environment where they are disposed (Gironi and Piemonte, 2011). Usually, PHAs are produced by SmF such that a large range of waste streams can be used to produce them with a numerous variety of microorganisms (Rodriguez-Perez et al., 2018; Tan et al., 2014). However, one of the main constraints of the process is the high cost of common substrates, which could represent up to 50% of the total fermentation cost (Du et al., 2012). Thus, the enzymes produced from BSG could serve to hydrolyse this leftover in an environmentally sustainable way, and then, using the fermentable sugars as a source for PHA production through specific microorganisms. Hence, obtaining value-added products from a low-cost substrate, achieving the revalorisation of a residue and presenting an advance on the development of the biobased plastic.

- **Antioxidants**

The demand to replace the synthetic antioxidants for natural-produced antioxidants in food additives has focused on the utilisation of vegetal wastes (Conde et al., 2011). The polyphenols are the most abundant antioxidants in the human diet. Lignocellulosic

materials have been on the focus to obtain antioxidants due to their properties and characteristics and the low-cost substrate to produce them. Especially, BSG has been reported in several studies to be a potential source to obtain antioxidants. (Aliyu and Bala, 2011; da Costa Maia et al., 2020; McCarthy et al., 2013). Furthermore, different authors have used lignocellulosic materials to obtain antioxidants while producing another product, such as lignocellulosic enzymes (Leite et al., 2019). The action of several enzymes is crucial to obtain antioxidants because it is necessary to hydrolyse the fibres of the substrate to release these phenolic compounds (da Costa Maia et al., 2020). In this sense, the co-production of lignocellulolytic enzymes and antioxidants is an interesting strategy to perform a better valorisation of the substrate.

1.3 Lignocellulolytic enzymes

Enzymes have gained interest as biocatalysts in many industrial sectors, including biorefineries converting lignocellulosic biomass, to replace the use of chemicals, which have a negative impact on humans and the environment (Kim, 2018). The production of enzymes from environmentally friendly feedstock has been in the spotlight of the industrial biotechnology to reduce production costs.

The enzymes allow the bioconversion of the biomass, and they are essential in many processes to obtain the desired product. Also, the hydrolysis process could be an important step of a multistep process that could determine the global process efficiency and costs (Pino et al., 2018). Consequently, lignocellulolytic enzymes are high value-added process intermediates and are critical to obtaining good yields in the process production (Paz et al., 2019).

Furthermore, the enzymatic hydrolysis process can be altered due to the releasing of inhibitory compounds, such as furfuraldehyde, vanillic acid, or syringic acid, which are

within the fibre network of the substrate and therefore, could affect the process performance by inhibiting the enzymes when they are released (Berlin et al., 2006; Kim et al., 2011). Also, these inhibitory compounds could remain on the hydrolysate affecting the downstream processing to obtain the desired products. In this sense, it is necessary to study this hydrolysis step to analyse if a detoxification is necessary to reduce the presence of these compounds.

Typically, commercial hydrolytic enzymes are produced by SmF starting from pure substrates implying high sugar consumption and high costs (Dodge, 2009). In this context, the search for more economical and sustainable sources and processes for producing these enzymes has become a current concern.

Different authors have proposed the SSF technology as a potential alternative to SmF for producing hydrolytic enzymes more sustainably and economically (de Castro and Sato, 2015; Díaz-Godínez et al., 2001).

Table 1.2 presents the advantages and disadvantages of the SSF over the SmF. Compared to SmF, SSF usually achieves higher productivities and end-concentration of products, requires lower water and energy consumption and limits the production of significant waste streams (Soccol et al., 2017; Yazid et al., 2017).

For instance, Castilho et al. (2000) analysed economically both SSF and SmF, on the production of 100 m^3 of *Penicillum restrictum* lipase concentrate per year. The study concluded that the total capital investment for SmF was 78% higher than the one for SSF, and the unitary product cost of SSF was 47% lower than the actual selling price, generally due to the low price of the raw material used as substrate in the SSF.

Table 1.2 Advantages and disadvantages of SSF over SmF (Rodríguez Couto and Sanromán, 2005)

Advantages	Disadvantages
Higher productivities	Problems on the scale-up
Higher yields	Lack of homogeneity inside the reactor
Process a wide range of low-cost substrates	Difficulties with the heat transfer when scale-up
Reduce the energy and cost of the process	Recovery product cost increase
Reproduces the natural living environment	Complications in controlling process parameters

One of the main advantages of SSF lies in the ability to process a wide range of solid organic residues as raw materials to obtain different bioproducts (Yazid et al., 2017). Some of the most relevant lignocellulolytic enzymes are briefly explained below.

- **Cellulases**

Cellulases (EC 3.2.1) comprise a large group of different enzymes that form the enzymatic system for the hydrolysis of the plant cell wall. The cellulase structure is typically composed of one catalytic domain and a binding module where a carbohydrate is attached to improve the contact of the enzyme with the cellulose. They hydrolyse glycosidic bonds between a carbohydrate and another molecule that can be a carbohydrate as well (Ramos and Xavier Malcata, 2017).

- **B-glucosidases**

β-glucosidases (3.2.1.21) are enzymes that catalyse the hydrolysis of the glycosidic bonds to residues with non-reducing residues terminal in beta-D-glucosides and oligosaccharides, with the release of glucose. They work together with cellulases and they are responsible of the last step of the hydrolysis of the cellulose to glucose (Molina et al., 2016).

- **Xylanases**

Xylanase (EC 3.2.1.8) is a class of enzymes that degrade the linear polysaccharide xylan into xylose, thus breaking down hemicellulose. Filamentous fungi are particularly interesting producers of xylanases from an industrial point of view, because they are very stable (Bajpai, 2014).

Table 1.3 summarises some studies from the literature that used different SSF systems to produce lignocellulolytic enzymes.

These studies used similar lignocellulosic waste from the food industry to analyse the xylanase, cellulase and β-glucosidase activity. As seen, many strains have been used to produce lignocellulolytic enzymes obtaining different activity levels. The fungal strains most commonly used in SSF to produce lignocellulolytic enzymes are from the genre *Aspergillus* and *Trichoderma*. *A. niger* and *T. reesei* are the most used strains of these two genres and have been reported in several studies as important lignocellulolytic enzymes producers. (Farinas, 2015; Hansen et al., 2015; Rodríguez Couto and Sanromán, 2005). These are filamentous fungi that extracellularly excrete enzymes in order to degrade the substrate and obtain nutrients. Also, Thermoascus aurantiacus is an interesting filamentous fungal strain used in many studies due to its characteristics as a thermophile organism, like the thermostability. Using such microorganisms could be an advantage in some processes where the temperature conditions are crucial because the denaturalisation of the enzymes and the consequent loss of production is reduced. Also, the reaction rate increases, which makes less enzyme necessary to obtain the same amount of product, consequently reducing the production cost (A. Sharma et al., 2019).

Table 1.3 Lignocellulolytic enzymes production in different SSF systems.

Strain	System	Substrate	Xylanase activity ($U \cdot g^{-1}DM$)	Cellulase activity ($FPU \cdot g^{-1}DM$)	β-glucosidase activity ($U \cdot g^{-1}DM$)	Reference
T. reesei	0.25 L E. flask	Sugar cane bagasse (SCB)	-	4.6 ± 0.2	21.5 ± 0.3	Sukumaran et al. 2009
A. fumigatus	0.25 L E. flask	Rice straw and wheat bran (WB)	444.0 ± 6.3	2.1 ± 0.3	243.7 ± 0.9	Soni et al. 2010
T. reesei A.oryzae	Static tray reactor	Soybean hull and WB	-	10.8 ± 0.1	10.7 ± 0.1	Brijwani et al., 2010
Trichoderma. Reesei and A. oryzae	Deep bed reactor	Soybean hull	242.0 ± 4.3	5.4 ± 0.1	18.4 ± 0.1	Brijwani and Vadlani, 2011
A. niger NS-2	0.25 L E. flask	Corn cob	-	3.1 ± 0.3	1.8 ± 0.2	Bansal et al. 2012
A. niger NS-2	0.25 L E.flask	Kitchen waste	-	10.3 ± 0.3	19.5 ± 0.2	Bansal et al. 2012
A. ibericus	0.5 L E. flask	BSG	50.0 ± 2.0	-	-	Sousa et al. 2018
Aspergillus. niger	Petri dishes	BSG	290.6 ± 0.1	-	3.9 ± 0.2	Leite et al. 2019
A. ibericus	Petri dishes	BSG	313.8 ± 5.3	-	4.1 ± 0.2	Leite et al. 2019
T. reesei	Plastic bags (12 cm x 20 cm)	SCB and WB	-	5.8	4.1	Frassatto et al., 2020
Thermoascus aurantiacus	Plastic bags (12 cm x 20 cm)	SCB and WB	-	10.1	35.7	Frassatto et al., 2020
A. niger	0.25 L Erlenmeyer (E.) flask	BSG	1400.8 ± 43.9	6.2 ± 0.2	-	Moran-Aguilar et al. 2021

DM: dry BSG; U: xylanase and B-glucosidase activity unit; FPU: filter paper unit. Values are presented as the mean ± the standard deviation.

However, most of the reported studies work with small volume reactors using few grams of the substrate, limiting their application in actual conditions. Although there are many studies related to the production of enzymes, most of the studies found in the literature

only focus on enzyme production and in obtaining higher productivities and yields. That makes that the application of these enzymes is not analysed, and therefore, the real use of this product is not studied, which is a key factor due to the effect of the inhibitor compounds released that could affect the process efficiency (Jönsson and Martín, 2016; Kim et al., 2011). In this sense, there is a growing need for complementing these studies with a methodology to analyse the application of the produced enzymes in real environments.

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Chapter 2. Research objectives

The main objective of this research is to study the production of lignocellulolytic enzymes by pure fungi strains using the solid-state fermentation and to assess their further application in the hydrolysis process, as an alternative to the valorisation of agro-industrial residues.

In order to reach this goal, different specific objectives were set and are presented below:

- To define the potential of three selected lignocellulosic-derived residues (Brewer's spent grain, grape pomace, and olive mill solid waste) to produce lignocellulolytic enzymes
- To assess the performance of three fungal strains (*Aspergillus niger, Thermoascus aurantiacus* and *Trichoderma reesei*) for producing lignocellulolytic enzymes via solid-state fermentation at lab-scale.
- To study the effects of some of the main operational variables affecting the enzyme production in the SSF system at lab-scale
- To test the SSF process for producing lignocellulolytic enzymes using different bioreactor configurations at bench-scale.
- To evaluate the efficiency of the obtained enzymatic extracts for hydrolyzing lignocellulosic materials to reach fermentable sugars.
- To evaluate the use of the obtained enzymatic extracts to obtain other value-added products, such as polyhydroxyalkanoates and antioxidants.

Chapter 3 Materials and methods

3.1 Materials

3.1.1 Strains and inoculum

Aspergillus niger (ATCC 16888) and *Trichoderma reesei* (ATCC 26921) were obtained from "Colección Española de Cultivos Tipo" (CECT, Spain). *Thermoascus aurantiacus (*ATCC 26904) from American Type Culture Collection (ATCC, Virginia). *B. cepacia* (CCM 2656) was purchased from the Czech Collection of Microorganisms, Brno, Czech Republic, and *Cupriavidus necator* (DSM428) used in the PHA production was purchased from the German Collection of Microorganisms and Cell Cultures **(**DSMZ**,** Braunschweig, Germany). Strains were maintained at -80ºC in cryovials (Brand, Germany). Inoculum preparation for the fungi strains consisted of adding one pearl into a 100 mL Erlenmeyer flask containing 50 mL of a glucose-starch media (GS) (peptone: 5 $g \cdot L^{-1}$, starch: 20 $g \cdot L^{-1}$ and glucose: 20 $g \cdot L^{-1}$). For *B. cepacia* and *C. necator*, the same procedure was performed but using a LB (Lysogeny Broth) media. Flasks were placed in an orbital shaker at 120 rpm keeping an aerobic environment for 24 h in the case of *B. cepacia* and *C. necator* and 96 h for the fungi strains. The growth temperature was set at 30ºC for *B. cepacia, C. necator, A. niger,* and *T. reesei*, and at 45ºC for *T. aurantiacus*. All reagents and materials have been previously sterilized at 121ºC for 15 min.

3.1.2 Substrates

Three different lignocellulosic residues were used: Brewer's spent grain (BSG), Grape pomace (GP) and, Olive-mill solid waste (OSW). BSG was provided by *Companyia Cervesera del Montseny* (Catalunya, Spain). GP was collected from the vineyard of *Celler Cooperatiu d'Espolla* (Catalunya, Spain). OSW was provided by *Davmus Fruits*, a local oil producer in Huesca (Aragon, Spain). The residues were dried at 60 °C overnight, and then they were stored at room temperature until used. The appearance of the dried residues is shown in 3.1.

For the SSF experiments, BSG was used as the substrate. The preparation of this substrate consisted of humidifying the BSG with a suitable solution (distilled water, phosphate buffer 0.1 N, citrate buffer 0.1N) until reaching the desired moisture content (MC) and pH. The prepared BSG was sterilized at 121ºC for 15 min and then cooled at room temperature. BSG was inoculated with the fungal culture (section 3.1.1) in a range of 5-15% inoculum load depending on the experiment.

For the hydrolysis experiments, the substrate was used in its dried form. The residues were sterilized at 121ºC for 15 min and then cooled at room temperature before their use.

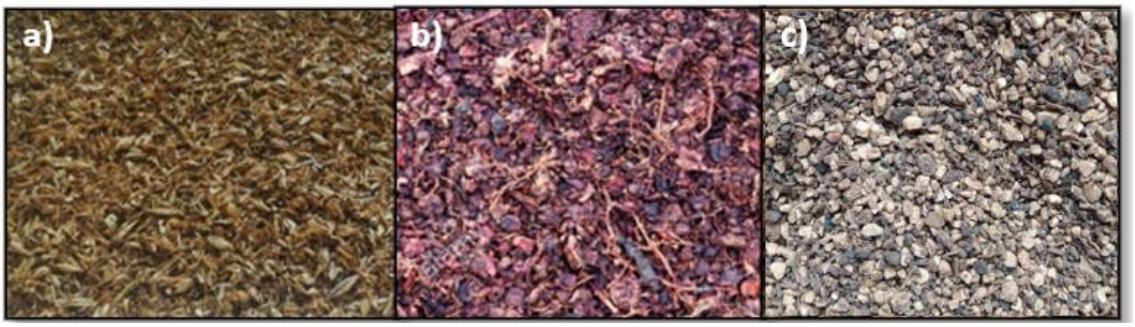

Figure 3.1 Brewer's spent grain (a), Grape pomace (b) and, Olive-mill solid waste (c) dry appearance.

3.2 Solid-state fermentation systems

3.2.1 Lab-scale set-up

SSF was performed in a self-built dynamic respirometric system composed of a temperature-controlled water bath in which reactors (0.5 L Erlenmeyer) were placed. The system was airtight and allowed supplying air with a mass flow meter from the top to the bottom (Figure 3.2) such that the air was forced to flow through the solid bed. The exhausted gases were conducted to oxygen sensors connected to an online acquisition system (Arduino-based). The dynamic respirometric index at 24h was calculated with the data collected with the online acquisition system, following the equations presented by

Gea et al. (2004). To prevent the drying of the substrate inside the reactors, the supplied air was previously saturated with water.

Reactors were filled with 100 ± 1 g of the prepared substrate (section 3.1.1).

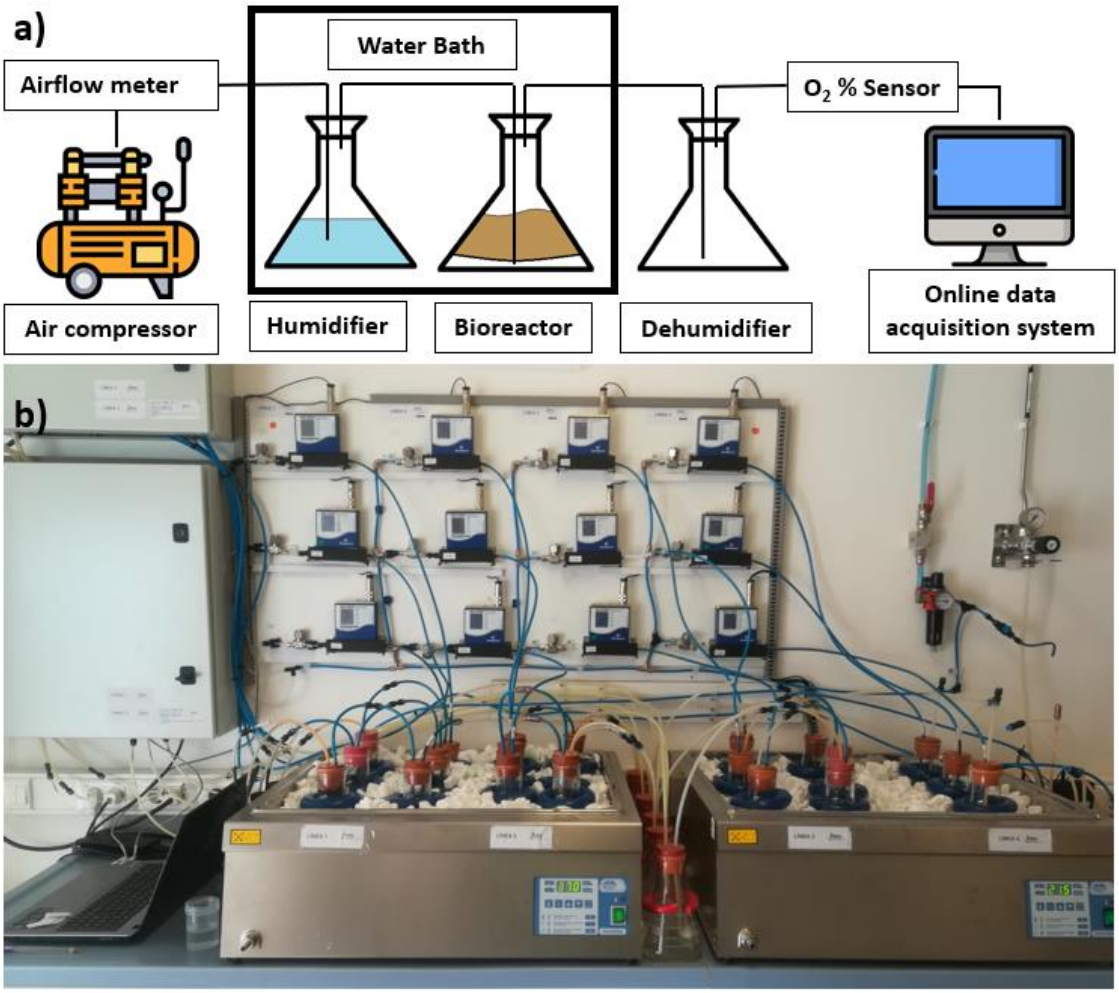

Figure 3.2 a) Diagram and b) picture of the respirometric system used for lab-scale solid-state fermentation.

The temperature for the experiments was set depending on the used strain, at 37 ºC for *A. niger* and *T. reesei* as suggested by Díaz-Godínez et al. (2001) and at 45ºC for *T. aurantiacus* as proposed by dos Santos et al. (2003). Fermentations were monitored up to 216 h.

3.2.2 Bench-scale cylindrical reactors

The respirometric system described in section 3.2.1 was also used to perform the fermentations at bench-scale. In this case, the reactors consisted of three cylindrical polyvinyl chloride (PVC) 3 L reactors (Figure 3.3) and three 5 L Dewar reactors (Figure 3.4). While the PVC reactors worked as not-isolated systems, the Dewar's served to simulate a near-adiabatic operation. In both cases, reactors were filled with 1100 ± 5 g of prepared substrate. For the analysis of the samples, 20 g of BSG were used, and the solid remaining in the reactor was used to continue the fermentation. Reactors contained an inlet to supply air from the bottom to the top forcing the air to flow through the solid bed. As detailed in section 3.2.1 the system was airtight such that the exhausted gases were conducted to an oxygen sensor connected to the same online acquisition system.

Figure 3.3 Bench-scale PVC reactors (not-isolated) used to perform SSF.

In these systems, the SSF was not temperature-controlled, and the reactors worked based on their own heat generation. However, the temperature of the solid bed was monitored using a temperature logger (i-Button). Fermentations were monitored up to 120 h.

Figure 3.4 Bench-scale Dewars reactors (near-adiabatic reactors) used to perform SSF.

3.2.4 Bench-scale tray-type reactor

The tray-type reactor consisted of an incubator with three 260x320 mm steel trays. The fermentation was performed with 900 ± 4 g of the prepared substrate (section 3.1.2 Substrates) in each tray. The incubator and the trays were sterilized before the fermentation. Fermentation was performed for 120 h, and the temperature of the incubator was set depending on the used strain as explained in section 3.2.1. The solid substrate was mixed every sample to oxygenate the solid bed, due to the impossibility of applying forced aeration.

Figure 3.5 Tray-type reactors used to perform SSF.

3.3 Enzyme extraction

Enzymes were extracted from BSG by adding 150 mL of citrate buffer (0.05 M, pH 4.8) to 10 g of fermented solid sample (1:15 ratio) in a 250 mL flask. The flasks were stirred for 30 min at room temperature. Then, the mixture was centrifuged at 5000 rpm for 10 min, and the supernatant was filtered using a 0.45 μm membrane filter. The obtained filtrate was used for enzymatic activity determination (Dhillon et al., 2012). Furthermore, the enzymatic extract obtained from the SSF of BSG was used for the hydrolysis experiments (section 3.4 and 3.6).

3.4 Liquid hydrolysis experiments

These experiments consisted of mixing 2 g of sterile and dried lignocellulosic substrates (section 3.1.2) in a 100 mL Erlenmeyer with the correspondent solution: the enzymatic extract obtained after the SSF process with the fungal strains (section 3.3), a commercial enzymatic cocktail Viscozyme L (Novozyme Inc., Copenhagen, Denmark) (1% dose)

used as a reference, or a citrate buffer 0.05 M as a blank control. In all cases, a 5% (w/v) substrate:extract ratio was used to perform the hydrolysis of the residue. The reducing sugar concentration of the produced sugar-rich hydrolysates was followed up to 48 h. All the samples were tested at two different hydrolysis temperatures, 37°C and 45°C using a controlled temperature shaker.

3.5 Steam explosion pre-treatment

The steam explosion process was conducted at *Parco Scientifico della Recerca* (Torino, Italy) using a system constituted of two different reactors: the biomass pressurization reactor (22 L) and the biomass expansion reactor (300 L). Biomass pressurization reactor was able to process up to 2 kg of biomass. The material to be pre-treated was put in contact with the vapor at a given pressure and time depending on the desired severity factor (R0). After this first step, the biomass was immediately expanded into the atmospheric pressure vessel. The sudden change of pressure induced a violent vapor expansion, resulting in the breaking up of the chemical linkages in the solid material. When the collecting vessel was completely de-pressurized, and the internal temperature dropped down to safety conditions, both liquid and solid fractions were collected.

3.6 Solid-state hydrolysis experiments

In these experiments, the lignocellulosic substrates and their steam exploded counterparts, were sterilized at 121ºC for 15 min and cooled at room temperature. Then, the crude enzymatic extracts obtained through SSF and Viscozyme L (Novozyme Inc., Copenhagen, Denmark) (1% dose) were added to the solid substrates to achieve the desired moisture content and to perform the hydrolysis with a high solid rate. As suggested by Martínez-Ávila et al. (2021) the moisture content for BSG and OSW was adjusted at 50% and for GP at 40%. Hydrolysis was followed up to 48 h by measuring the reducing sugars released by measuring the supernatant after a solid-liquid extraction

with water in a 1:15 (w/v) ratio. The hydrolysis was conducted at 35°C as suggested by Martínez-Ávila et al. (2021).

3.7 Submerged fermentation to produce PHA

PHA was produced via submerged fermentation by using 20 mL of the hydrolysates obtained in section (3.4) as substrate. The tested hydrolysates were placed into 100 mL Erlenmeyer flasks and complemented by adding two mineral media as described by Kucera et al. (2017). The substrates and all materials have been sterilized at 121°C for 15 min, and after cooling, the liquid substrate was inoculated by using 5% (v/v) of *B. cepacia* or *C. necator* as inoculum. Fermentation was conducted at 30ºC and 120 rpm in an orbital shaker assuring an aerobic environment. The system was monitored up to 72 h as suggested by Kucera et al. (2017) and the cells were harvested after 48 h for *C. necator* and after 72 h for *B. cepacia* for PHA analysis.

3.8 Analytical methods

3.8.1 Specific oxygen uptake rate

Specific oxygen uptake rate (sOUR) was calculated based on an on-line respirometric analysis, and as a mean for continuous monitoring of the system providing indirect information about the biological activity during the fermentation. sOUR was calculated as:

$$sOUR = F \cdot (0.209 - y_{O_2}) \cdot \frac{P \cdot 32 \cdot 60 \cdot 10^3}{R \cdot T \cdot DW \cdot 10^3} \qquad \text{(Eq. 1)}$$

where sOUR is the specific oxygen uptake rate (mg O_2 g^{-1} DM h^{-1}), F is the airflow rate used on the fermentation (mL·min^{-1}), y_{O2} is the oxygen molar fraction in the exhaust gases (mol O_2·mol^{-1}), P is the pressure of the system (101325 Pa), 32 is the molecular

weight of oxygen (g O_2 mol O_2 $^{-1}$), R is the ideal gas constant (8310 $Pa \cdot L \cdot K^{-1} \cdot mol^{-1}$), T is the temperature at which F is measured (K), and DW is the initial dry weight of the solids (g). 10^3 and 60 are conversion factors (Ponsá et al., 2010).

3.8.2 Cellulase, β-glucosidase, and xylanase activity

Total cellulases, β-glucosidase, and xylanase activities were measured (triplicate) using filter paper assay (FPase) and β-glucosidase assay (BGase), as recommended by IUPAC, according to Ghose (1987) and the method presented by Ang et al. (2013), respectively. FPase and xylanase activity assay are based on DNS method (Miller G.L, 1959).

a) Filter Paper Activity Assay (FPase)

The substrate for this assay was Whatman filter paper strips (6 x 1 x 1 cm). First of all, 1 mL of citrate buffer (0.05 M, pH 4.8) and 0.5 mL of the diluted enzyme were added to 25 mL test tubes. The strips were rolled up to fit in the test tubes, and then, one strip was added inside each test tube and correctly placed at the bottom to assure that it was covered by the liquid. Additional test tubes were prepared to perform the substrate and enzyme controls and these were measured by triplicate. Substrate and enzyme controls were prepared by replacing the enzyme volume or the substrate volume for citrate buffer (0.05 M, pH 4.8) respectively. A spectro blank was performed together with all the other samples, where 1.5 mL of citrate buffer (0.05 M, pH 4.8) were added.

All the test tubes were incubated in a water bath at 50°C. After 60 min of reaction, the tubes were taken out the bath and 3 mL of DNS reagent were added. After mixing, the test tubs were boiled for 5 min in a water bath. All test tubes of the samples, controls, and the spectro blank were boiled together. Then, the tubes were transferred to a cold-water bath and 20 mL of distilled water were added to each tube. After mixing, the samples were measured against the spectro blank at 540 nm.

One unit of FPase (FPU) was expressed as the amount of enzyme that releases 1 µmol of glucose per minute. Cellulase activity was expressed to the dry matter content as FPU·g^{-1}DM.

The cellulase activity was calculated using the following equation:

$$FPase\ (FPU\ g^{-1}\ DM) = \frac{(C_{sample})\cdot D\cdot E\cdot 100}{0.18\cdot 60\cdot DM} \qquad \text{(Eq. 2)}$$

where C_{sample} is the concentration of reducing sugars in g·L^{-1}, D is the dilution factor of the enzymatic extract, E is the extraction factor in (mL of buffer·g^{-1} of fermented solid) and DM is the dry matter content of the fermented solid. 0.18 is the molecular weight of glucose in kg/mol, 100 is a conversion factor to obtain the desired units and 60 is the reaction time.

b) β-glucosidase activity Assay

The substrate used in this assay is a solution of cellobiose 15 mM prepared using citrate buffer (0.05 M, pH 4.8). First of all, 1 mL of the cellobiose solution was mixed with 1 mL of the diluted enzyme in a 25 mL assay tube. Additional test tubes were prepared as controls for the substrate and the enzyme and these were measured by triplicate. Substrate and enzyme controls were prepared by replacing the enzyme volume or the substrate volume for citrate buffer (0.05 M, pH 4.8) respectively.

All the samples and controls were incubated at 50°C for 30 min. Then the tubes were placed in a cold-water bath and the glucose was determined using YSI 2700 Select Biochemistry Analyser.

One unit of β-glucosidase activity (U) was expressed as the amount of enzyme that releases 1 µmol of glucose per minute. β-glucosidase activity was expressed to the dry matter content as U·g^{-1}DM.

The β-glucosidase activity was calculated using the following equation:

$$BGase\ \ (U\ g^{-1}\ DM) = \frac{(C_{sample})\cdot D\cdot E\cdot 100}{0.18\cdot 30\cdot DM} \qquad \text{(Eq. 3)}$$

where C_{sample} is the concentration of reducing sugars in $g \cdot L^{-1}$, D is the dilution factor of the enzymatic extract, E is the extraction factor in (mL of buffer$\cdot g^{-1}$ of fermented solid) and DM is the dry matter content of the fermented solid. 0.18 is the molecular weight of glucose in kg/mol, 100 is a conversion factor to obtain the desired units and 30 is the reaction time.

c) Xylanase activity Assay

The substrate for this assay is a solution of xylan from beechwood. This solution was prepared by mixing 750 mg of xylan from beechwood with 40 mL of citrate buffer (0.05 M, pH 4.8). First of all, 1.5 mL of the solution of xylan from beechwood and 0.15 mL of the diluted enzyme were added to a 25 mL test tube. Additional test tubes were prepared as controls for the substrate and the enzyme and these were measured by triplicate. Substrate and enzyme controls were prepared by replacing the enzyme volume or the substrate volume for citrate buffer (0.05 M, pH 4.8) respectively. A spectro blank was performed together with all the other samples, where 1.65 mL of citrate buffer (0.05 M, pH 4.8) were added.

All the test tubes were incubated in a water bath at 50°C. After 20 min of reaction, the tubes were taken off the bath and 0.75 mL of DNS reagent were added. After mixing, the test tubs were boiled for 10 min in a boiling water bath. All test tubes of the samples, controls, and the spectro blank were boiled together. Then the tubes were transferred to a cold-water bath and 7.5 mL of distilled water were added to each tube. After mixing, the solution from the test tube was transferred into Eppendorf and centrifuged for 5 min at 14000 rpm. Then the samples were measured against the spectro blank at 530 nm.

One unit of xylanase activity (U) was expressed as the amount of enzyme that releases 1 µmol of xylose per minute. Xylanase activity was expressed to the dry matter content as $U \cdot g^{-1}$DM.

The xylanase activity was calculated using the following equation:

$$(U\ g^{-1}\ DM) = \frac{(C_{sample})\cdot D\cdot E\cdot 100}{0.18\cdot 20\cdot DM} \qquad \text{(Eq. 4)}$$

where C_{sample} is the concentration of reducing sugars in $g\cdot L^{-1}$, D is the dilution factor of the enzymatic extract, E is the extraction factor in (mL of buffer$\cdot g^{-1}$ of fermented solid) and DM is the dry matter content of the fermented solid. 0.18 is the molecular weight of glucose in kg/mol, 100 is a conversion factor to obtain the desired units and 20 is the reaction time.

Calibration curves

To perform the calibration curve, the volume of the substrate of the reaction was replaced by citrate buffer in the test tubes. The volume with the enzymatic extract was replaced with several glucose standards with different concentrations for cellulase activity. For xylanase activity, the same procedure was followed but using xylose standards. Finally, the same steps to analyse the enzyme activity were followed to build the calibration curve. For β-glucosidase activity, the calibration curve was not necessary because the equipment measured the glucose concentration directly.

3.8.3 Antioxidant activity

Antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Briefly, 2 mL of sample were mixed with 1 mL of DPPH. After 30 min in a dark space, absorbance was read at 517 nm. A calibration curve was prepared using known quantities of 6-hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid (Trolox). The results were expressed in µmol of Trolox-equivalent per gram of dry solid substrate (µmol Trolox equivalent$\cdot$g DM^{-1}).

3.8.4 Reducing sugars quantification

Reducing sugars were determined according to the DNS method (Miller G.L, 1959). The analysis was performed using the supernatant obtained after a solid-liquid extraction of

the substrate with distilled water in a 1:10 (w:v) extract ratio at 30°C for 15 min. The liquid fraction was filtered through a 0.45 μm membrane filter, and, when needed, it was properly diluted before the measurement. Then, 3 mL of DNS reagent were added to 1 mL of supernatant in 25 mL glass test tubes and the mixture was boiled for 5 min. The sample was cooled, and 20 mL of distilled water was added. Absorbance was measured at 540 nm. For the blank, 1 mL of distilled water was used instead of 1 mL of supernatant. A calibration curve was prepared using glucose with concentrations ranging from 0 to 2 $g \cdot L^{-1}$. The reducing sugar content was expressed as grams of glucose-equivalent per gram of dry matter according to the following equation:

$$Reducing\ sugar\ content(g \cdot g^{-1}DM) = \frac{C}{R} \cdot V \cdot D \quad \text{(Eq. 5)}$$

where C is the concentration of glucose-equivalents ($g \cdot L^{-1}$), R is the extraction ratio ($g \cdot L^{-1}$), V is the total volume of the supernatant (L), and D is the dilution factor.

3.8.5 PHA extraction

The biomass produced during the bacterial fermentation (section 3.7) was quantified by centrifuging 10 mL of the fermented sample at 5000 rpm for 5 min. The cells were washed with distilled water and centrifuged again at 5000 rpm for 5 min. While supernatant was collected and used to determine the reducing sugar content (section 3.8.4), the biomass pellet was dried at 60°C for 48 h and then weighed to obtain the cell dry weight (CDW) and used to determine the PHA content.

3.8.6 PHA quantification

PHAs were quantified by GC-FID after a solid-liquid extraction of the obtained dried pellet. The extraction was conducted as described by Brandl et al. (1988). Briefly, 8-12 mg of the dry pellet were mixed in a 2 mL vial with 1 mL of chloroform and 0.8 mL of methanol-sulfuric acid solution. Benzoic acid (0.1 $g \cdot mL^{-1}$) was also added as an internal standard. Vials were properly sealed and then placed in a thermostatic bloc at 94°C for 3

h. After cooling, the vial content was put in 4 mL vials and mixed with 0.5 mL of NaOH 0.05M through inversion for 5 min. The obtained organic phase was used to determine the PHA content of the extract. The GC system (Agilent 7820A) consisted of a Flame Ionization Detector with an HP-Innowax column (30mx0.53mmx1µm). The injection port was set at 250°C in splitless mode, and the column temperature was initially set at 70°C for 2 min, then temperature reached 190°C at 10°C min^{-1} and hold for 7 min. The detector temperature was set at 300°C. Identification and quantification were performed using calibration curves (internal standard) by comparing retention times of analytical grade standards (Sigma-Aldrich) P(3HB-co3HV) processed using the same conditions as the samples.

3.8.7 Inhibitory compounds quantification

Inhibitory compounds content on the liquid hydrolysates was determined by HPLC using a modified method from Chen (2006). Briefly, the HPLC system consisted of an Agilent 1920 Infinity UHPLC equipped with a UV-Vis Diode Array Detector G4212A and a Nucleosil 120C18 (3µmx125mmx4mm) column. The mobile phase (1 $mL \cdot min^{-1}$) was a mixture of 0.05% H_3PO_4 and acetonitrile: H_2O (90:10). A gradient allowed changing 100% of 0.05% H_3PO_4 until 100% acetonitrile: H_2O after 38 min. Then this condition was held for 10 min. Quantification was performed by comparing samples from the analytical standards of the selected inhibitory compounds at the same conditions by using external standard calibrations.

3.9 Standard methods

3.9.1 Moisture and dry matter content.

Moisture content (MC) and dry matter content (DM, equivalent to total solids) were determined gravimetrically by weighting 5 ± 1 g of the solid sample in crucibles. Samples

were dried in an oven at 105ºC for 24 h and then weighed to calculate the MC and the DM according to the following equations:

$$MC(\%) = \frac{(W_i - W_f)}{(W_i - W_o)} * 100 \qquad \text{(Eq. 6)}$$

$$DM(\%) = 100 - MC(\%) \qquad \text{(Eq. 7)}$$

where w_o is the weight of the dry capsule, w_i is the weight of the wet sample, and w_f is the weight of the dry sample.

3.9.2 Volatile solids

To determine the volatile solids (VS equivalent to organic matter content), the dried solid sample obtained from section 3.9.1 was submitted to ignition at 550ºC for 4 h. Volatile solids were calculated as follows:

$$VS(\%) = \frac{(W_i - W_a)}{(W_i - W_o)} * 100 \qquad \text{(Eq. 8)}$$

where w_o is the weight of the dry capsule, w_i is the weight of the dry sample, and w_a is the weight of the ashes, obtained after ignition.

3.9.3 pH

pH was determined by mixing a ratio of 1:5 w/v of the sample into distilled water. The sample was mixed at room temperature for 30 min to allow the salts to solubilize into the liquid phase. Then the solution was centrifuged at 3500 rpm for 10 min and the pH was measured using a pH-meter.

3.9.4 Water holding capacity

Water holding capacity (WHC) is defined as the ability of a solid material to retain water. First, approximately 10 g of the dried material were placed in a cylindrical tube containing a net at the bottom (to retain all the solid material), and it was saturated by adding distilled

water. The material was allowed to drain for 30 min. The saturated material was weighted to determine the amount of retained water. WHC was calculated with the following equation:

$$WHC\left(\frac{g_{H_2O}}{g_{susbtrate}}\right) = \frac{W_s - W_d}{W_d} \qquad \text{(Eq. 9)}$$

where w_s is the weight of the saturated sample (g) and w_d is the weight of the dried sample (g).

3.9.5 Fibre content

The cellulose, hemicellulose, and lignin content were determined by the gravimetric method (Cunniff, P. 1995). This method is based on two different methodologies, detergent acid, and neutral methods, which consists of the gravimetric determination of the residue previously treated with acid and neutral detergent solutions. These methods determine their soluble fraction and their components (Van Soest et al., 1991).

3.9.6 Carbon/Nitrogen content

The following methods were conducted as described by Thompson et al. (2001)

a) Total Kjeldahl nitrogen (TKN)

The TKN was determined using 0.5 g of the dried sample. The sample was triturated and then was transferred to a 100 mL Kjeldahl tubes. To perform the digestion step, 20 mL of concentrated sulphuric acid and a catalyst (Kjeldahl tablets (Catalyst with 6.25 % $CuSO_4 \cdot 5H_2O$)) were added, and the samples were digested for 1.5 h at 420°C using a Bloc Digester (J.P. Selecta S.A., Barcelona, Spain). After the digestion, the sample was distilled with an excess of NaOH (35%). The condensate was placed in a 250 mL Erlenmeyer with 100 mL of boric acid (4%) with a mixed phenolphthalein indicator. After distillation, titration with HCl was used to measure the amount of nitrogen formed. TKN was calculated using the following equation:

$$TKN\ \left(\frac{g\ N}{kg}\right) = \frac{(V_1 - V_0)\cdot N\cdot 1.4}{DW} \qquad \text{(Eq. 10)}$$

where V_1 is the volume of HCl consumed (mL) in sample titration, V_0 is the volume of HCl consumed (mL) in control titration, N is the normality of the HCl used in determination and DW, is the sample weight in dry basis (g).

b) Ammonium (NH_4^+)

This determination was similar to Kjeldahl-like distillation (section 3.9.6.a)) but in this method, the digestion step was not performed. This analysis allows quantifying the soluble N-NH_4^+ that is originally contained in the sample. The obtained extract was distilled and titrated as explained in the Kjeldahl determination. The following equation shows how to calculate the concentration:

$$NH_4{}^+\left(\frac{g\ N-NH_4{}^+}{kg}\right) = \frac{(V_1 - V_0)\cdot N\cdot 1.4}{DW} \qquad \text{(Eq. 11)}$$

where V_1 is the HCl volume consumed (mL) in sample titration, V_0 is the volume of HCl consumed (mL) in control titration, N is the normality of the HCl used in the determination and DW is the sample weight in dry basis (g).

c) Oxidizable carbon

To quantify the oxidable carbon, 0.2 g of the dried sample were added into 100 mL Kjeldahl tubes with 20 mL of $K_2Cr_2O_7$ (1/3 M). Then, the sample was digested for 10 min at 160°C using 26 mL of concentrated sulphuric acid using a Bloc Digester (J.P. Selecta S.A., Barcelona, pain). The blank was prepared only with the reagents mix. After the digestion, the sample was cooled at room temperature and after decanting for 12 h, it was titrated with $FeSO_4$ (0.2 M). Oxidizable carbon was calculated using the following equation:

$$\text{Oxidizable carbon } \left(g \frac{C}{kg\ DM}\right) = \frac{(V_B - V_S) \cdot M \cdot 3}{DW} \cdot D \cdot 10 \qquad \text{(Eq. 12)}$$

where V_s is the volume of $FeSO_4$ consumed (mL) in sample titration, V_B is the volume of $FeSO_4$ consumed (mL) in control titration, M is the effective molarity of $FeSO_4$ used in determination, D is the dilution and DW is the sample weight in dry basis (g).

3.10 Statistical analysis

Statistical differences of the experiments were evaluated by using one-way ANOVA ($p<0.05$) using the Tukey test. All the experiments were conducted in triplicates, and values were presented as mean ± standard deviation. Data were analysed using Minitab 18 software.

The response surface method experiments were designed and analysed with Minitab 18 software. A Box-Behnken design with 3 factors and 15 experiments was used to analyse the response on the xylanase activity. A model equation from the surface was obtained and the optimum point was calculated.

The experimental design to perform the steam-explosion pre-treatment (SE) experiments consisted of a experiment design of 8 runs and was used to analyse the effect of three different parameters (temperature, pressure, and time) on the lignocellulosic substrates during the steam explosion pre-treatment. The severity factor is calculated using the (R0) by the following equation:

$$\text{(R0)} \left[\text{R0} = \text{t} * e^{\frac{T-100}{14.75}}\right] \qquad \text{(Eq. 13)}$$

where "t" is the pre-treatment time in minutes and "T" is the temperature in Celsius degrees. The Severity Factor corresponds to the log (R0). Table 3.1 report the conditions and the severity factor in each run of the factorial experiment.

Table 3.1 Temperature, pressure, time conditions, and the severity factor of each run of the experiment.

Run	Substrate	Temperature (°C)	Pressure(bars)	Time (min)	Severity factor
1	BSG	186.1	12.4	4	3.25
2	BSG	186.1	12.4	10	4.14
3	BSG	217.8	23	4	3.65
4	BSG	217.7	23	10	4.53
5	GP	217.9	23	10	3.25
6	GP	197	15.6	10	3.94
7	GP	186.4	12.5	4	4.53
8	OSW	196.8	15.5	10	3.94

3.11 Efficiency of the self-produced extracts calculation

To analyse the efficiency of the self-produced extracts on the different substrates these were compared with the results obtained using Viscozyme, and the efficiency of the process was defined as:

$$Efficiency\ (\%) = \frac{Max\ reducing\ sugars\ released\ per\ DM\ using\ the\ extracts}{Max\ reducing\ sugars\ released\ per\ DM\ using\ Viscozyme} \cdot 100 \qquad \text{[Eq. 14]}$$

Where the maximum reducing sugars released correspond to the difference between the highest reducing sugars content, and the initial content at the beginning of the hydrolysis.

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Chapter 4 Brewer's spent grain biotransformation to produce lignocellulolytic enzymes and polyhydroxyalkanoates in a two-stage valorisation scheme

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Introduction

This chapter presents an initial selection of the most suitable substrate and strains to produce lignocellulolytic enzymes through SSF. In addition, in the context of the VALORA project, the obtained enzymatic extracts were tested to hydrolyse one of the tested substrates (BSG), obtaining a sugar-rich hydrolysate used as raw material for PHA production.

The main objective of this chapter was to establish the basis of the enzymatic production process and the application of the extracts to obtain sugars for future optimization and scaling up of this process.

4.1 Selection of the most suitable lignocellulosic residue to perform SSF.

First of all, three lignocellulosic-derived wastes proposed within the framework of the VALORA project were selected as potential substrates for producing lignocellulolytic enzymes via SSF. In this case, such a selection was based on their physic-chemical characteristics. As seen in Table 4.1, the tested residues comprised BSG, GP, and OSW. All of them, lignocellulosic wastes derived from the local food industry, and available in large quantities within Catalonia region.

As it can be seen in Table 4.1, BSG contained a significant amount of hemicellulose and cellulose, making this leftover a potential source of sugars after a hydrolysis process and as a potential substrate for the SSF, inducing enzyme production. In contrast, GP and OSW had a small amount of hemicellulose, and GP also had a small amount of cellulose. OSW had higher amount of cellulose than BSG, but its lignin content resulted very high.

Furthermore, BSG lignin content was particularly low, which could be considered a positive aspect since this would limit the adverse effects of lignin on the availability of hemicellulose and cellulose (Mussatto et al., 2008).

Table 4.1. Characterization of the three lignocellulosic residues

Parameters	Brewer's spent grain (BSG)	Grape pomace (GP)	Olive-mill solid waste (OSW)
Hemicellulose (% DM)	40.1 ± 0.3	3.5 ± 0.1	17.8 ± 0.7
Cellulose (% DM)	21.6 ± 0.2	11.0 ± 0.4	29.5 ± 0.6
Lignin (% DM)	7.7 ± 0.1	16.9 ± 0.6	36.0 ± 0.9
Moisture content (%)	79.0 ± 1.3	45.4 ± 0.8	8.6 ± 0.3
Volatile Solids (% DM)	96.0 ± 0.1	81.6 ± 0.4	80.7 ± 0.2
Water holding capacity ($gH_2O \cdot g^{-1}$)	4.3 ± 0.3	0.64 ± 0.01	0.89 ± 0.05
Bulk density at WHC ($kg \cdot L^{-1}$)	0.23 ± 0.03	0.18 ± 0.04	0.50 ± 0.03
pH	5.8 ± 0.1	3.6 ± 0.3	5.4 ± 0.2
Reducing sugars ($g \cdot g^{-1}$DM)	0.03 ± 0.00	0.07 ± 0.01	0.01 ± 0.00
TKN ($g \cdot kg^{-1}$DM)	30.4 ± 4.3	8.1 ± 1.5	9.7 ± 2.0
Oxidable carbon (OXC) ($g \cdot kg^{-1}$DM)	585 ± 92	624 ± 23	437 ± 5
C/N	19.2 ± 2.1	77.0 ± 3.2	45.1 ± 1.5
Dynamic respirometric index 24 h ($g_{02} \cdot kg^{-1} \cdot h^{-1}$)	5.7 ± 0.7	1.7 ± 0.3	0.50 ± 0.03

DM: dry matter

In general, these BSG values are in accordance with those previously found by other authors such as del Río et al. (2013) but slightly different compared to Mussatto et al. (2008) and Paz et al. (2019), wherein they found higher lignin contents for BSG. Besides, in other studies such as in Russ et al. (2005), cellulose, hemicellulose, and lignin of the used BSG were 23-25%, 30-35%, and 7-8%, respectively, while in Mussatto and Roberto (2005) they were 16.8%, 28.4%, and 27.8% respectively. Thus, compared with the characterization presented here, some differences can be attributed to the inherent

dependence on factors such as the quality of the used cereal or the specific conditions of the beer processing (Santos et al., 2003). Hence, it is expected that BSG could be a potential source of fermentable sugars after the hydrolysis of its cellulose and hemicellulose fractions.

On the other hand, moisture content, water holding capacity, and the bulk density of the substrate can be considered as important parameters for the SSF of fungal strains. As Table 4.1 details, GP could support a moisture content up to 50%, while the maximum MC in OSW was still too low to enable fungal growth. In contrast, BSG showed a suitable maximum moisture content (79.0% ± 1.3) and a higher water holding capacity value among the three substrates, which is an essential condition for fungal growth. The bulk density of BSG was also suitable to perform the SSF, avoiding compacting issues which could generate problems in the air distribution inside the reactor. In contrast, the OSW lacked enough porosity to enable the fungal growth.

Moreover, BSG had the highest value of the dynamic respirometric index, directly related to the substrate's biodegradability potential, which serves as an initial parameter to evaluate the potential of the substrate for a SSF process. Furthermore, Martínez-Ávila et al. (2021) analysed the inhibitory compounds in these three lignocellulosic substrates and recommended previous detoxification of GP and OSW to limit the effect of the inhibitory compounds on the fungal strains during the SSF. In the same report, it was found that BSG contained fewer inhibitory compounds making negligible the effect of limitation on the fungal strains.

For all these reasons, BSG appeared as the substrate with the highest potential of the evaluated set, and it was chosen to be used as a model substrate for enzyme production via SSF.

4.2 Assessment of fungal strains for enzyme production

The initial experiments consisted of assessing the ability of each of the selected strains to produce xylanases and cellulases through SSF using BSG as substrate. These strains are commonly used for enzyme production (as mentioned in Chapter 1). Table 4.2 summarizes the main results obtained for each fungal strain at lab-scale. As observed, it was found that *A. niger* reached the maximum xylanase activity of the evaluated set (268 ± 24 $U \cdot g^{-1}DM$) after 168 h of fermentation. However, this strain presented a second peak of xylanase activity (217 ± 30 $U \cdot g^{-1}DM$) after 48 h of fermentation with no significant difference compared to the first one (p 0.086) (Figure 4.1). It can be observed that, from the productivity standpoint, the peak at 48 h (4.5 ± 0.6 $U \cdot g^{-1}DM \cdot h^{-1}$) was better than the 168 h peak (1.6 ± 0.1 $U \cdot g^{-1}DM \cdot h^{-1}$), making the first more interesting to produce xylanases. In terms of cellulase activity, *A. niger* reached up to 1.9 ± 0.1 $FPU \cdot g^{-1}DM$ after 48 h of fermentation. Regarding *T. aurantiacus*, the maximum xylanase activity was reached after 72 h (241 ± 10 $U \cdot g^{-1}DM$) and the maximum cellulases activity (3.0 ± 0.2 $FPU \cdot g^{-1}DM$) after 168 h. Similarly, with *Trichoderma reesei*, the maximum xylanase activity was reached after 168 h (150 ± 24 $U \cdot g^{-1}DM$) and the maximum cellulases activity (3.0 ± 0.1 $FPU \cdot g^{-1}DM$) after 120 h of fermentation.

Table 4.2 Maximum enzymatic activities obtained from the evaluated fungal strains and commercial Viscozyme L (1%).

Parameter	*A. niger*	*T. aurantiacus*	*T. reesei*	Viscozyme L (1%)
Max xylanase activity ($U \cdot g^{-1}DM$)	217 ± 30^{A} (48 h)	241 ± 10^{A} (72 h)	150 ± 24^{B} (168 h)	277 ± 58^{A}
Max cellulase activity ($FPU \cdot g^{-1}DM$)	1.9 ± 0.1^{C} (48 h)	3.0 ± 0.2^{D} (168 h)	3.0 ± 0.1^{D} (120 h)	$2.4 \pm 0.4^{C,D}$
Max sOUR ($mgO_2 \cdot g^{-1}DM\ h^{-1}$)	4.7	10.2	5.6	-

sOUR: Specific oxygen uptake rate; DM: dry matter; FPU: filter paper activity unit; U: xylanase activity unit. Capital letters (A, B) in superscripts denote significant differences between the evaluated groups ($p<0.05$) on each row based on the Tukey test analysis.

Comparing the enzyme activities found here against a commercial multi-enzyme complex (Viscozyme L) with a standard dose for enzymatic hydrolysis (1%) (Kucera et al., 2017; Min et al., 2006), the produced extracts reached similar activity levels (Table 4.2). As observed, *A. niger* (p 0.986) and *T. aurantiacus* (p 0.584) extracts reached the same xylanase activity levels as Viscozyme L (1%) (p 0.986). Only *T. reesei* was significantly different (p 0.008), but in terms of cellulases, all extracts resulted similarly to Viscozyme L (1%) (p 0.183). Although the obtained cellulase activities were not as high as in other studies using similar feedstocks, they were in the same order of magnitude as Viscozyme L (1%). Such a result suggests that there was enough potential for using these enzymes for the hydrolysis processes. This aspect could be highly dependent on another factor such as the selection of the substrate for the fermentation. For instance, Dhillon et al. (2012) have used apple pomace as a substrate with *A. niger* reaching cellulase activities as high as 384 FPU·g^{-1}DM, while authors such as Ortiz et al. (2015) using wheat bran and *T. reesei* have reached activities around 96 FPU·g^{-1}DM. Also, Paz et al. (2019) reached cellulase activities of 14.8 FPU·g^{-1}DM using BSG as substrate, while Leite et al. (2019) reached between 50 and 60 FPU·g^{-1}DM using *A. niger* and *A. ibericus* with the same leftover. Although variability among substrates is significant, the obtained results were at the same levels as previous reports using BSG, such as in Leite et al. (2019).

Similarly, by using other substrates, the xylanase activity levels could be as high as 1000 U·g^{-1}DM (Dhillon et al., 2012), but typical values have been found between 200 and 800 U·g^{-1}DM (Mansour et al., 2016) as occurred here. Also, with BSG as a substrate, Leite et al. (2019) reached xylanase activities between 250 and 310 U·g^{-1}DM with *A. niger* and *A. ibericus*. These values are in concordance with the results obtained in this study using the same fungal strain. Moreover, the xylanase activity obtained by Leite et al. (2019) with BSG resulted similar to those obtained in this study with *T. auranticus*.

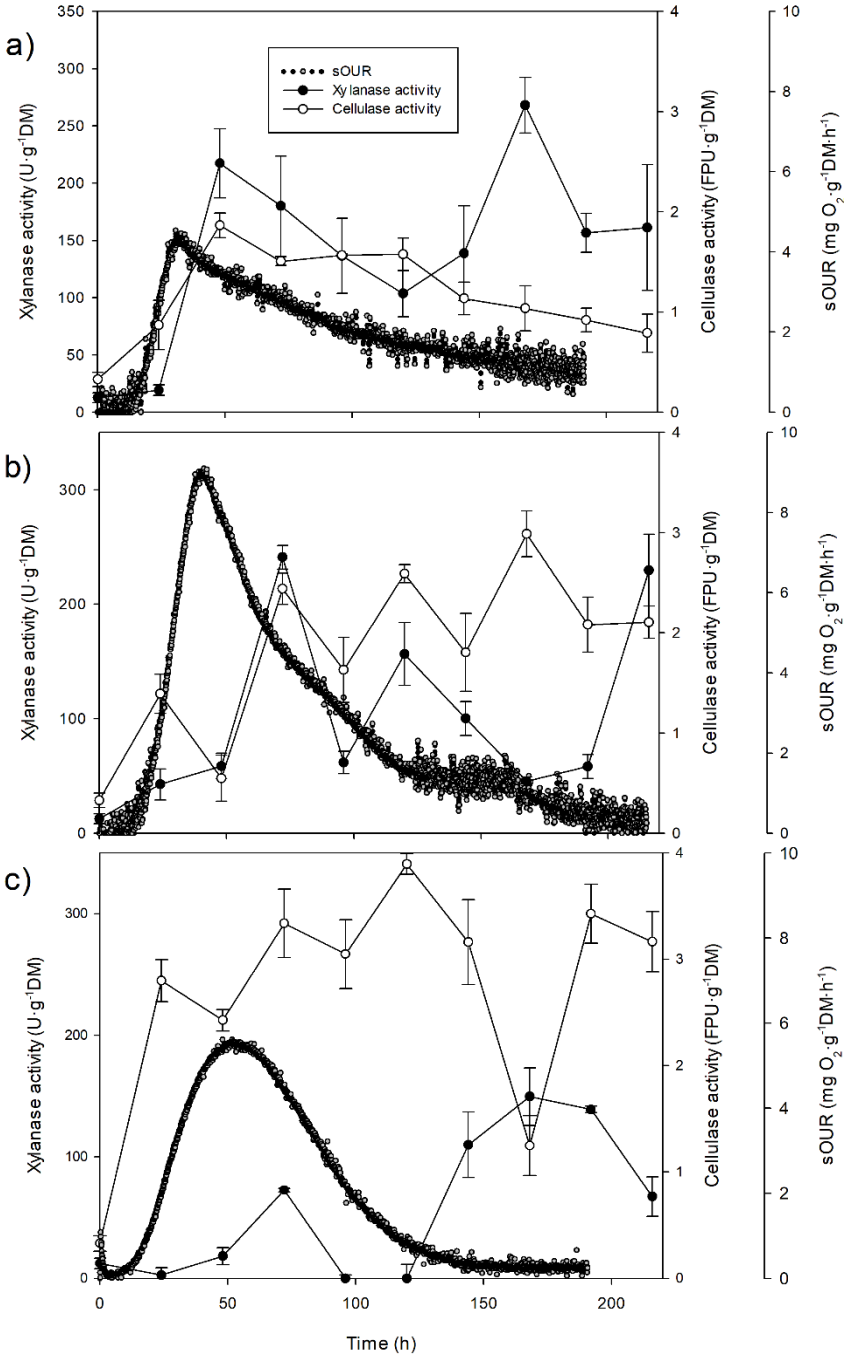

Figure 4.1 Time-course of the solid-state fermentation of brewer's spent grain (BSG) for producing xylanases and cellulases with (a) *A. niger*, (b) *T. aurantiacus*, and (c) *T. reesei*. sOUR: Specific oxygen uptake rate; DM: dry BSG; FPU: filter paper activity unit; U: xylanase activity unit.

Figure 4.1 details the time-course of the xylanases and cellulases activities from the SSF of the BSG of the three fungal strains. With *A. niger* (Figure 4.1a), the maximum enzyme activities were obtained after the peak of maximum microbiological activity (indirectly measured as sOUR) (32 h), and the maximum cellulases activity coincided with the first

peak of xylanases activity. Similarly, with *T. aurantiacus* (Figure 4.1b), it can be observed that, as occurred with *A. niger*, the peaks of activities have occurred some hours after the peak of maximum sOUR, which suggests that could exist a relationship among enzyme activities and the respiration activity as it was found in other SSF processes (Cerda et al., 2017). Hence, sOUR could be potentially used in further development stages as a monitoring and control parameter for producing these enzymes (Puyuelo et al., 2010).

From Figure 4.1, it can also be seen that, in the evaluated period, xylanases activity had a pseudo-cyclic behavior for both strains. For *A. niger,* there were two characteristic peaks separated by 120 h, and the levels achieved in both points were significantly higher than in the rest of the fermentation.

Similar behaviour can be found when using *T. aurantiacus*. This time, peaks were split by 144 h, and there were no significant differences among them (p 0.585). Finally, for *T. reseei*, the same pseudo-cyclic behavior was found (Figure 4.1c).

However, in this case, the xylanase activity levels were significantly lower, and the activity of the second peak was higher than the first one, leading to a decrease in the productivity of the process.

During the fermentation of the three fungal strains, no significant change in the MC was detected (MC changes were below 3%), assuring that neither drying of the bed nor the leaching of the products affects the process.

According to these results, fermentation time for maximum productivity corresponds to 48 h for *A. niger* and 72 h for *T. aurantiacus* and *T. reseei*. Given these results, it could be stated that *T. auranticus* and *A. niger* were the fungal strains with higher potential to produce lignocellulolytic enzymes from BSG from the evaluated group. Also, the mesophilic and thermophilic behaviour of each strain made them interesting to work with

them to analyse the different potential. Thus, the extracts obtained from these fermentations were selected for further hydrolysis tests.

4.3 Hydrolysis tests

In these experiments, the enzymatic extracts obtained from the SSF have been used to hydrolyse BSG to determine their initial efficiency. As seen in Figure 4.2a, these tests have been conducted at 37 and 45°C (Mussatto et al., 2008; Paz et al., 2019) using the extracts from the three fungal strains, the commercial enzyme cocktail Viscozyme L (1%) and a blank control. From these tests, it was clear that there was a significant difference between the obtained extracts and the commercial enzymes during the first 12 h (p = 000). Independently of the hydrolysis temperature, the commercial enzymes reached the highest reducing sugar release after 12 h reaching almost 35 $g \cdot L^{-1}$.

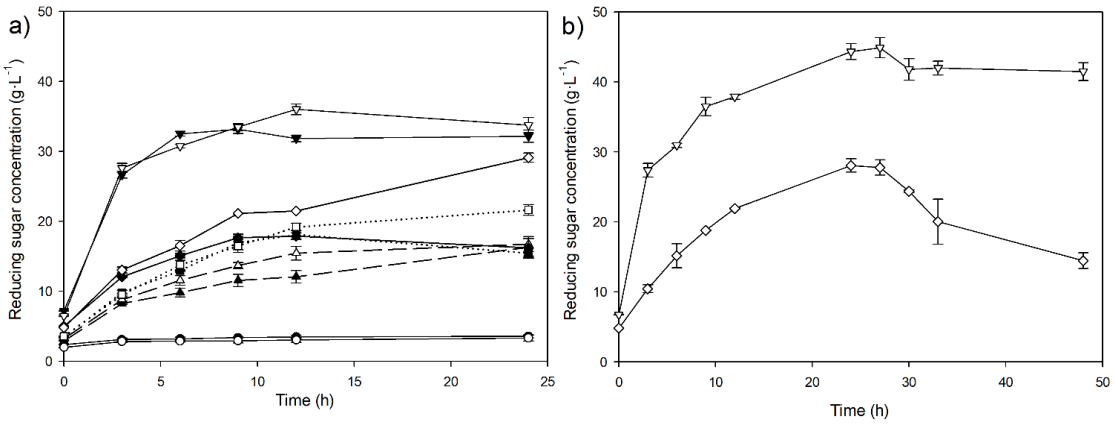

Figure 4.2 Hydrolysis tests to release fermentable sugars from dried brewer's spent grain (BSG) using the enzymatic extracts obtained via solid-state fermentation. (a) BSG hydrolysis at two temperature levels (37°C, 45°C) during 24 h, (b) BSG hydrolysis at 37°C during 48 h using *A. niger*. —●—: Blank control 37°C; —○—: blank control 45°C; —▲—: *T. aurantiacus* 37°C; —△—: *T. aurantiacus* 45°C; ···■···: T. reesei 37°C; ··□··: T. reesei 45°C; —◆—: *A. niger* 37°C; —◇—: *A. niger* 45°C; —▼—: Viscozyme L 37°C; —▽—: Viscozyme L 45°C.

On the other hand, *A. niger* extract at 45ºC showed the best performance among the evaluated extracts and temperatures, reaching 86-90% of the reducing sugar release

obtained by the commercial enzymes at 45°C and 37ºC respectively after 24 h. In general, these results suggest that working at 45°C is better than at 37°C for both the commercial enzymes and those produced from BSG.

After 12 h, a small decrease in the release of reducing sugars was detected. In this sense, some fungal growth was observed after 24 h of hydrolysis in the extracts from BSG (samples were inoculated in Petri dishes, and fungal growth was confirmed), suggesting that these could be consuming part of the sugars released. Thus, based on these results, the second set of experiments was conducted at 45 ºC using only *A. niger* extracts for an extended period to identify the effect of this reduction due to the potential fungal growth. As Figure 4.2b details, the maximum reducing sugar release in this scenario was obtained after 24 h with 28.1 ± 1.0 $g \cdot L^{-1}$, which was a 36% lower than the maximum achieved by the commercial enzymes at the same conditions. Hence, the maximum sugars released corresponded to 0.56 g of reducing sugar g^{-1}DM. After reaching the maximum, a significant decrease in reducing sugar content occurred, down to 51% after 48 h of hydrolysis.

Consequently, it is expected that, at the evaluated conditions, the hydrolysis could be conducted just for 24 h such that the potential loss of sugars can be minimized. Besides, due to the potential loss of sugars, future developments using such an enzymatic extract should include a purification step to limit this adverse effect. For instance, Liu and Xia (2006) suggest using ultrafiltration processes with 30 kDa membranes capable of retaining the contaminants (Liu and Xia, 2006).

In general, the levels of reducing sugar found here (28.1 ± 1.0 $g \cdot L^{-1}$) are similar to those found by other authors such as Mussatto and Roberto (2005) that reached concentrations between 21 and 35 $g \cdot L^{-1}$ by using acid hydrolysis of the BSG. Paz et al. (2019), using enzymatic extracts from *A. niger*, reached a total sugar content of almost 23 $g \cdot L^{-1}$ with

raw BSG. Besides BSG hydrolysates resulted competitive compared to other residues used to obtain fermentable sugars. For instance, Buzała et al. (2017) reached concentrations of 16.5 $g \cdot L^{-1}$ using poplar pulp, 14.5 $g \cdot L^{-1}$ for pine pulp, 16.9 $g \cdot L^{-1}$ for beech pulp, 15.9 $g \cdot L^{-1}$ for birch pulp, 17.7 $g \cdot L^{-1}$ for wheat straw pulp and 11.1 $g \cdot L^{-1}$ for hemp hard pulp using a commercial multi-enzyme preparation NS-22086. Similarly, Saucedo-Luna et al. (2011), using a commercial multi-enzyme mixture from *A. niger and T. reesei*, reached 17.5 $g \cdot L^{-1}$ of fermentable sugars after 48 h of hydrolysis of agave bagasse. Also, Wei et al. (2009), using the commercial enzymatic mixture Celluclast (Novozyme Inc., Denmark), reached 17.3 $g \cdot L^{-1}$ of fermentable sugars after 48 h of hydrolysis of rice hull. However, in many of these studies, chemical pre-treatments have been performed before the enzymatic hydrolysis to enhance the sugar release, or synthetic substrates have been added as substrates, making these alternatives more expensive. Thus, using BSG without any pre-treatment appears as an interesting option for obtaining fermentable sugars economically and sustainably.

4.4 PHA production from sugar-rich hydrolysates

The BSG sugar-rich hydrolysates obtained from the enzymatic extracts of *A. niger* and the commercial enzyme cocktail Viscozyme L (1%) had been used to evaluate the PHA production in liquid cultures. The solution obtained after the hydrolysis of BSG with citrate buffer was also tested as a control (blank). The PHA production was analysed by quantifying the produced poly-3-hydroxybutyrate (P3HB) and the polyhydroxyvalerate (P3HV) as the most common forms of polyhydroxyalkanoates produced starting from sugars. In this case, the P3HV levels were negligible (data not shown), so the results only considered the produced P3HB.

As seen from Figure 4.3a, the P3HB accumulation was higher when using *C. necator* compared to using *B. cepacian,* independently of the enzymatic extract used. With *A.*

niger extracts as hydrolysis agent, it was obtained 0.15 ± 0.03 and 0.13 ± 0.01 g P3HB·g^{-1} CDW with *C. necator* and *B. cepacia* respectively (0.41 ± 0.05 g P3HB·L^{-1} and 0.35 ± 0.03 g P3HB·L^{-1}). On the other hand, by using the commercial enzymes as hydrolysis agent, the P3HB contents were higher, achieving 0.25 ± 0.03 and 0.21 ± 0.03 g P3HB·g^{-1} CDW with *C. necator* and *B. cepacia* respectively (1.19 ± 0.14 g P3HB·L^{-1} and 1.01 ± 0.17 g P3HB·L^{-1}).

In all cases, *C. necator* had slightly higher production per biomass than *B. cepacia*. This result suggests that *C. necator* is a better PHA producer than *B. cepacia* from hydrolysed BSG. Previously, Rodrigues et al. (2019) compared the PHA production using *C. necator* and *B. cepacia* from soybean as a substrate. In that study, *C. necator* resulted better than *B. cepacia* to produce PHA, reaching up to 0.84 g P3HB·L^{-1}. Also, the PHA content in the biomass was higher with *C. necator*, between 20-23 %, compared to *B. cepacia*, which only reached 7-10 %, suggesting that *C. necator* is better accumulating PHA from this kind of hydrolysates.

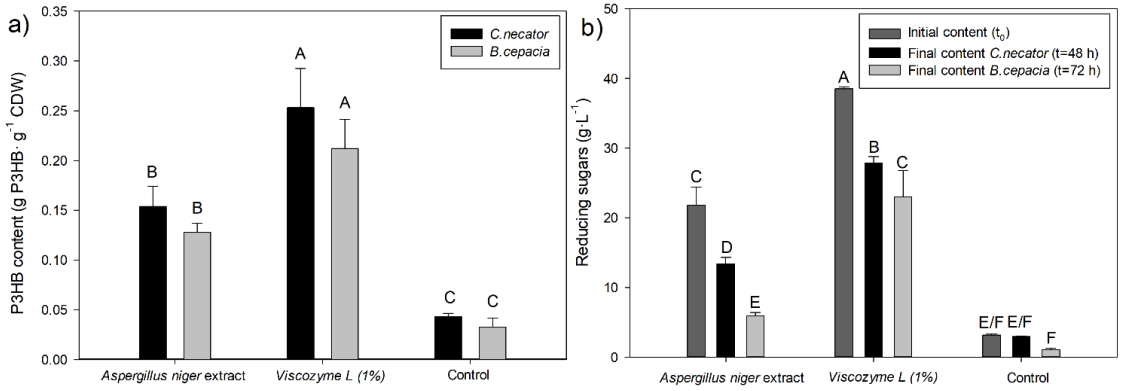

Figure 4.3 (a) P3HB production in liquid culture using different enzymatic extracts, and (b) initial and final reducing sugars content during P3HB production. CDW: cell dried weight; P3HB: poly-3-hydroxybutyrate. Different capital letters indicate significant differences between the evaluated groups ($p<0.05$) based on the Tukey test analysis.

Figure 4.3b summarizes the reducing sugars content of the BSG sugar-rich hydrolysates at time zero of the fermentation and the end of the fermentation. The reducing sugars

content obtained after 24 h of hydrolysis with each enzymatic extract was considered as the initial value (corresponding to time zero of fermentation). As seen, after hydrolysis of the BSG, the commercial enzymes and *A. niger* extract released 38.9 $g \cdot L^{-1}$ and 21.8 $g \cdot L^{-1}$ of reducing sugars, respectively, in agreement with the results found before. As detailed, it was found that *B. cepacia* consumed more sugars than *C. necator* in all the evaluated scenarios: *A. niger* extract (72.9% and 38.7% of consumed sugars, respectively), the commercial enzymes (40.2% and 27.4% of consumed sugars, respectively) and control (68.3% and 6.3% of consumed sugars respectively) (Figure 4.3b). However, P3HB production with *C. necator* resulted better than using *B. cepacia*. Thus, even though reducing sugars consumption was higher for *B. cepacia*, consumption was not reflected in higher P3HB production.

A higher sugars consumption was observed in *A. niger* extracts than in the commercial enzyme's samples, and it could be attributed to the potential fungal growth observed before. Comparing *A. niger* extracts and the commercial enzymes as hydrolysis agents on the *C. necator and B. cepacia* P3HB production, *A. niger* samples obtained 9.0 ± 0.4 and 7.0 ± 0.6 mg P3HB$\cdot g^{-1}$ DM respectively, and the commercial enzymes samples 23.4 ± 1.8 and 19.7 ± 3.5 mg P3HB$\cdot g^{-1}$ DM respectively.

Additionally, *A. niger* extracts reached between 35-40% of P3HB production reached with the commercial enzymes with both strains. This result suggests that an optimization of the hydrolysis step to obtain higher sugar levels and then, potentially increase the production of P3HB from the BSG is required. However, the obtained P3HB levels could also be altered by the inhibitory compounds produced during the hydrolysis step and the possible competition between the bacterial strains and the fungal strains observed before. In this sense, some authors (Jönsson and Martín, 2016) suggested that the formation of furan aldehydes and some soluble substances like hemicellulose-derived and cellulose-

derived carbohydrates could interfere with the cellulolytic enzymes altering the hydrolysis step. Other compounds such as acetate, furfural, vanillin, and levulinic acid could also affect bacterial fermentation, decreasing the PHA content (Pan et al., 2012). Table 4.3 summarizes the concentration of some of the most common inhibitory compounds found in the hydrolysates used here (from Viscozyme L and *A. niger*).

Table 4.3 Concentration ($mg \cdot L^{-1}$) of some potential inhibitory compounds in the evaluated hydrolysates.

Inhibitory compound	**Hydrolysate**	
	Viscozyme L (1%)	***A. Niger* extract**
Formic acid	<50	<50
Acetic acid	150 ± 1	157 ± 2
Levulinic acid	<100	<100
Furfuryl alcohol	<0.5	<0.5
5-HMF	0.64 ± 0.04	0.48 ± 0.03
Furfuraldehyde	4.9 ± 0.1	3.5 ± 0.1
Vanillic acid	0.53 ± 0.03	0.42 ± 0.02
Syringic acid	0.65 ± 0.04	0.51 ± 0.03
Vanillin	<0.5	<0.5
Syringaldehyde	<0.2	<0.2
Coumaric acid	0.98 ± 0.05	0.80 ± 0.04

Mean values are presented as the mean ± the standard deviation. "<" means below the detection limit of the method.

In general, it could be stated that the commercial enzymes produced hydrolysates with a higher content of inhibitory compounds such as 5-HMF, furfuraldehyde, vanillic acid, or syringic acid compared to hydrolysates obtained from *A. niger*. Although the levels found in both hydrolysates allowed the growth of both bacterial strains, these could limit the transformation of the available sugars into PHA. In future developments, a detoxification step could reduce the presence of such compounds, promoting a higher sugar consumption. However, higher PHA contents were obtained with the commercial extract

even more inhibitory compounds were found using it. Therefore, the lack of purification step in the self-produced extracts, generating the possible competition between the bacterial and the fungal could affect the PHA final production on the *A. niger* hydrolysates.

On the other hand, the results obtained here were in line with other reports using alternative raw material for producing PHA. For instance, Kucera et al. (2017) reached 0.30 g PHB·g^{-1} CDW using *B. cepacia* and up to 0.88 g PHB·g^{-1} CDW with *Burkholderia sacchari* starting from spruce sawdust as a substrate. Also, Tripathi et al. (2012), using sucrose as a carbon source, obtained 0.35 g PHA·g^{-1} CDW with *Pseudomonas aeruginosa.*

4.5 Conclusions

BSG was selected as a potential source of sugars after a hydrolysis process and as a potential substrate for the SSF for enzyme production. *A. niger* produced the highest enzymatic activities from the SSF of BSG, reaching up to 268 ± 24 U·g^{-1}DM of xylanase activity, obtaining an enzymatic extract capable of producing significant fermentable sugars contents. Although *A. niger* reached similar xylanase and cellulase activities than Viscozyme L (1%), its efficiency to hydrolyse the BSG resulted significantly lower. However, the BSG hydrolysates obtained from *A. niger* enzyme extract have supported the P3HB production using *C. necator* and *B. cepacia*, reaching 9.0 ± 0.4 and 7.0 ± 0.6 mg P3HB·g^{-1} DM, respectively. These results suggest that BSG can be used as a low-cost raw material for obtaining both lignocellulolytic enzymes and PHA as value-added products resulting in a valorisation approach in two stages.

Remaining challenges will focus on optimizing the parameters of the production of the lignocellulolytic enzymes through SSF process to increase the enzymatic activities and the change of scale. Also, additional research on the hydrolysis step needs to be done to

improve the release of sugars from BSG and other lignocellulosic substrates and to analyse the application of the obtained enzymatic extracts. Furthermore, the addition of a pre-treatment strategy coupled with the hydrolysis step could serve to improve the overall performance of the proposed approach.

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Chapter 5 Optimization of the lignocellulolytic enzyme production process parameters through SSF with *Aspergillus niger* and *Thermoascus aurantiacus*

Part of this chapter is included in an article, along with Chapter 6, that will be submitted to the journal Biochemical Engineering Journal.

Introduction

Once the fundamentals of the enzymatic production process and the application of the enzymatic extracts were stablished in the first steps of the experimentation, the production process was optimised to improve the SSF performance. Before the optimization, different fermentations were performed to assess the influence of the airflow rate on the enzyme production. Considering the previously obtained results, that present *A. niger* and *T. aurantiacus* as potential producers of lignocellulolytic enzymes through SSF from BSG, the optimization was carried out with both strains aiming to maximize the xylanase activity. The xylanase activity was chosen as the response factor to optimize because the previous results obtained in Chapter 4, presents the xylanase activity values as more promising and to have more potential than the cellulase activity values obtained. Three process parameters (pH, MC and inoculum load) were chosen to evaluate the effect on the obtained xylanase activity.

5.1 Airflow rate influence on the enzyme production

The supplied airflow rate was studied to assure the correct aerobic environment needed for fungal growth, and to check if this variable induces a significant effect on the enzymatic activity, while keeping in mind the need for minimizing the air consumption of the process. As Figure 5.1 details, for each fungal strain, four airflow rates (0.03, 0.07, 0.1, and 0.12 $L \cdot h^{-1} \cdot g^{-1}$DM) were evaluated considering the effect on the xylanase activity. The airflows were evaluated at 48 h and 72 h of fermentation for *A. niger* and *T. aurantiacus* respectively (maximum productivity time found in Chapter 4). The tests were performed with the lab-scale set-up. For both strains (*A. niger* and *T. aurantiacus*), 0.07 $L \cdot h^{-1} \cdot g^{-1}$DM was the lowest airflow rate ensuring aerobic conditions, finding no significant differences with the other evaluated levels ($p<0.05$).

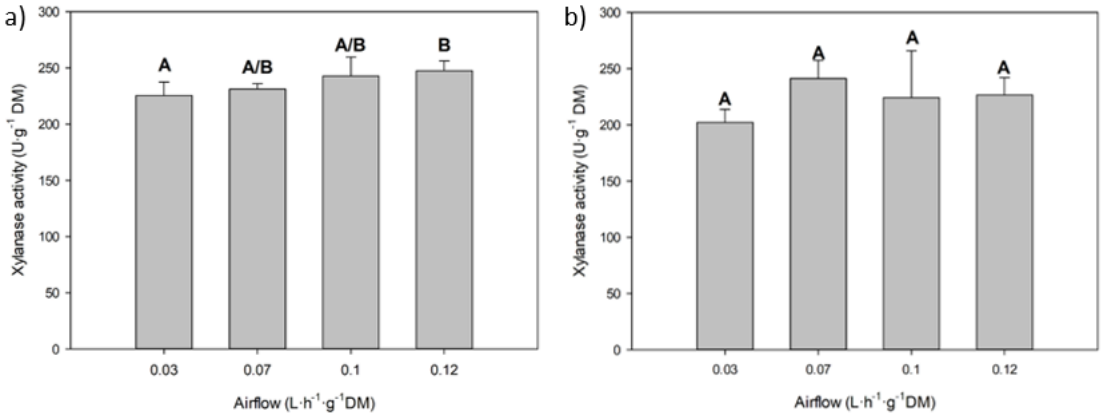

Figure 5.1 Airflow influence on the xylanase activity with (a) *A. niger*, (b) *T. aurantiacus*. DM: dry matter; U: xylanase activity unit. Capital letters (A, B) in superscripts denote significant differences between the evaluated groups ($p<0.05$) on each row based on the Tukey test analysis.

Furthermore, above that point, no particular growth improvement was seen because of excess of air, coinciding with previous reports in literature (Mejias et al., 2017). On the contrary, further increasing the airflow rate would only increase the processing cost. Thus, for the response surface methodology (RSM) optimization the airflow rate was kept constant at 0.07 $L \cdot h^{-1} \cdot g^{-1}$ DM.

5.2 Optimization of the process parameters using Response Surface Methodology

The RSM optimization was carried out to evaluate the effect of three parameters at three different levels: pH (4.6, 5.7, 6.8), moisture content (53%, 64.5%, 76%), and inoculum load (5%, 10%, 15% (w/w)) on xylanase activity as the response factor. The initial pH and the moisture content of the substrate are important parameters in SSF processes because they directly influence the fungi growth on the substrate. Similarly, inoculum load (In) is also an important parameter prone to be studied to maximize the production, while avoiding potential problems for a rapid substrate depletion. Besides, an inefficient use of inoculum could affect the operational costs of the process. The pH evaluation range was selected to cover the natural pH of the BSG (5.5-6) and the inoculum load was set in typical levels used in previous experiments (Chapter 4). Moisture content levels were set

based on the maximum saturation of the BSG (around 80%), above this value the BSG couldn't absorb the additional water.

5.2.1 *A. niger*

Figure 5.2 shows the response surface obtained after optimization using *A. niger*. The *A. niger* model equation (Eq. 15) of the response surface, with an r^2=0.99, is presented below:

[Eq. 15]

$$Xyl_{act} = 1048 - 48.9\,\mathrm{Mc} + 251.7\,\mathrm{pH} - 56.4\,\mathrm{In} + 0.29\mathrm{Mc}^2 - 34.4\,\mathrm{pH}^2 + 1.64\,\mathrm{In}^2 + 2\,\mathrm{Mc} * \mathrm{pH} + 0.17\,\mathrm{Mc} * \mathrm{In} + 2.79\,\mathrm{pH} * \mathrm{In}$$

From the p values of the *A. niger* model equation terms it can be inferred that all of them significantly affected the xylanase activity ($p < 0.05$). Table 5.1 shows the design matrix of the Box-Behnken model and the experimental and predicted values of xylanase activity for *A.niger*.

Table 5.1 Design matrix of the Box-Behnken model and the values of the experimental and predicted xylanase activity for A. niger.

Mc	pH	In	Experimental xylanase activity ($U \cdot g^{-1}DM$)	Predicted xylanase activity ($U \cdot g^{-1}DM$)
63	5.6	10	39.1 ± 5.0	18.0
76	5.6	5	117.57 ± 6.4	90.3
53	5.6	15	100.7 ± 21.9	87.8
76	5.6	15	172.6 ± 22.6	139.7
63	6.8	5	20.2 ± 13.6	3.4
63	6.8	15	83.8 ± 10.3	64.2
53	6.8	10	8.4 ± 4.6	-7.6
76	4.6	10	13.8 ± 2.3	-6.7
76	6.8	10	107.3 ± 10.1	80.1
53	5.6	5	85.2 ± 9.3	77.4
63	4.6	15	25.9 ± 6.5	4.0
63	4.6	5	23.7 ± 4.7	4.5
53	4.6	10	15.9 ± 1.4	6.9

The model suggests that the maximum xylanase activity is found at pH 6.5, MC 76%, and inoculum load 15%. At that point, the predicted xylanase activity was 191.4 $U \cdot g^{-1}$DM. During the fermentation, the pH decreased in all the samples until reaching values around 3 after 48 h of fermentation. This could occurs due to the releasing of acids by *A. niger* during the SSF such acetic acid and other organic acids (Show et al., 2015).

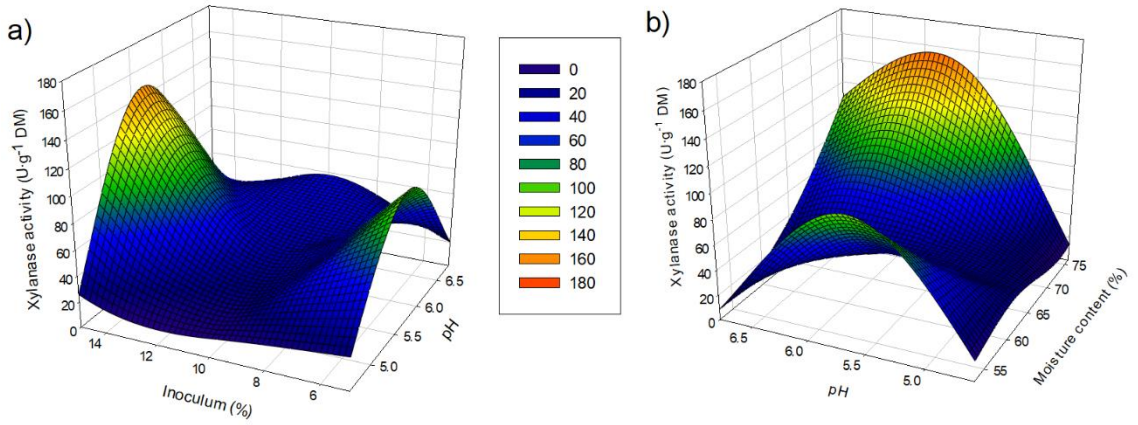

Figure 5.2 Response surface for xylanase activity from *A. niger* and BSG as a function of (a) pH and inoculum (%) and (b) pH and moisture content (%). DM: dry BSG; U: xylanase activity unit.

From Figure 5.2 it can also be seen that xylanase activity was enhanced at high moisture contents, and below 70%, the activity significantly decreased. High inoculum loads promoted the fungal growth resulting in higher values of xylanase activity and only a small range of initial pH was suitable to obtaining competitive xylanase activity results. Previous reports of similar processes have shown comparable pH optimum points and profiles. Soliman et al. (2012) found that pH 5.5 was the optimum for xylanase production by *A. niger* using agriculture residues. Furthermore, they stated that xylanase decreased below pH 4 and above 6.5. Also, Betini et al. (2009) found that pH values between 5.5 and 6 were the optimum for xylanase production by *A. niger* using a mixture of agro-industrial substrates. Rosés and Guerra (2009) found that 75% was the optimum MC with *A. niger* using sugarcane bagasse as substrate, similar to the optimum moisture content

found. Regarding the inoculum load, the lack of reports showing such information has make impossible to compare the obtained results.

5.2.2 *T. aurianticus*

Figure 5.3 shows the results obtained with *T. aurianticus.* In this case, the model equation (Eq. 16) of the response surface, with an r^2=0.94, is presented below:

$$\mathrm{Xyl}_{act} = -2262 - 2.8\,\mathrm{Mc} + 798\,\mathrm{pH} + 8.3\,\mathrm{In} + 0.09\,\mathrm{Mc}^2 - 62.7\,\mathrm{pH}^2 + 0.56\,\mathrm{In}^2 - 0.79\,\mathrm{Mc} * \mathrm{pH} - 0.28\,\mathrm{Mc} * \mathrm{In} + 0.04\,\mathrm{pH} * \mathrm{In} \quad \text{[Eq. 16]}$$

From the p values of the *T. aurianticus* model equation terms it can be induced that the pH (p=0.007) and pH^2 (p=0.001) terms were significant ($p < 0.05$**)**. The remaining terms, MC, and inoculum load were not statistically significant. Table 5.2 shows the design matrix of the Box-Behnken model and the experimental and predicted values of xylanase activity for *T. aurianticus.*

Table 5.2 Design matrix of the Box-Behnken model and the values of the experimental and predicted xylanase activity for T. aurianticus.

Mc	pH	In	Experimental xylanase activity ($U \cdot g^{-1}DM$)	Predicted xylanase activity ($U \cdot g^{-1}DM$)
53	4.6	10	5.5 ± 5.9	-13.7
76	4.6	10	40.0 ± 11.2	41.0
53	6.8	10	76.5 ± 5.3	78.2
76	6.8	10	70.9 ± 14.3	92.8
53	5.6	5	76.4 ± 2.2	92.9
76	5.6	5	161.6 ± 19.4	161.6
53	5.6	15	146.5 ± 36.1	141.7
76	5.6	15	168.3 ± 14.2	146.0
63	4.6	5	4.4 ± 2.0	2.2
63	6.8	5	93.8 ± 8.9	76.2
63	4.6	15	6.9 ± 2.4	22.6
63	6.8	15	97.1 ± 8.6	97.5
63	5.6	10	110.63 ± 8.3	107.5

In this case, the model equation shows that the maximum xylanase activity is reached at pH 5.9, moisture content 76%, and inoculum 15%. At that point, the optimum was 162.8 U·g^{-1}DM. The xylanase activity decreased below an initial pH of 5. During the *T. aurianticus* fermentation, the pH increased to values around 7-8 in all runs except the ones that started at pH 4.6. In these samples, the final pH value was always below 5, which suggests that the pH was not suitable for fermentation. The optimum inoculum load was 15% but it can be seen that there were no significant differences between the results and the inoculum had no significant effect on xylanase activity.

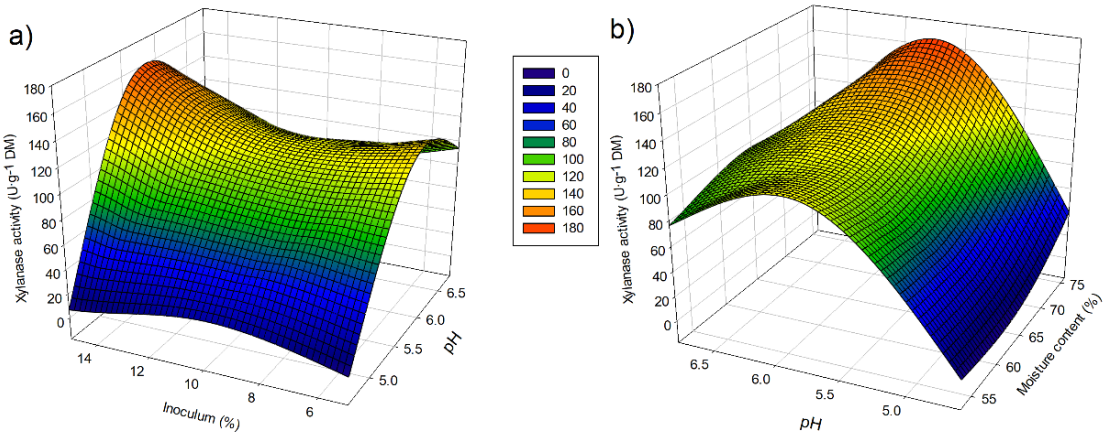

Figure 5.3 Response surface for xylanase activity from *T. aurianticus* and BSG as a function of (a) pH and inoculum (%) and (b) pH and moisture content (%). DM: dry BSG; U: xylanase activity unit.

Figure 5.3 shows a different surface profile than the obtained with *A. niger*. The inoculum load and the moisture content of the substrate did not have an important effect on the xylanase activity. However, the pH was a determinant parameter to obtain competitive values of xylanase activity, which was hindered with pH values below 5.5. In a range of pH from 5.5 to 6.5, the xylanase activity values were more stable, with a maximum close to a value of 6.

These results aligned to other studies following similar pH profiles. Alam et al. (1994) found the optimum pH value at 5 with *T. aurianticus* using lignocellulosic residues. Da

Silva et al. (2005) found that for xylanase activity, optimum pH was between 5.0-5.5 with *T. aurianticus* using agricultural residues. On the contrary, Kalogeris et al. (2003) found that pH 4 was the optimum for cellulase production by *T. aurianticus* using lignocellulosic wastes. De Oliveira et al. (2016) using wheat bran found that the optimum pH for amylase activity was 6.

Comparing both strains, it could be seen that the surface profiles were different. In the case of *A. niger*, the moisture content, the pH and the inoculum load had an important effect on the xylanase activity, as opposed to *T. aurianticus*. With the latter, only the pH played an important role, and the moisture content and the inoculum effect were not significative in the studied range. This implied that each strain had different potential to give robustness at the production process, depending on the process conditions or the substrate characteristics. However, the xylanase activity was higher in the case of *A. niger*. These facts helped that both strains had a potential for enzyme production through SSF. Also, the different operational temperature made both strains interesting regarding the future change of scale of the process. At large scale, with high solids loads the heat transfer problems affect the SSF and the enzyme production (Figueroa-Montero et al., 2011; Singhania et al., 2009). Furthermore, the denaturalization of the enzymes at high temperatures reduces the total production and increase the total cost of the process. In this sense, the thermostability of the enzymes produced by thermophile fungi could be an advantage at larger scales. However, a more detailed research is needed to verify these results, and to bring technology closer to the real scale that the industry needs.

5.3 SSF time-course at the optimal conditions

After the optimization of the operational parameters, a time-course at the optimal conditions was performed to obtain more information of the enzymatic production process. In this case, the systems were monitored using additional analyses to follow

different parameters during the fermentation such as the sugars levels, the enzymatic activities, pH and sOUR. In this way, it could be allowed to observe the relations between the different parameters and the obtained results.

Figure 5.4 summarizes the time course with both fungal strains. In the *A. niger*'s fermentation (Figure 5.4a), the pH decreased in the first 24 h of fermentation to 3, remaining almost constant afterwards, coinciding with results found in chapter 4. Between 24 h and 34 h, the reducing sugars decreased rapidly, coinciding with the maximum oxygen uptake rate.

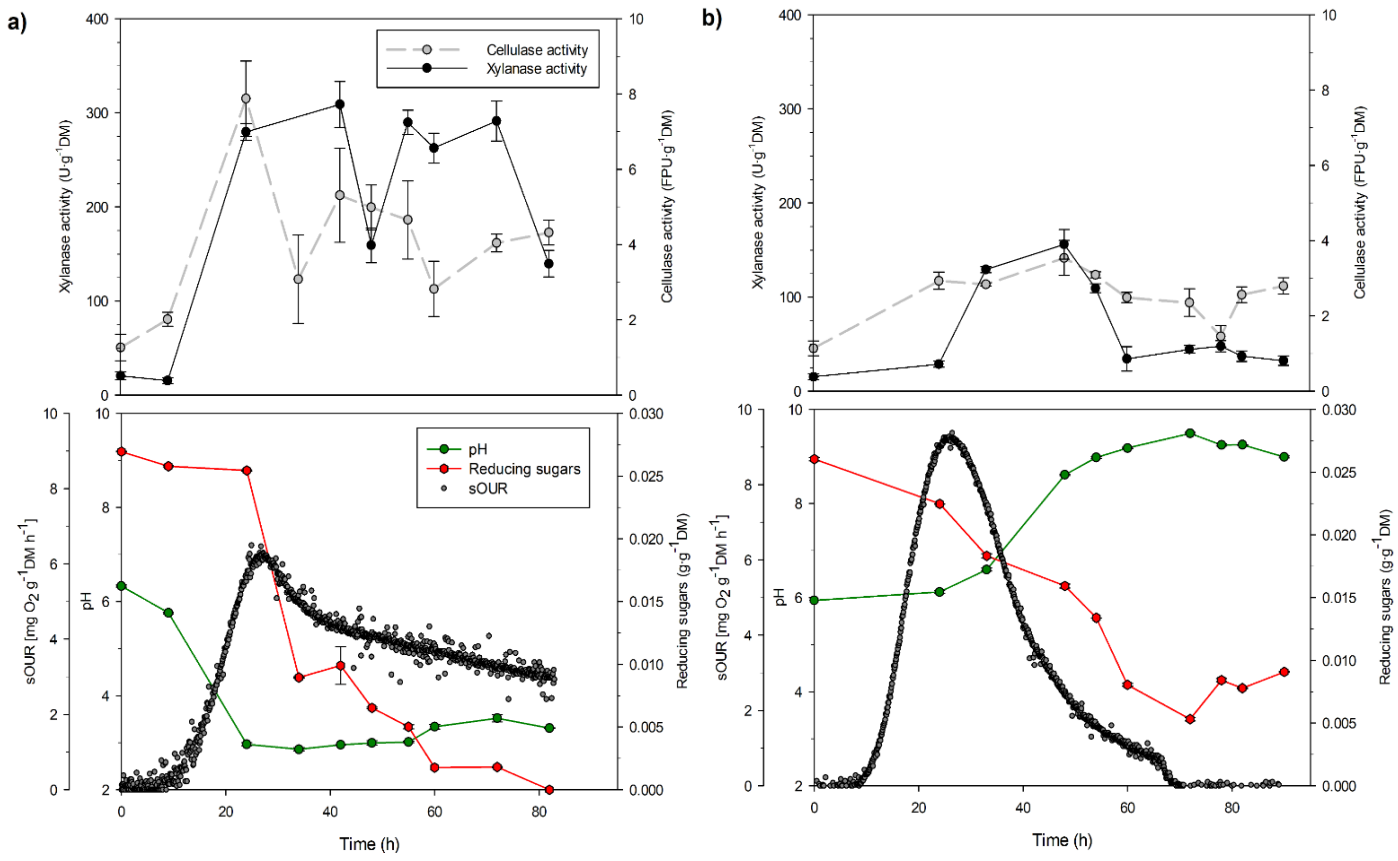

Figure 5.4 Time-course of the solid-state fermentation of brewer's spent grain (BSG) for lignocellulolytic enzyme production at the optimal conditions with *A. niger* (a) and *T. aurantiacus* (b) at lab-scale. sOUR: Specific oxygen uptake rate; DM: dry BSG; FPU: filter paper activity unit; U: xylanase or β-glucosidase activity unit.

In contrast, in the *T. aurantiacus*‘ fermentation, the pH increased during the fermentation reaching values around 9, similar to those reported in section 5.2.2. Sugar consumption in the *T. aurantiacus*‘ fermentation was slower than in the *A. niger*‘s fermentation. The sOUR profile reached different maximum values, around 7 and 9 mg $O_2 \cdot g^{-1}DM \cdot h^{-1}$ for *A. niger* and *T. aurantiacus* respectively. These differences could be mainly due to the growth temperature of each strain. At thermophilic temperatures the metabolism is more accelerated than at mesophilic temperatures, and more oxygen is typically consumed (Maheshwari et al., 2000). However, after the peak of maximum oxygen consumption, in the case of *A. niger*, the respiration rate dropped to values around 4 mg $O_2 \cdot g^{-1}DM \cdot h^{-1}$ to remain almost constant, and with *T. aurantiacus*, the respiration fell after the peak till it stopped consuming oxygen. In this sense, the total oxygen consumption was higher in *A. niger* fermentation, and it could be related to the sugar consumption, that was higher in the *A. niger*‘s fermentation.

In terms of xylanase activity, the level obtained with *T. aurantiacus‘s* at 48 h of fermentation (156.1 ± 15.8 $U \cdot g^{-1}DM$) was statistically similar ($p<0.05$) to the one predicted with the RSM (162.8 ± 1.0 $U \cdot g^{-1}DM$) with a productivity of 3.3 ± 0.3 $U \cdot g^{-1}DM \cdot h^{-1}$. On the contrary, *A. niger* produced higher xylanase activity than predicted. The maximum xylanase activity value (309.0 ± 24.5 $U \cdot g^{-1}DM$) was obtained at 42 h, being 1.5 times higher than the predicted value at 48 h. The productivity at this time was 7.36 ± 0.6 $U \cdot g^{-1}DM \cdot h^{-1}$, that is 2.2 times the productivity obtained with *T. aurantiacus.*

In terms of cellulase activity, *A. niger* appeared as a more suitable producer than *T. aurantiacus. A. niger* obtained a maximum value of 7.9 ± 1.0 $FPU \cdot g^{-1}DM$ after 24 h of fermentation (0.3 ± 0.01 $FPU \cdot g^{-1}DM \cdot h^{-1}$ of productivity). With *T. aurantiacus* the cellulase activity remained around a value of 3.0 $FPU \cdot g^{-1}DM$ during the fermentation and never exceeded the 4.0 $FPU \cdot g^{-1}DM$ or 0.2 $FPU \cdot g^{-1}DM \cdot h^{-1}$.

In previous studies using the same SSF system, lower maximum xylanase and cellulase activities were found with *A. niger* (217.0 ± 30.0 U·g^{-1}DM and 1.9 ± 0.1 FPU·g^{-1}DM at 48 h of fermentation) (Chapter 4). Considering these results, the optimization of the SSF to produce lignocellulolytic enzymes with *A. niger* was successful, increasing productivity 1.6 times compared to the fermentation in the previous chapter (Chapter 4).

However, the maximum xylanase activity found in Chapter 4 with *T. aurantiacus* (241.0 ± 10.0 at U·g^{-1}DM 72 h of fermentation) was higher than the reached after the optimization. In terms of maximum cellulase activity in Chapter 4 (3.0 ± 0.2 FPU·g^{-1}DM at 168 h of fermentation) was lower than the obtained here and it was obtained after a longer period, decreasing the productivity.

Table 5.3 Summary of the different tested systems to obtain lignocellulolytic enzymes.

Fermentation	**Xylanase activity (U·g^{-1}DM)**	**Cellulase activity (FPU·g^{-1}DM)**	**Xylanase productivity (U·g^{-1}DM·h^{-1})**	**Cellulase productivity (FPU·g^{-1}DM·h^{-1})**
A. niger				
Chapter 4 (Proof of concept)	217.0 ± 30.0	1.9 ± 0.1	4.5 ± 0.6	0.04 ± 0.0
Optimum predicted value	191.4	-	4.0	-
SSF at optimum conditions	309.0 ± 24.5	7.9 ± 1.0	7.4 ± 0.6	0.33 ± 0.0
T. aurantiacus				
Chapter 4 (Proof of concept)	241.0 ± 10.0	3.0 ± 0.1	3.4 ± 0.1	0.02 ± 0.0
Optimum predicted value	162.8	-	3.4	-
SSF at optimum conditions	156.1 ± 15.8	3.5 ± 0.5	3.25 ± 0.3	0.07 ± 0.0

However, from Table 5.3 it can be seen that the xylanase productivity of *T. aurantiacus* remained constant in the different fermentations, and in the value obtained in the optimization, therefore the selected parameters did not have a significant influence on the xylanase activity. The cellulase activity reached at optimum conditions was higher than

the obtained in Chapter 4. However, there was not a significant difference ($p<0.05$) between the results.

From the literature, it could be retrieved that using BSG as a substrate, Leite et al. (2019) reached xylanase activities between 250 and 310 $U \cdot g^{-1}DM$ with *A. niger* and *A. ibericus*. These values are in concordance with the results obtained so far in this thesis with the same fungal strain, *A. niger*. The cellulase activity (6.2 ± 0.2 $FPU \cdot g^{-1}DM$) levels that were obtained by Moran-Aguilar et al. (2021) using *A. niger* and BSG were in the same range than the results obtained in this study.

However, it must be taken into account that the working temperature was different in each strain. It is interesting evaluating to work under mesophilic and thermophilic conditions considering that at large scale, different conditions can be found. As previously discussed, the amount of lignocellulosic wastes generated in the industry is usually hundreds of kilos or even tons. Thus, the SSF working at a commercial scale must be able to process high amounts of feedstock. With large amounts of wastes the temperature inside the solid bed is expected to increase due to metabolic heat released in the fermentation (Figueroa-Montero et al., 2011; Singhania et al., 2009). In this sense, working with a thermophilic organism could be an advantage al large scales, reducing the cooling needs and the associated operating cost.

5.4 Conclusions

The undertaken optimization increased xylanase yield-productivity 1.6-fold for *A. niger*. That strain obtained higher enzymatic yield than *T. aurantiacus* after the optimization of the process. The best conditions obtained at lab-scale from the RSM of *A. niger* were pH 6.5, moisture content 76%, and inoculum 15%. The *A. niger* SSF performed at the optimal

conditions found a maximum xylanase activity value of 309.0 ± 24.5 $U \cdot g^{-1}$DM at 42 h and a maximum cellulase activity value of 7.9 ± 1.0 $FPU \cdot g^{-1}$DM at 24 h.

Despite this, the future change of scale that must be carried out to deal with the large amounts of lignocellulosic wastes produced to solve the real problem must be taken into account. In this case, with larger amounts of waste self-heating problems can appear, and therefore, make temperature control difficult and alter the behaviour of the fermentation found at lower temperatures. In this sense, working with a thermophilic organism as *T. aurantiacus* is interesting to observe its behaviour on a larger scale.

The best conditions obtained at lab-scale from the RSM of *T. aurantiacus* were pH 5.9, moisture content 76%, and inoculum 15%. The *T. aurantiacus* SSF performed at the optimal conditions found a maximum xylanase activity value of 156.1 ± 15.8 $U \cdot g^{-1}$DM at 48 h and a maximum cellulase activity value of 3.5 ± 0.5 $FPU \cdot g^{-1}$DM at 48 h.

These results present the enzyme production process through SSF with BSG as a substrate, as a potential alternative to the submerged fermentation but further research at larger scale should be performed to verify the feasibility of this approach.

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Chapter 6 Effect of scale and bioreactor configuration on the lignocellulolytic enzyme production process through SSF with *Aspergillus niger* and *Thermoascus aurantiacus*

Part of this chapter is included in an article, along with Chapter 6, that will be submitted to the journal Biochemical Engineering Journal.

Introduction

After the optimal conditions were tested (Chapter 5), a change of scale of the SSF to produce enzymes was performed, using two different reactor configurations, PVC reactors (non-adiabatic conditions) and Dewar reactors (near-adiabatic conditions). The difference between the reactors is the heat transfer between the inside and the outside environment of the reactor and this difference could influence the temperature dynamics and thus, the enzymatic production process. Even though *A. niger* obtained better results at lab-scale, the change of scale was performed also with *T. aurantiacus*, as commented in the previous chapter, due to their thermophilic behaviour and the future scale-up of the process, since it could be interesting to observe the differences with the mesophilic behaviour during the SSF. Another reactor configuration (tray-type reactor), commonly used in SSF, was tested to have a deeper knowledge of the enzyme production process.

Xylanase and cellulase are enzymes that are usually analysed in the SSF process targeting lignocellulolytic enzymes, but the fungal strains can produce simultaneously other types of enzymes and other value-added products like the antioxidants (Leite et al., 2019). B-glucosidase is one of the enzymes contributing to total cellulase activity. Beta-glucosidase is a rate-limiting enzyme of cellulase enzyme complex because it is responsible for the final step hydrolysis of lignocellulose completing the hydrolysis of cellulose into glucose (Naraian and Gautam, 2018). Also, these enzyme, along with the xylanases and other cellulases, have an important influence on the mobilization of phenolics compounds from the lignocellulosic materials, increasing the antioxidant activity (Leite et al., 2019). In this sense, new analyses were implemented (β-glucosidase and the antioxidant activity) to have a better knowledge of the process, to obtain new products, and to check if there is any relationship or synergy between the different enzymes production.

6.1 Bench-scale SSF (PVC reactors)

Operation scale was increased 10-fold by performing the SSF in the 3-L PVC reactors working with 1.1 kg BSG.

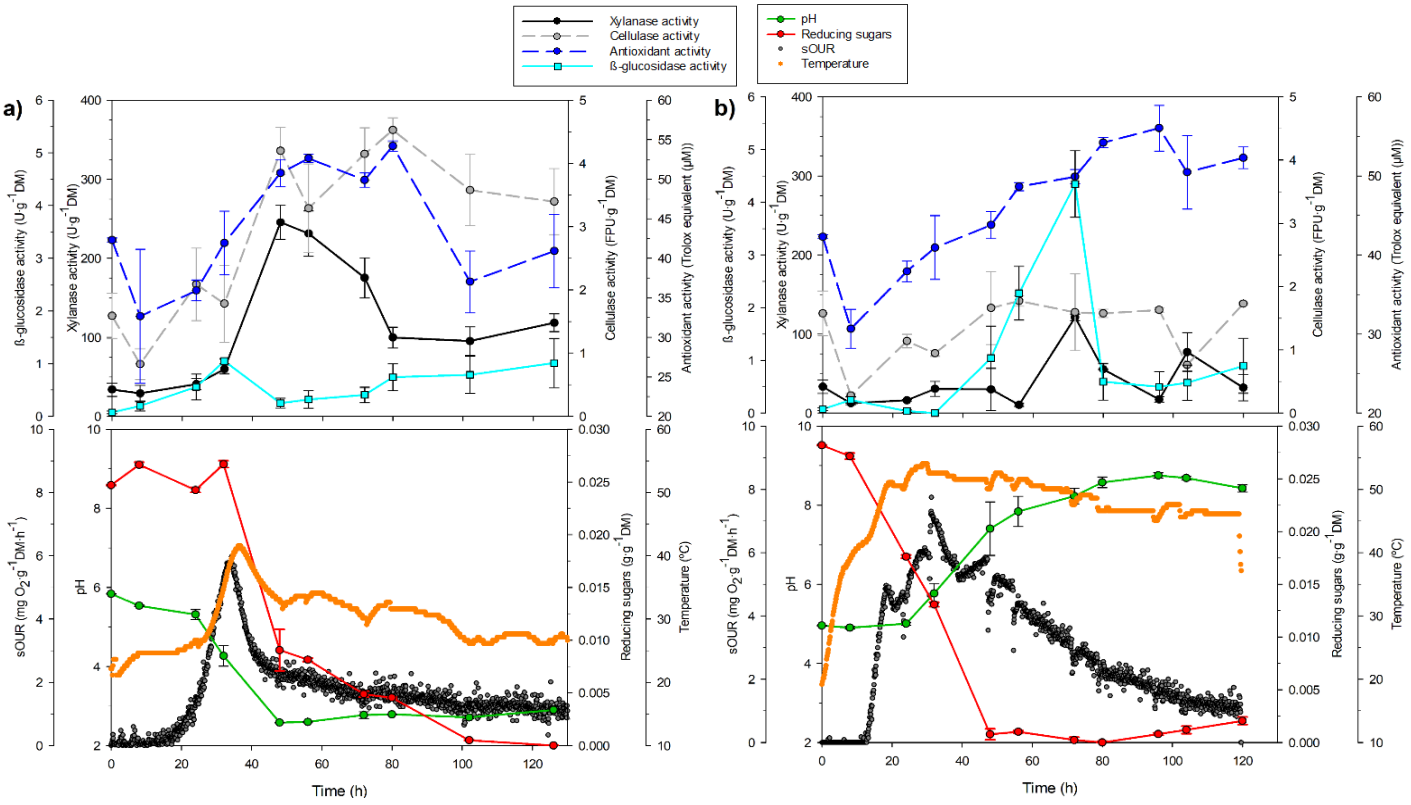

Figure 6.1 Time-course of the solid-state fermentation of brewer's spent grain (BSG) for lignocellulolytic enzyme production at the optimal conditions with *A. niger* (a) and *T. aurantiacus* (b) at bench-scale using PVC reactors. sOUR: Specific oxygen uptake rate; DM: dry BSG; FPU: filter paper activity unit; U: xylanase or β-glucosidase activity unit.

In the case of *T. aurantiacus*, a thermophilic organism, the PVC reactors were placed inside an incubator at 45ºC, increasing the initial temperature of the residue to the growth temperature of the strain. However, in the case of *A. niger* the PVC reactors were at room temperature. *A. niger* optimal growth temperature is 37 ºC but has a growth range from 20-40 °C. Working without temperature control reduce the operational cost and the self-heating capacity derived from the fungal metabolic activity maintain the temperature inside the reactor.

The pH of the fermentations of each strain was in accordance with the previous SSF performed at lab-scale (Chapter 4). During *A. niger* fermentation the pH decreased to values around 3 and, in contrast, during *T. aurantiacus* the pH increased to values around 9 as in the lab-scale tests (chapter 4).

A. niger showed a similar sOUR and sugar profiles (Figure 6.1) as at lab scale, with maximum sugar depletion observed at the period of max microbial activity. The metabolic activity produced heat that accumulated in the solid matrix providing a temperature rise over room temperature in a profile parallel to that of sOUR.

In the case of *T. aurantiacus*, the temperature profile inside the reactor did not fit perfectly with the oxygen consumption because the reactors were placed inside a temperature-controlled incubator and, differently than the previous lab-scale experiments. In this fermentation was observed an increasing of the sugar consumption rate compared with the one performed at lab-scale. This fact could be due to the increment of the temperature at the beginning of the fermentation because the self-heating capacity of the strain and the larger amount of substrate than in the lab-scale, that reached temperatures above the 50ºC.

There was a general reduction of enzymatic activities working in these fermenters compared to lab scale fermentations. *A. niger* showed higher cellulase and xylanase activity than *T. aurantiacus*. *A. niger* reached its maximum of xylanase activity after 48 h with a value of 245.5 ± 21.6 $U \cdot g^{-1}DM$ (productivity 5.1 ± 0.5 $U \cdot g^{-1}DM \cdot h^{-1}$), and at the same time, 4.2 ± 0.4 $FPU \cdot g^{-1}DM$ of cellulase activity (0.09 ± 0.01 $FPU \cdot g^{-1}DM \cdot h^{-1}$). This is not the maximum cellulase activity value obtained with *A. niger* (4.5 ± 0.2 $FPU \cdot g^{-1}DM$ at 80 h of fermentation, 0.06 ± 0.00 $FPU \cdot g^{-1}DM \cdot h^{-1}$) but is is statistically equal and the best in terms of productivity. *T. aurantiacus*'s fermentation reached 121.2 ± 4.2 $U \cdot g^{-1}DM$

at 72 h of fermentation of xylanase activity (1.68 ± 0.06 $U \cdot g^{-1}DM \cdot h^{-1}$ of productivity). The maximum cellulase activity value obtained with *T. aurantiacus* was 1.8 ± 0.1 $FPU \cdot g^{-1}DM$ at 56 h of fermentation (0.03 ± 0.00 $FPU \cdot g^{-1}DM \cdot h^{-1}$ of productivity).

These results suggest that, compared with the optimal conditions of each strain at lab-scale, the increase of scale resulted more suitable for *A.niger* than for *T. aurantiacus*. Applying the change of scale, the reduction on the productivity was lower in the case of *A. niger*, maintaining 69% and 30% of the xylanase and celulase productivity respectively in contrast with only 51% and 15% in the case of *T. aurantiacus*. The temperature of *A. niger* fermentation was always within a range of values between 25ºC and 40 ºC during the fermentation, fact that could affect the enzyme stability and activity, in contrast with the constant temperature worked at lab-scale. As commented before, the self-heating capacity could increase the temperature and create temperature gradients between different zones inside the reactors. In the case of *T. aurantiacus*, these gradients could be even more extreme and effect even more the enzymatic activities (Maheshwari et al., 2000). Also, some compaction of the residue was observed that generates a loss of porosity in the solid bed and affect the results obtained. Furthermore, leached was observed in both fermentations. The leachate could indicate that the optimum humidity found on a laboratory scale is excessive once the scale had been changed and also altere the enzymatic activities results.

β-glucosidase activity in the *A. niger*'s fermentation were always equal or below to 1.0 $U \cdot g^{-1}DM$, resulting in low levels of this enzyme obtained. However, in the *T. aurantiacus*'s fermentation was observed a peak of 4.3 ± 0.6 $U \cdot g^{-1}DM$ at 72 h of fermentation, that is the same fermentation time of the xylanase peak observed. This fact could be due to the synergy of the enzymes to hydrolyse the lignocellulosic structure (Naraian and Gautam, 2018). The antioxidant activity started increasing after 8 h of

fermentations and this trend continued during the fermentations. However, a significant decrease of the antioxidant activity was observed at the last hours of the *A. niger*'s fermentation.

The hydroxycinnamic acids are normally correlated with the antioxidant activity of the extracts from BSG (McCarthy et al., 2013). These acids are linked to lignin and hemicellulose, thus the enzymes produced by the fungal strains release the free phenolics, increasing the antioxidant activity (McCarthy et al., 2013). The antioxidant activity values obtained before the 40 h of fermentation were lower than the initial value. This fact may be due to the biodegradation of the antioxidants in the first hours of the fermentations by the fungal strains (Sharma and Arora, 2013). However, when the cellulase and the xylanase activities increased, the lignocellulolytic enzymes produced started to hydrolyse the fibres releasing phenolic compounds from the BSG (Leite et al., 2019). This fact increased the antioxidant activity until the last hours of fermentation, when the lignocellulolytic enzymes activities started to decrease and the releasing of phenolics compounds decreased too. The antioxidant activity remained constant at the end of the *T. aurantiacus*'s fermentation. However, in *A. niger*'s fermentation a significant decrease of the antioxidant activity could be observed at the end of the fermentation, that could be a result of the degradation of these compounds by the laccases produced by the fungal strain (Andlar et al., 2018; Iqbal et al., 2018).

6.2 Bench-scale SSF (Dewar reactors)

Operation scale was increased 10-fold by performing the SSF in the 5-L Dewar reactors working with 1.1 kg BSG. The Dewar® vessels are near adiabatic reactors, implying that the heat losses through the vessel walls are negligible and heat is exchanged only through the lid. This configuration is attractive considering that the self-heating of the solid bed serves as a driving force to promote the fungal growth, requiring limited or null external

heat supply. Furthermore, such a configuration resembles the conditions found in large-scale SSF process where the thermal conductivity of the bed limits the heat transfer to the surroundings. This reproduces the behaviour within the core of a larger volume of solid, in other words, the core of a larger reactor. Also, Dewar reactors are commonly used for composting or SSF systems and there are several studies that demonstrate their efficiency in these processes (Cerda et al., 2017; Gea et al., 2007; Mejias et al., 2018; Santis-Navarro et al., 2011).

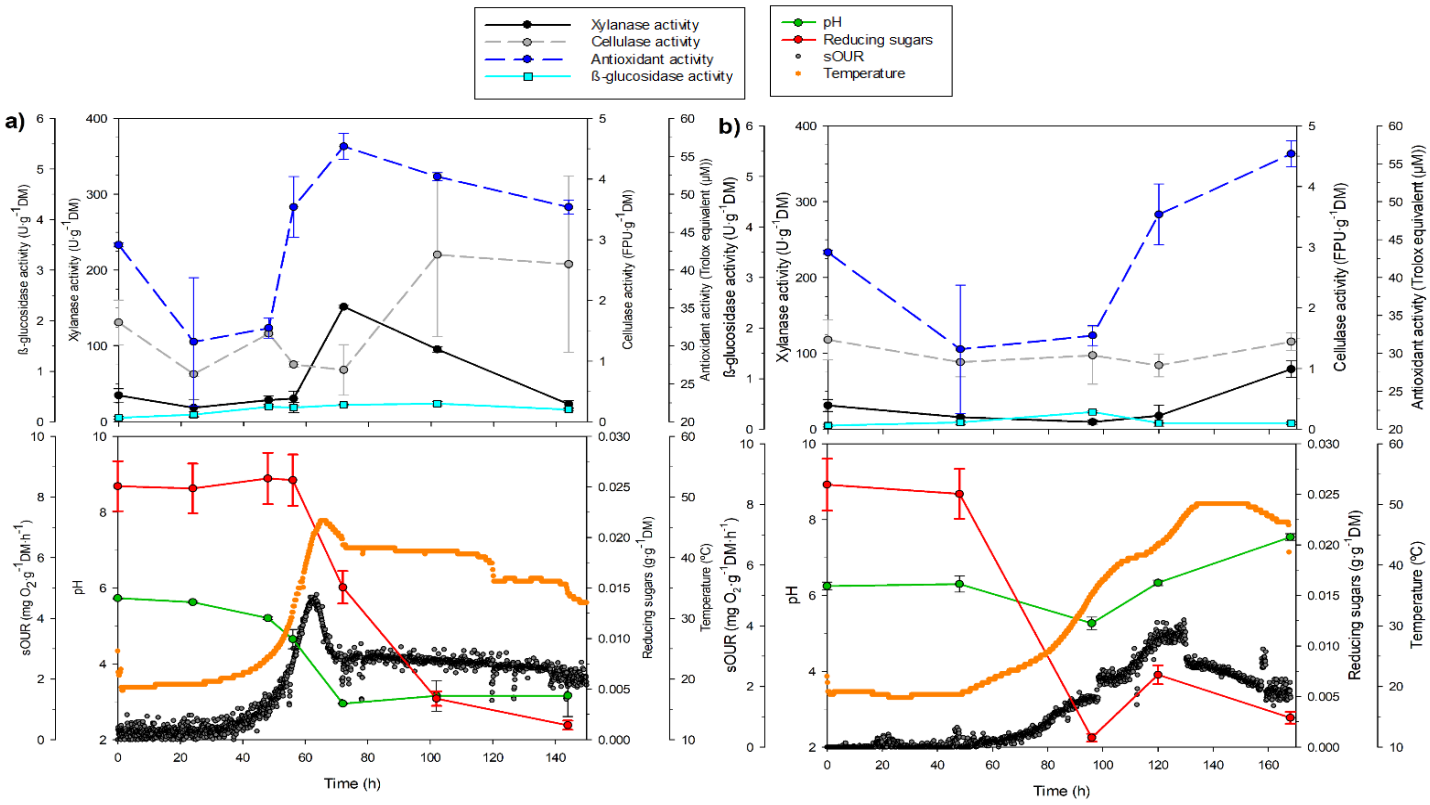

Figure 6.2 Time-course of the solid-state fermentation of brewer's spent grain (BSG) for lignocellulolytic enzyme production at the optimal conditions with *A. niger* (a) and *T. aurantiacus* (b) at bench-scale using Dewar reactors. sOUR: Specific oxygen uptake rate; DM: dry BSG; FPU: filter paper activity unit; U: xylanase or β-glucosidase activity unit.

As detailed in Figure 6.2, compared with the PVC reactors, Dewar reactors presented a longer lag phase especially in the case of T. auranticus, that took almost 80 h to start the fermentation. This fact directly affected the levels of enzyme obtained, and the results were lower than with the PVC reactors (section 6.1). The longer lag phase of the Dewar

reactors may could be affected by the initial substrate temperature. The substrate was inoculated and prepared at room temperature, and on winter months (when the Dewar reactors SSF were conducted) this could affect even more the initial temperature of the substrate, and therefore hindering the growth of the fungus and delaying the start of the fermentation. Also, during the winter months the laboratory temperature changes during the day and the night could affect the fungal growth too. It is important to consider that the minimum growth temperature for T. auranticus is 30ºC (Deploey, 1999). However, some studies had observed the maintenance of T. auranticus growth at 25ºC (Maheshwari et al., 2000). In order to take advantage of the self-heating capacity of the strains and reduce the operational cost of the process no temperature control was applied in both strains SSF. For future experiments working with this type of reactor, the effect of the initial temperature of the substrate should be assessed. Increasing the initial temperature of the substrate could be an important step to reduce this lag-phase of the oxygen consumption.

On the other hand, unlike with the PVC reactors, working with T. auranticus with Dewar reactors did not need to be temperature controlled to reach the desired growth temperature. This is because Dewar reactors are near adiabatic and then the self-heating capacity can properly maintain the temperature for biological growth and the fermentation can start, even though it needs more time.

The cellulase and xylanase enzymatic activities were lower than those obtained at lab-scale and with PVC reactors, although in the case of T. auranticus, the fermentation was not followed after 168 h and the trend was upward. However, in terms of productivity, the initial results were significantly lower after 160 h of fermentation, no needing to extend the fermentation monitoring further.

In *A. niger's* fermentation the maximum xylanase activity was reached at 72 h with 151.8 ± 1.9 $U \cdot g^{-1}DM$ (2.1 $U \cdot g^{-1}DM \cdot h^{-1}$), and the maximum cellulase activity was close to 3.0 $FPU \cdot g^{-1}DM$. From the time-course of the *A. niger's* fermentation (Figure 6.2 a)) the relation of the temperature with the oxygen consumption by the fungal was clearly seen. The two parameters follow the same profile, making obvious the interaction between them and the effect of the fungal growth on the temperature due to the metabolic heat.

In T. auranticus's fermentation cellulase activity results were practically constant during the fermentation and the xylanase activity did not exceed the 100.0 $U \cdot g^{-1}DM$. These results suggest that, in this scale, the PVC reactors were more suitable than the Dewar reactors for xylanase and cellulase production, however the initial temperature of the residue is an important factor to take into account. Furthermore, having the scale-up in mind, the Dewar reactors represents the core of a larger reactor, so they are an interesting tool to emulate the behaviour at larger scales and analysed it, before the scale-up. The β-glucosidase activity results in both fermentations with the Dewar reactors were practically zero, and the antioxidant activity followed a similar profile to PVC fermentations, were the maximum value were observed at the last stages of the fermentations.

These results present Dewar reactors to be suitable to produce lignocellulolytic enzymes through SSF from BSG, specially with the perspective of a future scale-up. Moreover, the use of Dewar reactors could be interesting while working with thermophile microorganism to reduce the loss of heat and promote the growth with the self-heating. However, the initial temperature condition of the material had an important impact on the SSF with the Dewar reactors. This fact should be studied in future research, taking into account that at higher scales, with higher quantities of solid material, the SSF behaviour could change.

6.3 Bench-scale SSF (Tray-type reactors)

Another reactor configuration was tested to observe the differences between different configurations. Three trays, with 900 ± 4 g were placed inside the incubator, without applying forced aeration. This fact did not allow the saturation of the inlet air, and there was not any control system implemented to avoid the evaporation, and therefore, the alteration of the moisture content of the sample couldn't be controlled correctly.

During the fermentation with both strains, the temperature inside the incubator (37 or 45 ºC depending on the strain) caused the evaporation of the water and even the drying of the residue. This fact altered the enzyme production process in the case of *A. niger* and affected the behaviour of the fermentation. With *T. aurantiacus*, due to the higher working temperature, the BSG was dried in a few hours, hindering the fungal growth. Figure 6.3 shows the time-course of the *A. niger* SSF with the tray-type reactor.

Although working conditions were no set appropiately, we, still, aimed to assess the low-cost performace of this configuration. From Figure 6.3 it can be concluded that the xylanase activity levels for *A. niger* with the tray-type reactor were lower than the obtained with the PVC reactors, and the xylanase activity maximum peak (87.9 ± 3.7 $U \cdot g^{-1}$DM) was found at later stages of the fermentation (82 h). Dewar reactors had a similar behaviour with a higher maximum xylanase activity value of 151.8 ± 1.9 $U \cdot g^{-1}$DM at 72 h of fermentation. The cellulase activity levels found were higher than the obtained with the Dewar reactor, with a maximum of 3.7 ± 0.5 $FPU \cdot g^{-1}$DM, resulting in lower levels than the obtained with the PVC reactor.

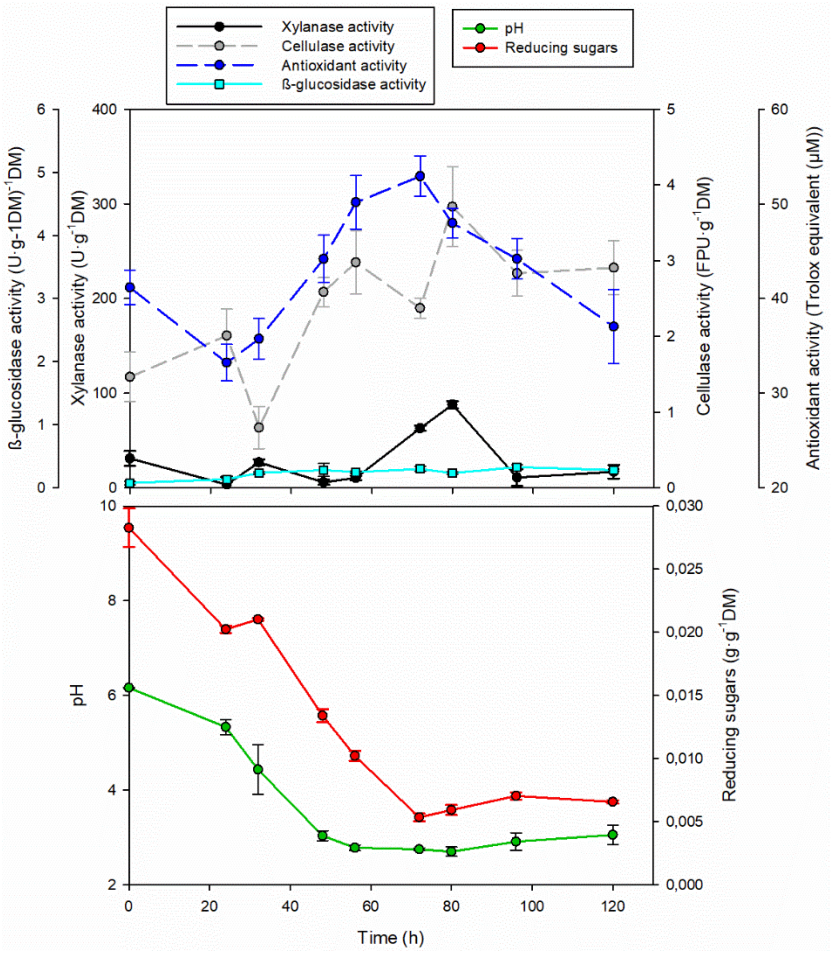

Figure 6.3 Time-course of the solid-state fermentation of brewer's spent grain (BSG) for lignocellulolytic enzyme production at the optimal conditions with *A. niger* at bench-scale using Tray-type reactors. DM: dry BSG; FPU: filter paper activity unit; U: xylanase or β-glucosidase activity unit.

The β-glucosidase activity levels were practically zero, similar to the Dewar reactors. Finally, the antioxidant profile was similar to the previous reactors, which suggests that BSG is an important source of this component with different reactors configurations.

For future research, a control system to minimize the evaporation rate should be implemented. Similar studies have used systems to control the evaporation rate as reported by Khanahmadi et al. (2018).

6.4 Comparative and analysis of the reactor configurations

Finally, Table 6.1 summarizes the results obtained in this study and compare these with some other studies. The aim of this table is to include studies that have used similar

producing strains and also BSG to produce lignocellulolytic enzymes to facilitate the comparison between the activities. The selected strain in a SSF process determine the specific enzymes produced. Also, the characteristics and the properties of the substrate are important factors because it is determinant to obtain the specific enzymes due to their fibres composition and furthermore, to be able to compare the results obtained (Novy et al., 2019).

Table 6.1 Lignocellulolytics enzymes production in different SSF systems using BSG as a substrate.

Strain	Reactor	Amount of substrate	Xylanase activity ($U \cdot g^{-1}DM$)	Cellulase activity ($FPU \cdot g^{-1}DM$)	B-glucosidase activity ($U \cdot g^{-1}DM$)	Reference
Aspergillus ibericus	Petri dishes	2 g	313.83 ± 5.25	-	4.06 ± 0.2	Leite et al. 2019
Aspergillus ibericus	0.5 L Erlenmeyer flask	40 g	50.0 ± 2.0	-	-	Sousa et al., 2018
Aspergillus awamori	Tray-type reactor	15 g	547.9	-	-	Costa et al., 2021
A. niger	Petri dishes	2 g	290.6 ± 0.1	-	3.9 ± 0.2	Leite et al. 2019
A. niger	0.25 L Erlenmeyer flask	2 g	1400.8 ± 43.9	6.2 ± 0.2	-	Moran-Aguilar et al. 2021
A. niger	0.5 L Erlenmeyer flask	100 g	230 ± 10	5.3 ± 1.3	-	Martínez-Avila et al., 2021
A. niger	0.5 L Erlenmeyer flask	100 g	309.0 ± 24.5	7.9 ± 1.0	-	Present study
A. niger	3.5 L PVC reactor	1100 g	245.5 ± 21.6	4.5 ± 0.2	1.0 ± 0.1	Present study
A. niger	5 L Dewar reactor	1100 g	151.8 ± 1.9	2.8 ± 1.4	0.4 ± 0.1	Present study
A. niger	Tray-type reactor	900 g	87.9 ± 3.7	3.7 ± 0.5	0.3 ± 0.1	Present study
T. aurantiacus	0.5 L Erlenmeyer flask	100 g	156.1 ± 15.8	3.5 ± 0.5	-	Present study
T. aurantiacus	3.5 L PVC reactor	1100 g	121.2 ± 4.2	1.8 ± 0.1	4.3 ± 0.6	Present study
T. aurantiacus	5 L Dewar reactor	1100 g	79.2 ± 11.0	1.5 ± 0.3	0.3 ± 0.0	Present study

DM: dry BSG; U: xylanase and B-glucosidase activity unit; FPU: filter paper unit. Values are presented as the mean ± the standard deviation.

Table 6.1 highlights the little amount of substrate used in the majority of the SSF studies using BSG nowadays, which does not correspond with the real problem of the large production of agro-industrial waste. It can be seen that the values of xylanase, cellulase and β-glucosidase activities obtained in other studies, with a few grams of BSG, are in the same range than the obtained in this present study, that used higher quantities of substrate. This suggests that the production of these products could be a real alternative to valorise the lignocellulosic substrates, that are a by-product of the food industry. Therefore, more efforts to change the scale of the SSF to produce lignocellulolytic enzymes are needed to obtain more robust results and consolidate SSF as a possible real alternative technology.

6.5 Conclusions

After the change of scale of the enzyme production process from BSG at the optimum conditions, the best results were obtained using *A. niger* with the PVC reactors with a maximum xylanase activity (245.5 ± 21.6 $U \cdot g^{-1}DM$, 5.1 ± 0.5 $U \cdot g^{-1}DM \cdot h^{-1}$ of productivity) at 48 h and a maximum cellulase activity (4.5 ± 0.2 $FPU \cdot g^{-1}DM$, 0.06 ± 0.00 $FPU \cdot g^{-1}DM \cdot h^{-1}$ of productivity) at 80 h. However, in terms of productivity, the best value obtained was at 48 h of fermentation with a value of 4.2 ± 0.4 $FPU \cdot g^{-1}DM$ of cellulase activity (0.09 ± 0.01 $FPU \cdot g^{-1}DM \cdot h^{-1}$ of productivity). For both strains, the PVC reactors worked better than the Dewar reactors, and in the case of *A. niger* the tray-type reactor, to produce enzymes in this scale. However, the possible heat transfer problems at larger scale presents Dewar reactors as a potential system to produce enzymes and *T. aurantiacus* as a potential source. Especially, because the performance of Dewars can be further improved controling the initial temperature of the substrate. Moreover, during the SSF the antioxidant activity of BSG increased in each strain, and in each reactor, making the BSG an interesting residue to obtain different value-added products. Also, the load of

solids used in the fermentations, gives robustness to the obtained results and makes them that come closer to the reality of the needs of the industry. The utilization of SSF combined with the BSG as a substrate presents a sustainable and economical alternative to the submerged fermentation in enzyme production, however, further research should be done at larger scales.

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Chapter 7 Efficiency of the obtained enzymatic extracts in the hydrolysis of different lignocellulosic substrates

Part of this chapter will be submitted to the journal Waste Management.

Introduction

Although several studies can be found on enzyme production through SSF, most of them focus on the productive step, and just a few on the applicability of the obtained extracts. Since defining the efficiency of these extracts is essential for future developments, in this chapter some of the enzymatic extracts obtained in Chapter 6 have been used to perform a hydrolysis using the same three lignocellulosic substrates proposed within the VALORA project: brewer's spent grain (BSG), grape pomace (GP), and olive-mill solid waste (OSW). As commented before, these substrates are derived from the local food industry and they are produced in large amounts. Also, their different characteristics made them interesting substrates to be tested to obtain fermentable sugars (Table 4.1).

Furthermore, the enzymatic extracts were tested on the hydrolysis of the solid fraction of the steam exploded substrates (BSG, GP, OSW) as an attempt to define their efficiency in a wider set of solid materials. The steam explosion (SE) pre-treatment is commonly used to fractionating lignocellulosic biomass and enhancing its enzymatic digestibility (Duque et al., 2016; Kemppainen et al., 2016). This pre-treatment uses thermal, chemical and mechanical effects to solubilize the hemicellulose fraction, break the lignocellulose fibres, alter the cellulose crystallinity and the lignin structure (Duque et al., 2016). It is an interesting technology due to the low energy consumption compared with the conventional milling techniques (Jacquet et al., 2015). In the SE pre-treatment, two factors mainly influence the efficiency of the process: the retention time and the pressure. The retention time is correlated with the hydrolysis of the hemicellulose fraction, and the pressure is correlated with the temperature and affect the hydrolysis of the cellulose fraction. After the process, a sugar-rich liquid stream and the remaining solid fraction are obtained. Commonly, the solid fraction of the SE is not valorised considering that the focus of the pre-treatment is the solubilization of the hemicellulose and cellulose fraction

into the liquid fraction (Jacquet et al., 2015). In that way, further exploiting that solid fraction appears as an attractive alternative to provide a more complete valorisation of these materials.

The hydrolysis on the lignocellulosic substrates was performed using two approaches: a conventional liquid enzymatic hydrolysis and solid-state enzymatic hydrolysis. While the liquid enzymatic hydrolysis is commonly used to obtain fermentable sugars from biomass, the solid-state enzymatic hydrolysis (SSEH) present attractive characteristics for the development of the process (Da Silva et al., 2020). SSEH is conducted using enzymes with high solid loads in the absence or near absence of free water (Modenbach and Nokes, 2013). SSEH requires low water and energy inputs, decreasing operating costs while working with small size equipment compared to conventional hydrolysis in a liquid phase (Koppram et al., 2014; Modenbach and Nokes, 2013). Furthermore, the similarities between SSF and SSEH made interesting to integrate these two processes to obtain a solid with high sugar content to use it then coupled with the SSF to produce the desired product (Chen and Liu, 2017; Guo et al., 2017; Martínez-Avila et al., 2021).

Figure 7.1 shows a block diagram of the processes performed in chapter 7 and the principal parameters analysed.

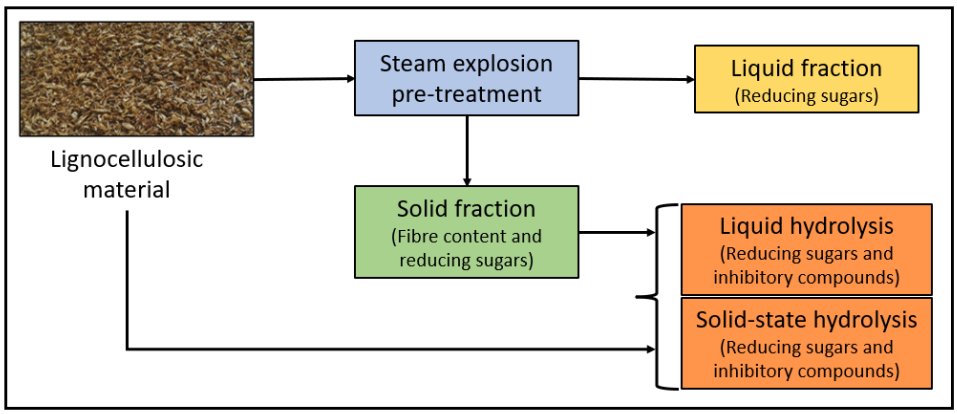

Figure 7.1 Block diagram of the processes performed in chapter 7. Between brackets the principal parameters analysed.

7.1 Steam explosion pre-treatment on the lignocellulosic substrates

As seen in Table 7.1, a screening at different levels of SF was performed (8 tests), evaluating different conditions of temperature, pressure, and time. These parameters are directly related to the severity factor (SF), calculated as mention in section 3.10 (Eq. 13), which is the main variable influencing the behaviour of the SE test. At the same time, SF is related to the degradation of the substrate, and the higher SF, the more severe is the process, with the expected higher degradation of the substrate. However, achieving the maximum degradation is not always the aim of this pre-treatment. In general, the goal of the vapor explosion is to solubilize hemicellulose and cellulose and inducing a change in the structure of lignin to promote enzyme-cellulose binding. The parameters that affect this pre-treatment are the origin of the biomass, the particle size, temperature, residence time and moisture content (Jacquet et al., 2015).

Table 7.1 Steam explosion tests using three lignocellulosic substrates and their severity factor.

Test	Substrate	SF
1	BSG	3.14
2	BSG	3.54
3	BSG	4.07
4	BSG	4.47
5	GP	4.47
6	GP	3.86
7	GP	3.15
8	OSW	3.85

The different tests for the SE pre-treatment were chosen based on the potential of each lignocellulosic substrate for releasing sugars, directly associated to their characteristics

(as mentioned in chapter 4). Thus, BSG appeared as the more promising substrate due to their low lignin content followed by GP and then by OSW. Consequently, 4 tests included BSG (1-4), 3 tests included GP (5-7), and the last one was devoted to OSW (8).

After the SE pre-treatment, two fractions were collected for each test. On one side, a liquid fraction in which the main characteristic was the reducing sugar content consequence of the solubilization of the hemicellulose and the cellulose. On the other side, the exploded solid fraction, containing the remains of the pre-treatment. Figure 7.2 summarises the sugar content found in the liquid fraction of each test.

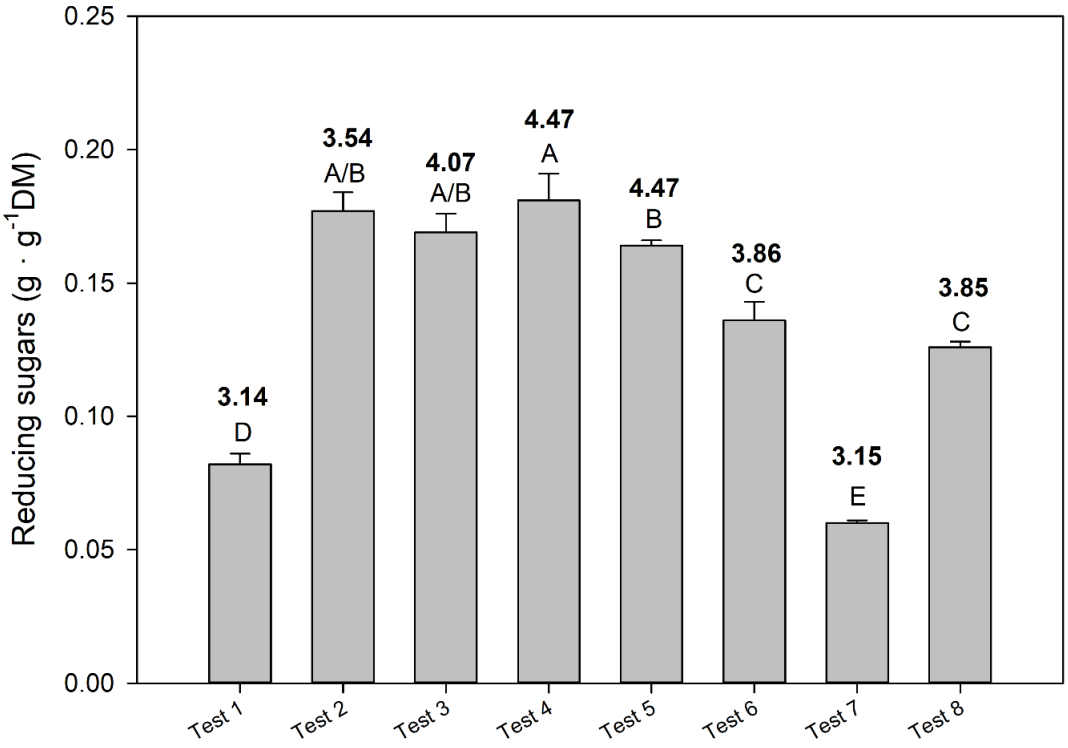

Figure 7.2 Reducing sugars content in the liquid fraction of the steam explosion pre-treatment. Different capital letters indicate significant differences between the evaluated groups ($p<0.05$) based on the Tukey test analysis. Numbers in bold font indicate the SF of each sample.

As observed, three of the tests using BSG (2, 3 and 4) released similar reducing sugars levels reaching up to 0.18 $g \cdot g^{-1}$DM. On the contrary, test 1, characterized by the lowest SF, only released around 50% of the sugars obtained in the rest tests with BSG. The

results are in concordance with Ravindran et al. (2018), that performed a steam explosion pre-treatment to BSG. These results show a proportional behaviour among SF and sugars released, something expected in lignocellulosic material of this characteristic. However, it was not a linear proportionality.

On the other hand, increasing the SF in GP resulted in the opposite result compared to BSG. In this case, the highest sugars released was obtained at the lowest SF (test 5) reaching up to 0.16 $g \cdot g^{-1}$DM. Despite the stringent conditions, in this substrate the increase in SF could be promoting the solubilization of other compounds different than sugars, affecting its efficiency. Finally, in the test with OSW using an intermediate SF, the release of sugars reached up to 0.13 $g \cdot g^{-1}$DM. Although was significantly different compared to the other substrates, it made sense considering the big differences in the fibres content among the tested substrates. Along with the change in the sugar's composition, the SE also affect the remaining solid fraction. This can be directly seen from the appearance of the exploded materials as it can be observed in Figure 7.3.

Figure 7.3 Appearance of the lignocellulosic substrates before and after the SE pre-treatment. a) raw BSG; b) raw GP; c) raw OSW; d) dry pre-treated BSG; e) dry pre-treated GP; f) dry pre-treated OSW.

However, the main change of this fraction after SE corresponds to the fibres content. Figure 7.4 shows the cellulose, hemicellulose and lignin content of the original substrates and their exploded counterparts.

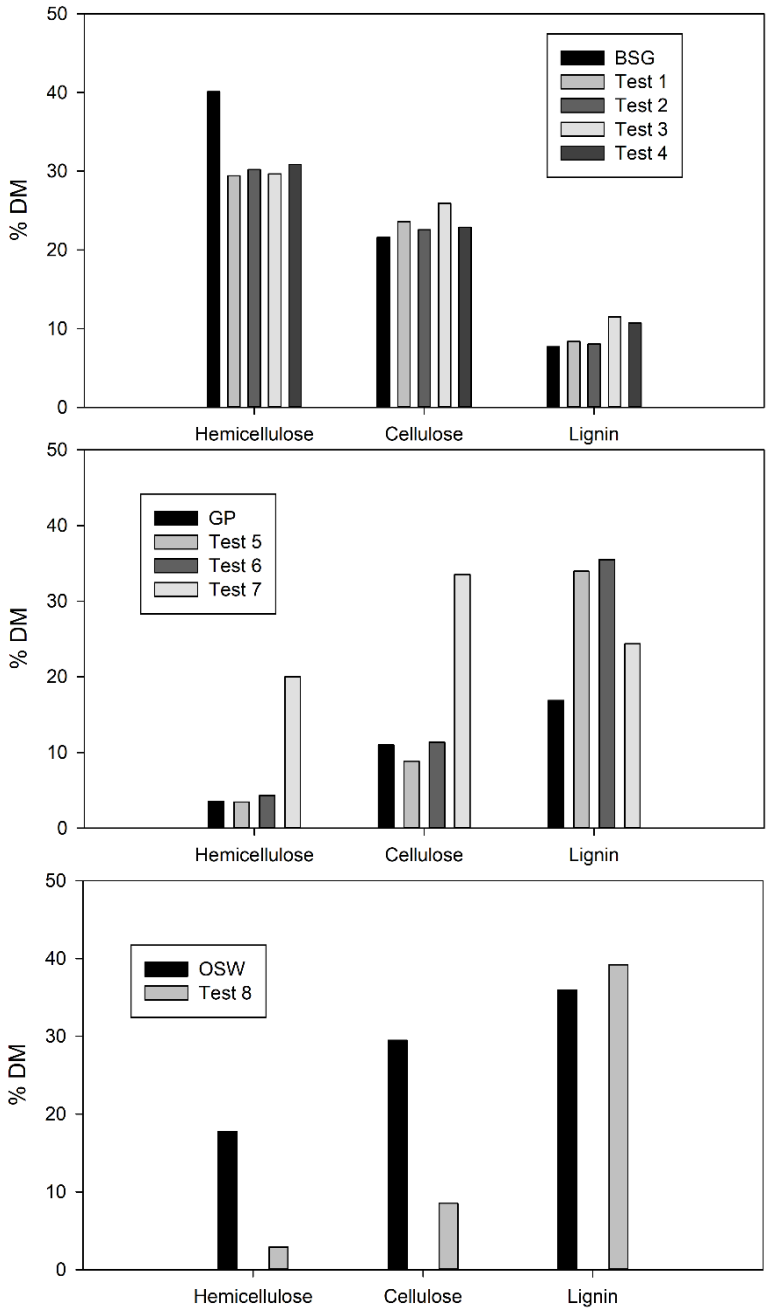

Figure 7.4 Fibre content of the lignocellulosic substrates before and after the SE pre-treatment.

Based on these results, it can be stated that the best hemicellulose solubilization yields (into the liquid fraction) were observed in the BSG and OSW (initially these two

substrates contained 40% and 17% hemicellulose, respectively). In the case of BSG, only the solubilization of hemicellulose was observed, while the cellulose and lignin levels have remained almost invariable regardless of the severity factor. The SE process also solubilized a significant fraction of cellulose when OSW was pre-treated, observing a decrease in hemicellulose and cellulose in the solid fraction of 84% and 71%, respectively. This solubilization was not such evident in the case of SE with GP. Test 5 alone showed a slight decrease of 2% in hemicellulose and 19% in cellulose. It is important to note that the initial hemicellulose and cellulose content of GP was lower than that of other substrates. The variation in the lignin composition may depend on the severity of the pre-treatment: a relative increase in the lignin composition is observed when the severity of the pre-treatment increases, due to depolymerization/ repolymerization of the lignin structure (Kim, 2018). In test 7, with higher SF, a significant increase in the content of hemicellulose and cellulose was observed, probably due to a rearrangement of the fibres. Also, higher content of crude fibre was found in the fibre analysis for Test 7.

After the change in their structure, the solid exploded substrates ended up with a reducing sugars content higher than the one in their not-pre-treated counterparts (see Table 4.1). Table 7.2 presents the content of reducing sugars obtained in the solid fraction of the SE.

Table 7.2 Reducing sugars content on the not-pre-treated and pre-treated with SE solid samples.

Sample	BSG	GP	OSW	T1	T2	T3	T4	T5	T6	T7	T8
Reducing sugars ($g \cdot g^{-1}DM$)	0.03	0.07	0.01	0.04	0.06	0.05	0.05	0.23	0.24	0.23	0.03

As seen, GP pre-treated samples had the higher reducing sugar content with 0.23-0.24 $g \cdot g^{-1}DM$. BSG pre-treated samples had lower values between 0.04-0.06 $g \cdot g^{-1}DM$ and

OSW had the lowest value among the pre-treated substrate with 0.03 $g \cdot g^{-1}$DM. These were the initial values of reducing sugars from which hydrolysis started.

7.2 Liquid enzymatic hydrolysis

As commented before, the efficiency of the selected enzymatic extracts was tested in the substrates with and without pre-treatment (11 in total) using the same conditions found in chapter 4, with a 5% (w/v) substrate:extract ratio, 45ºC, and monitoring the reducing sugar content on the hydrolysates up to 48 h.

On the other hand, the selected enzymatic extracts included two of the self-produced extracts obtained in chapter 6, as well as a commercial enzymatic mixture (Viscozyme 1%) as a manner of control. The self-produced extracts were obtained using *A. niger* in the PVC reactors configuration. In particular, extracts were collected at two fermentation times aiming to obtain a first extract richer in xylanases (Extract 1) and a second one richer in cellulases (Extract 2). Self-produced extracts were filtered using a 0.45 mm membrane. It is relevant to highlight that these extracts were not further purified in contrast with the commercial enzymatic extract used as a control. Table 7.3 presents the xylanase and cellulase activities of each extract used in the hydrolysis experiments.

Table 7.3 Xylanase and cellulase activity of the enzymatic extracts used in the hydrolysis.

Enzymatic activity	Viscozyme (1%) (Vis)	Extract 1 (E1)	Extract 2 (E2)
Xylanase activity (U·mL)	33421 ± 861	22560 ± 66	20875 ± 728
Cellulase activity (FPU·mL)	333 ± 5	140 ± 14	283 ± 6

The initial enzymatic activities from Vizcozyme were slightly higher than in the self-produced extracts. However, these were in the same order of magnitude, in line with observations in chapter 4.

Figure 7.5 shows the monitoring of the hydrolysis in liquid phase for the BSG samples. It can be seen that the self-produced extracts resulted more efficient to release sugars on the non-pre-treated BSG. As expected, the control using Viscozyme showed a significant release of sugars in all the tested materials. From pre-treated BSG, test 4 obtained the best sugar levels using Viscozyme reaching up to 0.35 ± 0.01 $g \cdot g^{-1}$DM. However, the non-pre-treated sample obtained higher reducing sugar levels than the pre-treated samples regardless of the extract used obtaining 0.5 ± 0.04 $g \cdot g^{-1}$DM using Viscozyme. Such a result was somehow expected, considering that part of the sugars in the pre-treated samples ended up into the liquid fraction of the exploded material.

In the case of the not-pre-treated BSG, the self-produced extracts obtained slightly lower reducing sugar levels than Viscozyme. However, the sugars released per unit of xylanase activity of the extracts (Vis, E1 and E2) used resulted in similar level with each extract ($1.2 \cdot 10^{-5}$x g $RS \cdot g^{-1}DM \cdot UA^{-1}$ approx.).

The efficiency per unit of xylanase activity compared to Viscozyme found at the maximum reducing sugar levels was 96.8% for E1 and 110.7% for E2. This presents the self-produced extracts as a potential competitive system to obtain enzymatic extracts to hydrolyse the BSG. Similarly, the maximum productivity obtained with the not-pre-treated BSG sample was found at 13 h of fermentation with 0.021 ± 0.002 g $RS \cdot g^{-1}DM \cdot h^{-1}$ using Viscozyme. The pre-treated BSG samples maximum productivity was found between 13h and 18 h of fermentation with values between 0.010 g $RS \cdot g^{-1}DM \cdot h^{-1}$ and 0.015 g $RS \cdot g^{-1}DM \cdot h^{-1}$ using Viscozyme.

E1 and E2 reached a maximum productivity with the not-pre-treated BSG sample at 13 h of fermentation with 0.013 ± 0.002 g $RS \cdot g^{-1}DM \cdot h^{-1}$ and 0.012 ± 0.002 g $RS \cdot g^{-1}DM \cdot h^{-1}$

respectively. For the pre-treated samples, the maximum productivity obtained by E1 and E2 was with Test 4 at 13 h of fermentation with 0.005 ± 0.001 g RS·g^{-1}DM·h^{-1}.

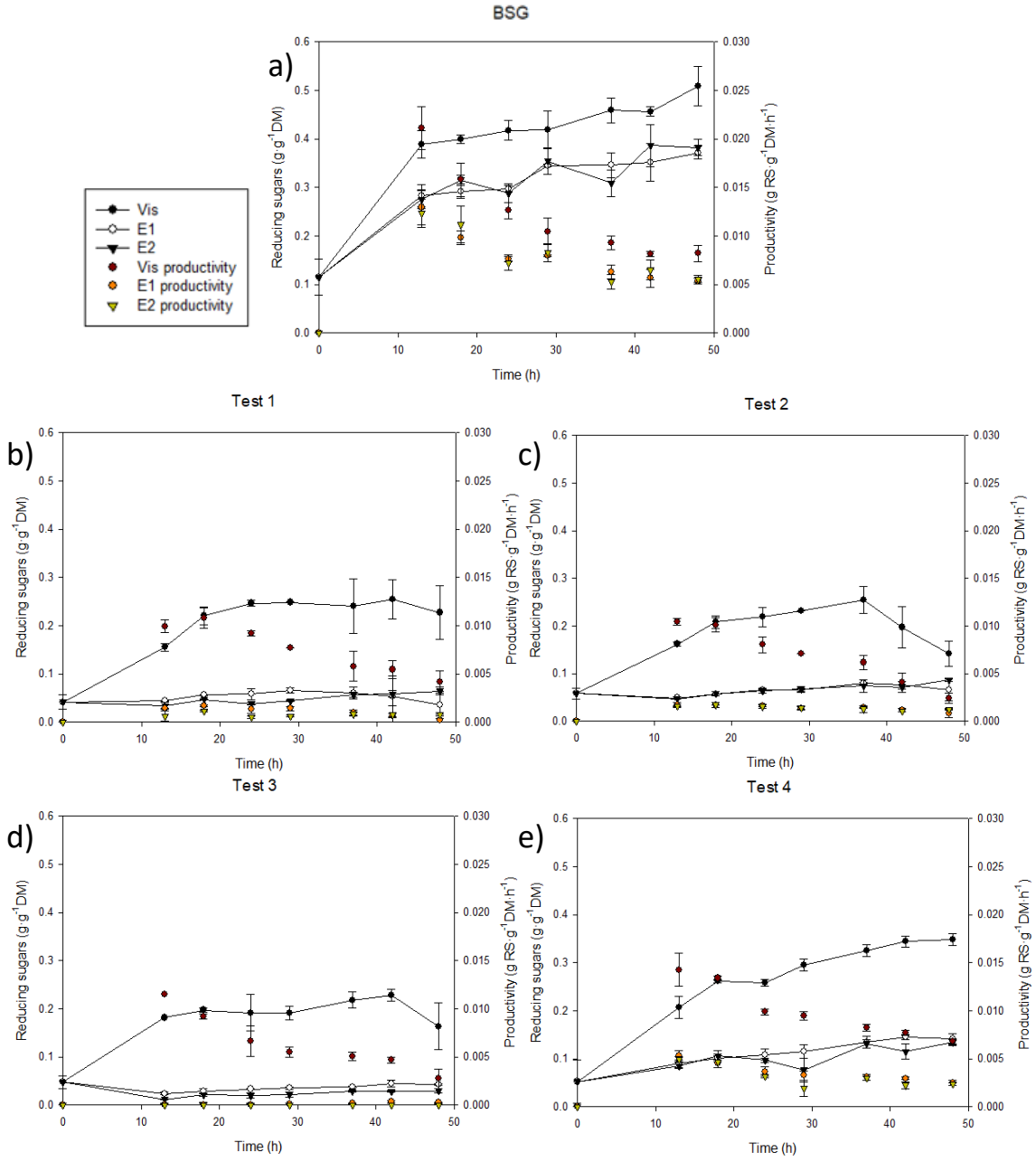

Figure 7.5 Reducing sugars evolution and productivity of the pre-treated and not-pre-treated BSG samples during the liquid hydrolysis. a) not-pre-treated BSG; b) BSG Test 1; c) BSG Test 2; d) BSG Test 3; e) BSG Test 4; RS: reducing sugars; Vis: Viscozyme; E1: extract 1; E2: extract 2.

Figure 7.6 presents the liquid hydrolysis with GP samples. In this case, similar trends were found compared to the hydrolysis of the BSG samples. While the self-produced

extracts performed better on the not-pre-treated GP compared to the pre-treated ones, the control with Viscozyme resulted more effective than the self-produced extracts in all the evaluated samples. Also, the non-pre-treated GP sample presented a similar behaviour to the non-pre-treated BSG sample, with similar results per unit of xylanase activity for the three extracts tested ($1.3 \cdot 10^{-5}$x g RS·g^{-1}DM·UA^{-1} approx.). The efficiency per unit of xylanase activity compared to Viscozyme found at the maximum reducing sugar levels was 96.0% for E1 and 91.0% for E2. For the pre-treated GP samples, the Test 5 obtained the best released sugars results with slightly lower levels than the non-pre-treated using Viscozyme.

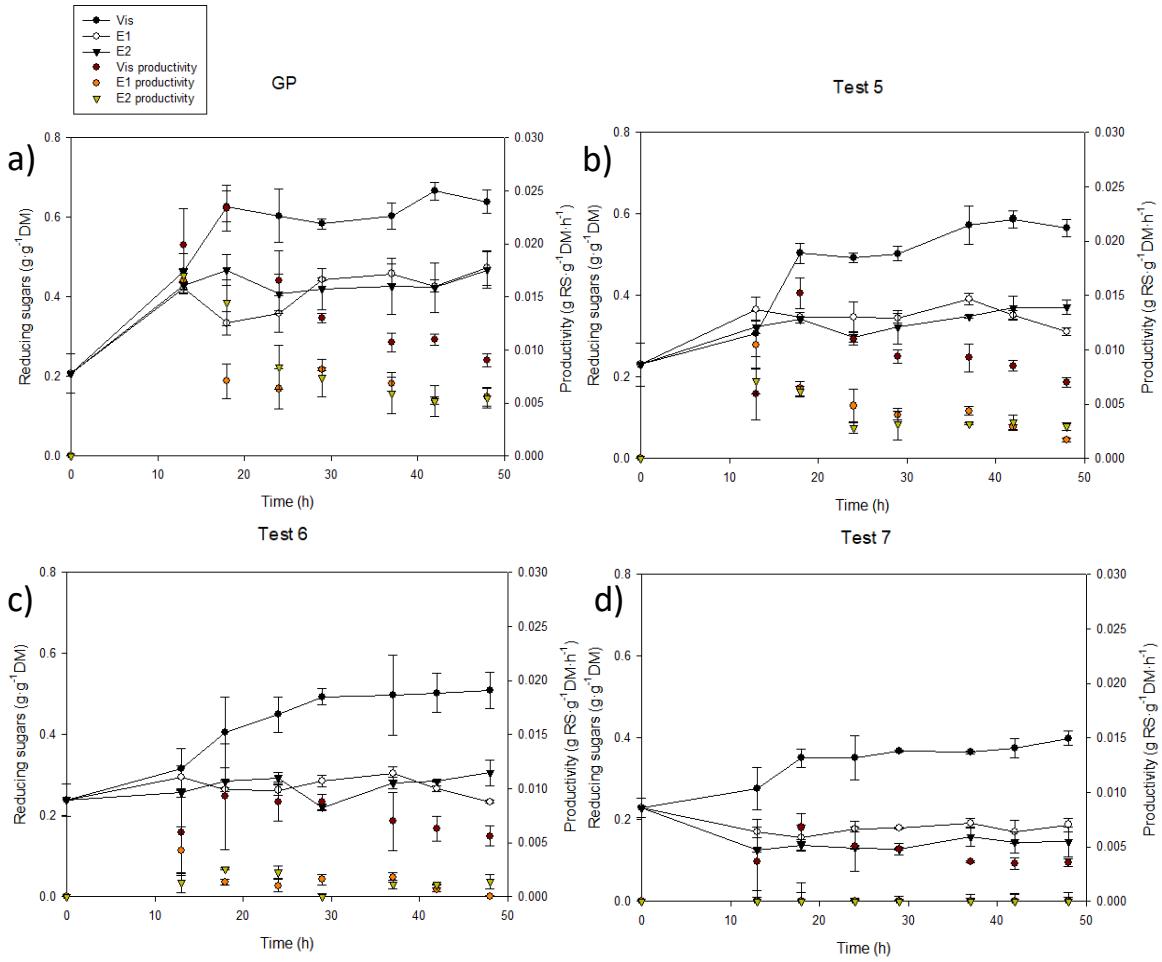

Figure 7.6 Reducing sugars evolution and productivity of the pre-treated and not-pre-treated GP samples during the liquid hydrolysis. a) not-pre-treated GP; b) GP Test 5; c) GP Test 6; d) GP Test 7; RS: reducing sugars; Vis: Viscozyme; E1: extract 1; E2: extract 2.

Regarding the productivity, the maximum was reached with the not-pre-treated GP at around 18 h of fermentation with 0.023 ± 0.002 g RS·g^{-1}DM·h^{-1} using Viscozyme. Among the pre-treated GP, Test 5 obtained the higher productivity that was found at 18 h of fermentation with 0.015 ± 0.001 g RS·g^{-1}DM·h^{-1} using Viscozyme. Test 6 and Test 7 obtained values of maximum of productivity below 0.01 g RS·g^{-1}DM·h^{-1}.

E1 and E2 reached a maximum productivity with the not-pre-treated GP sample at 13 h of fermentation with 0.017 ± 0.003 g RS·g^{-1}DM·h^{-1} and 0.017 ± 0.002 g RS·g^{-1}DM·h^{-1} respectively. For the pre-treated samples, the maximum productivity obtained by E1 and E2 was with Test 5 at 13 h of fermentation with 0.011 ± 0.002 g RS·g^{-1}DM·h^{-1} and 0.007 ± 0.001 g RS·g^{-1}DM·h^{-1}.

Finally, only the Viscozyme obtained significant reducing sugar levels on the OSW samples (Figure 7.7) and the self-produced extracts resulted inefficient to release sugars from this substrate. Both samples (pre-treated and non-pre-treated) obtained similar results and no significant differences were detected.

In this case, the maximum productivity with the not-pre-treated OSW sample was found at 13 h of fermentation with 0.012 ± 0.002 g RS·g^{-1}DM·h^{-1} using Viscozyme. The pre-treated GP sample obtained a similar maximum productivity at 13 h of fermentation with a value of 0.010 ± 0.002 g RS·g^{-1}DM·h^{-1} using Viscozyme.

The productivity of the self-produced extracts on the OSW was always below 0.003 g RS·g^{-1}DM·h^{-1}, for the not-pre-treated and the pre-treated sample.

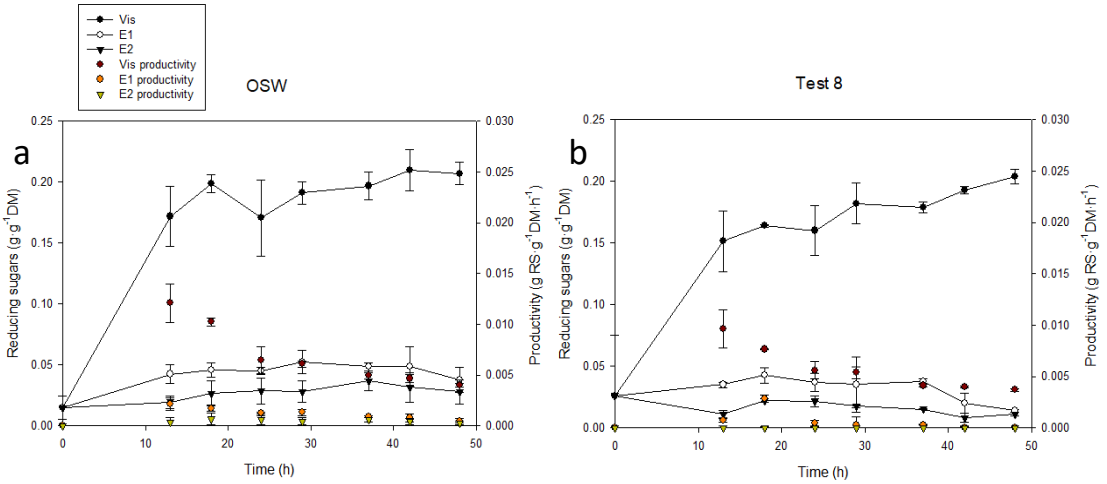

Figure 7.7 Reducing sugars evolution and productivity of the pre-treated and not-pre-treated OSW samples during the liquid hydrolysis. a) not-pre-treated OSW; b) OSW Test 8; RS: reducing sugars; Vis: Viscozyme; E1: extract 1; E2: extract 2.

In general, the self-produced extracts only obtained competitive results (compared to Viscozyme) on the not-pre-treated samples of BSG and GP. As expected, Viscozyme obtained significant reducing sugars levels on the pre-treated samples of the three lignocellulosic substrates. Since the self-produced extracts were obtained from a SSF using BSG as a substrate, it could be expected that the obtained enzymes were influenced by the BSG fibre structure (Malherbe and Cloete, 2002). In this sense, the produced enzymes were more suitable to hydrolyse the BSG, but they did not work on the OSW, maybe due to the high lignin composition of this substrate (36.0 ± 0.9 % DM) compared with the BSG (7.7 ± 0.1% DM), that did not allow the access of the enzymes to the cellulose or the hemicellulose, or enzymes were trapped within lignin (Leite et al., 2021). The low lignin content of the BSG entailed that the group of enzymes responsible to degrade the lignin were not produced. Also, in the midpoint, GP, with a lower lignin composition than OSW (16.9 ± 0.6 % DM) made this lignocellulosic substrate more suitable to be degraded by the self-produced extracts.

Regarding the maximum sugars released among the tested substrates, BSG and GP obtained the best results reaching up to 0.39 ± 0.04 and 0.45 ± 0.02 g RS ·g^{-1}DM respectively in the case of the non-pre-treated sample. These results confirm that BSG and GP were more suitable to obtain sugars than OSW. From the pre-treated samples, the highest reducing sugars levels were found in Test 4 (BSG) with 0.30 ± 0.01 g RS ·g^{-1}DM and Test 5 (GP) with 0.36 ± 0.02 g RS ·g^{-1}DM. All these maximum sugars released were obtained using Viscozyme.

With the self-produced extracts, the maximum sugars released with BSG and GP obtained 0.27 ± 0.01 and 0.27 ± 0.04 g RS ·g^{-1}DM with E1 and E2 respectively in the case of the non-pre-treated sample. From the pre-treated samples of these substrates, the highest reducing sugars levels were found in Test 4 (BSG) with 0.09 ± 0.01 g RS ·g^{-1}DM and Test 5 (GP) with 0.16 ± 0.02 g RS ·g^{-1}DM using E1.

Table 7.4 presents the summary of efficiencies for the self-produced extracts compared to Viscozyme on the different lignocellulosic substrates studied. Efficiency should be assessed keeping in mind that self-produced extracts are not purified like the commercial extract. However, getting good efficiencies without the downstream part could economically improve the process.

Table 7.4 Efficiency of the self-produced extracts on the releasing of sugars on the different substrates respect to Viscozyme on the liquid hydrolysis

Self-produced extract	**BSG**	**GP**	**OSW**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**
E1 (%)	66.7	58.7	20.0	9.5	10.0	0.0	30.0	44.4	25.9	0.0	27.8
E2 (%)	69.2	56.5	10.0	9.5	15.0	0.0	26.7	3.9	25.9	0.0	0.0

From table 7.4, it can be concluded that the two self-produced extracts have a similar efficiency in almost all the substrates although the actual reducing sugars levels are

different. The most relevant differences between the two self-produced extracts were the Test 5 and the Test 8, that were pre-treated samples of GP and OSW respectively. The higher efficiency results of the self-produced extracts were obtained using the not-pre-treated BSG and GP, with 67-69% of efficiency for the BSG and 57-59% of efficiency for the GP. Results show that the pre-treated samples were significantly less efficient than the not-pre-treated counterparts, always below the 50%. The best performance among the pre-treated samples was using E1 on the Test 5, that obtained an efficiency of 44%. The self-produced extracts had a very low efficiency on the OSW samples.

Figure 7.8 shows the total reducing sugars released in the combined process SE + hydrolysis (*i.e.*, liquid fraction of the SE + liquid hydrolysate) compared to the raw residues.

In the hydrolysis of BSG using Viscozyme, only the Test 4 released more reducing sugars than the raw BSG. Test 2 and 3 reached similar reducing sugars release to the non-pre-treated BSG. Using the self-produced extracts, only Test 4 reached similar reducing sugars levels to the raw BSG.

For GP, when Viscozyme and E1 were used, Test 5 and 6 reached similar reducing sugars levels to the raw GP, or maybe higher in the case of Test 5 using Viscozyme. For E2, only Test 6 reached similar reducing sugars levels to the raw GP.

Finally, in the case of OSW, the pre-treated samples reached higher reducing sugars levels than the non-pre-treated OSW regardless of the extract used (Viscozyme, E1 or E2). However, in this case, most of the reducing sugars were from the SE liquid fraction, due to the hydrolysis of this lignocellulosic substrate was not successful with the self-produced extracts.

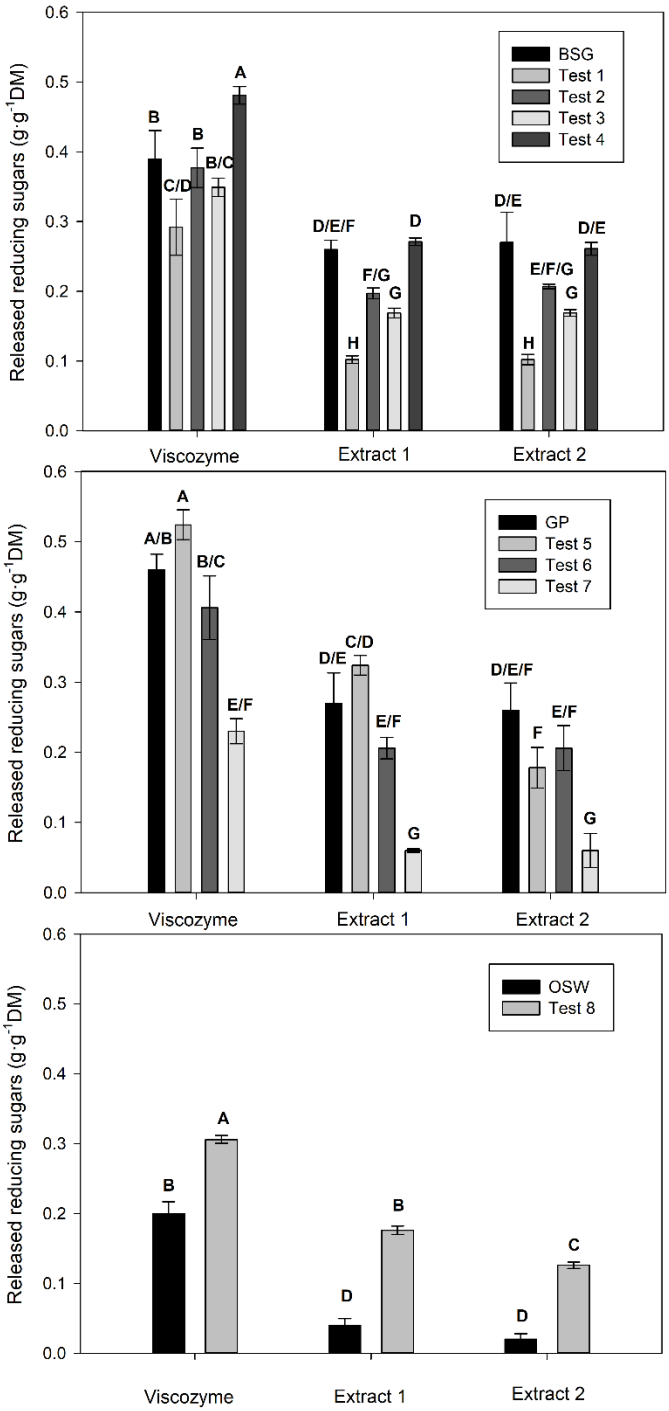

Figure 7.8 Reducing sugars released during the liquid enzymatic hydrolysis + the liquid fraction of the SE. Different capital letters indicates significant differences between the evaluated groups ($p<0.05$) based on the Tukey test analysis. For raw BSG, GP and OSW the values represent their original content.

These results showed that with the SE pre-treatment could be used to achieve more reducing sugars in the case of BSG and GP, in the Viscozyme samples. This suggests that

improving the purification or extraction of the self-produced extracts could lead to results similar to those found with Viscozyme.

7.3 Solid-state enzymatic hydrolysis

The solid-state hydrolysis was conducted with the same extracts used in the liquid hydrolysis. As mentioned in chapter 3 (section 3.6), the hydrolysis was followed up to 48 h by measuring the reducing sugars released in the supernatant after a solid-liquid extraction with water in a 1:15 (w/v) ratio. The hydrolysis was conducted at 35°C as suggested by Martínez-Ávila et al. (2021).

For the BSG (Figure 7.9), the solid-state hydrolysis only worked in the non-pre-treated sample and only Viscozyme had a significant release of sugars. Test 2 hydrolysed with Viscozyme was the best performer among the pre-treated samples, but the levels of released sugars were consistently low. Compared with the liquid hydrolysis, the solid-state hydrolysis had not a correct performance to hydrolyse the BSG.

The maximum productivity obtained with the not-pre-treated BSG sample was found at 18 h of fermentation with a value of 0.006 ± 0.001 g $RS \cdot g^{-1}DM \cdot h^{-1}$ using Viscozyme. The pre-treated BSG samples maximum productivity was found between 6h and 18 h of fermentation with values always below 0.005 g $RS \cdot g^{-1}DM \cdot h^{-1}$ using Viscozyme.

The productivity of the self-produced extracts on the BSG was always below 0.005 g $RS \cdot g^{-1}DM \cdot h^{-1}$, for the not-pre-treated and the pre-treated sample.

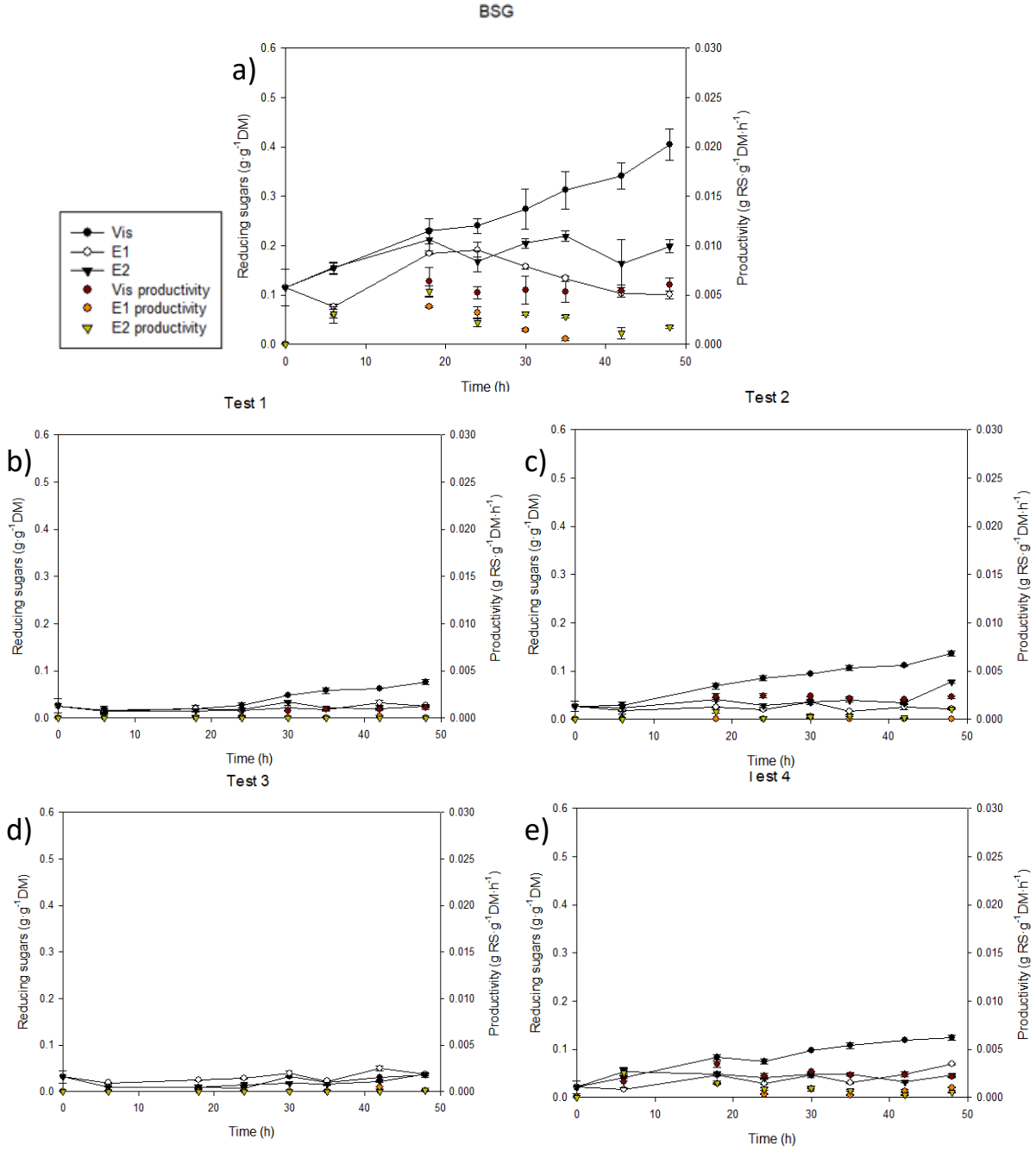

Figure 7.9 Reducing sugars evolution and productivity of the pre-treated and not-pre-treated BSG samples during the solid-state hydrolysis. a) not-pre-treated BSG; b) BSG Test 1; c) BSG Test 2; d) BSG Test 3; e) BSG Test 4; RS: reducing sugars; Vis: Viscozyme; E1: extract 1; E2: extract 2.

Figure 7.10 present the solid-state hydrolysis of the GP samples. In the case of GP, similar reducing sugar levels to the liquid hydrolysis were obtained in the non-pre-treated sample and the Test 5. However, Test 6 and 7 did not achieve the same sugar levels. The self-

produced extract worked similar to the Viscozyme in the non-pre-treated sample and in the Test 5.

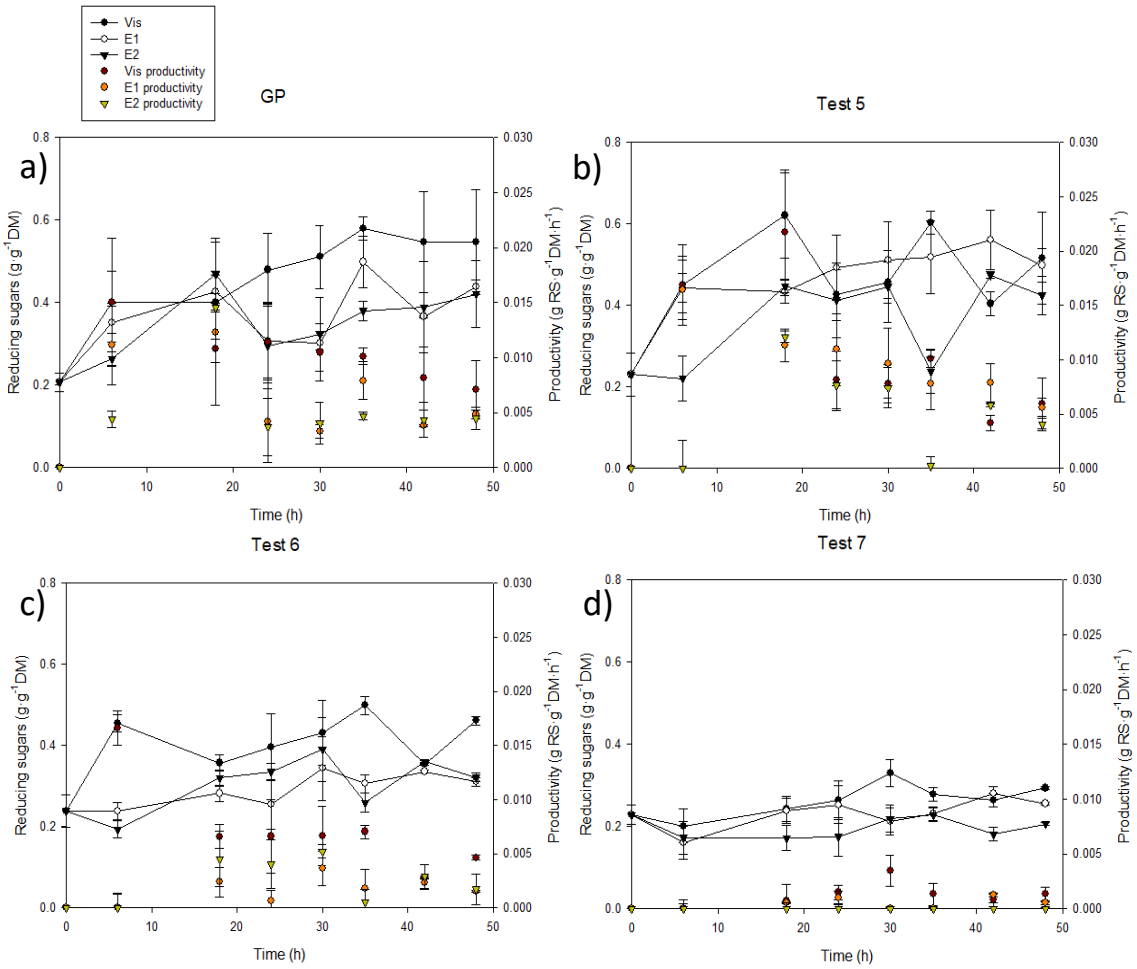

Figure 7.10 Reducing sugars evolution and productivity of the pre-treated and not-pre-treated GP samples during the solid-state hydrolysis. a) not-pre-treated GP; b) GP Test 5; c) GP Test 6; d) GP Test 7; RS: reducing sugars; Vis: Viscozyme; E1: extract 1; E2: extract 2.

The maximum productivity obtained with the not-pre-treated GP sample was found at 6 h of fermentation with a value of 0.015 ± 0.005 g RS·g^{-1}DM·h^{-1} using Viscozyme. Among the pre-treated GP samples Test 5 obtained the higher value of maximum productivity that was found at 18 h of fermentation with a value of 0.021± 0.005 g RS·g^{-1}DM·h^{-1} using Viscozyme. Test 6 and Test 7 obtained values of maximum of productivity below 0.01 g RS·g^{-1}DM·h^{-1}. E1 and E2 reached a maximum productivity with the not-pre-treated GP

sample at 6 and 18 h of fermentation with 0.016 ± 0.002 g RS·g^{-1}DM·h^{-1} and 0.012 ± 0.002 g RS·g^{-1}DM·h^{-1} respectively. For the pre-treated samples, the maximum productivity obtained by E1 and E2 was with Test 5 at 18 h of fermentation with 0.012 ± 0.002 g RS·g^{-1}DM·h^{-1} and 0.014 ± 0.002 g RS·g^{-1}DM·h^{-1}.

As occurred with the liquid hydrolysis, the self-produced extracts did not work to hydrolyse the OSW samples (Figure 7.11). Only Viscozyme achieved a correct hydrolysis of the OSW. Also, the non-pre-treated sample obtained better results with Viscozyme than Test 8. The sugar levels achieved in the solid-state hydrolysis were slightly lower than the obtained in the liquid hydrolysis. The maximum productivity reached with the not-pre-treated OSW and the pre-treated OSW sample was always below 0.005 g RS·g^{-1}DM·h^{-1}.

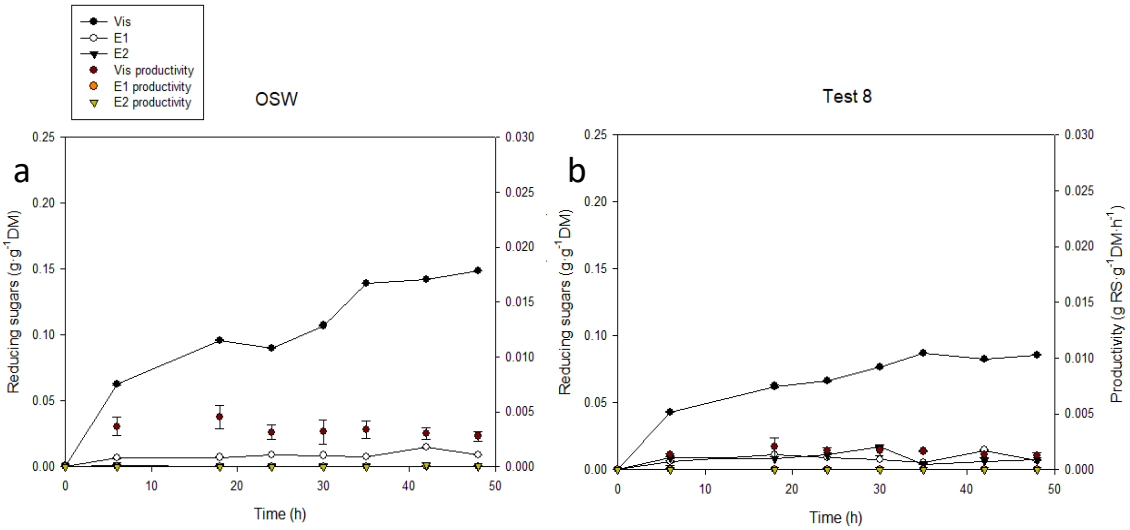

Figure 7.11 Reducing sugars evolution and productivity of the pre-treated and not-pre-treated OSW samples during the solid-state hydrolysis. a) not-pre-treated OSW; b) OSW Test 8; RS: reducing sugars; Vis: Viscozyme; E1: extract 1; E2: extract 2.

Compared to the liquid hydrolysis, a similar performance was observed in the solid-state enzymatic hydrolysis but with lower reducing sugars levels obtained in each sample. However, the solid-state hydrolysis with the non-pre-treated GP sample, obtained similar

sugars results with the liquid hydrolysis of the same sample. Nevertheless, in general the liquid hydrolysis of the samples obtained more reducing sugars levels than the solid-state hydrolysis.

Regarding the maximum sugars released and comparing the three lignocellulosic substrates, the non-pre-treated BSG and GP obtained 0.29 ± 0.03 and 0.37 ± 0.02 g RS·g^{-1}DM respectively. However, for GP the pre-treated samples (Test 5) obtained higher reducing sugars with 0.39 ± 0.08 g RS ·g^{-1}DM, but the results obtained were statistically similar ($p<0.05$). The other pre-treated sample with higher reducing sugars was BSG Test 2 with 0.08 ± 0.02 g RS ·g^{-1}DM. Similar to the liquid hydrolysis, OSW were not a suitable substrate to obtain sugars, compared with BSG and GP. All these maximum sugars released were obtained using Viscozyme.

With the self-produced extracts the maximum sugars released with BSG and GP obtained 0.10 ± 0.01 and 0.29 ± 0.05 g RS ·g^{-1}DM with E2 and E1 respectively in the case of the non-pre-treated sample. From the pre-treated samples of these substrates, the highest reducing sugars levels were found in Test 4 (BSG) with 0.02 ± 0.00 g RS ·g^{-1}DM and Test 5 (GP) with 0.33 ± 0.02 g RS ·g^{-1}DM using E1.

Table 7.5 presents the efficiency of the self-produced extracts compared to Viscozyme on the different lignocellulosic substrates studied in the solid-state hydrolysis. The calculation of the efficiency was made as it was made in the liquid hydrolysis. Efficiency results of liquid hydrolysis method that complement these following results are presented in table 7.5.

Table 7.5 Efficiency of the self-produced extracts on the releasing of sugars on the different substrates respect to Viscozyme on the solid-state hydrolysis

Self-produced extract	BSG	GP	OSW	T1	T2	T3	T4	T5	T6	T7	T8
E1	27.6	78.4	0.0	0.0	25.0	0.0	28.6	84.6	38.5	50.0	0.0
E2	34.5	70.3	7.7	0.0	25.0	0.0	0.0	61.5	57.7	0.0	0.0

Compared to the liquid hydrolysis, the efficiency of the majority of the samples was lower, however in the case of the not-pre-treated GP and the Test 5 and 6, the efficiency was higher. The GP obtained higher efficiency values (70-78%) in the solid-state hydrolysis than in the liquid hydrolysis (57-59%). In the Test 5, the E1 obtained an efficiency value of 85%, that is the higher in all cases. In other cases, the efficiency was low enough that the difference between the liquid or the solid-state hydrolysis was not significant. Regarding the substrate, the results suggests that the solid-state hydrolysis performed better to hydrolyse the GP, and the liquid hydrolysis performed better to hydrolyse the BSG, using the self-produced extracts. The self-produced extracts did not obtain higher efficiency values hydrolysing OSW samples.

Figure 7.12 shows the total reducing sugars obtained in the combined process SE + SSEH (*i.e.*, liquid fraction of the SE + solid hydrolysate) compared to the raw residues.

In the case of the BSG samples that using Viscozyme, Test 2 and 4 released similar reducing sugars to the non-pre-treated BSG. Test 1 and 3 reached lower released reducing sugars to the non-pre-treated BSG. Using the self-produced extracts, Test 2, 3 and 4 reached higher reducing sugars levels than the non-pre-treated BSG. However, the solid-state hydrolysis efficiencies using BSG were low, so most of the sugars in the pre-trated samples were from the SE liquid fraction.

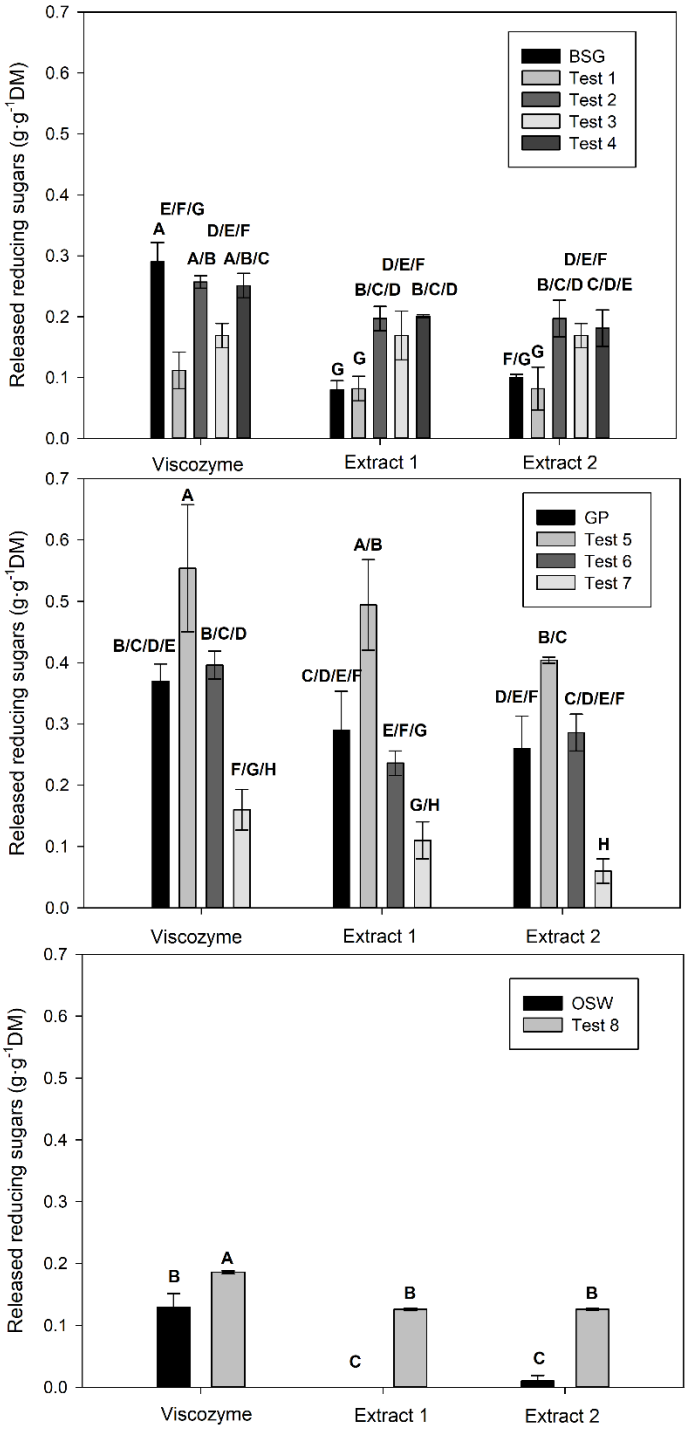

Figure 7.12 Reducing sugars released during the solid-state enzymatic hydrolysis + the liquid fraction of the SE. Different capital letters indicates significant differences between the evaluated groups ($p<0.05$) based on the Tukey test analysis. For raw BSG, GP and OSW the values represent their original content.

In the case of GP samples, Test 5 reached higher reducing sugars levels than the non-pre-treated GP and Test 6 reached similar reducing sugars levels to the non-pre-treated GP, regardless of the extract used.

Finally, the case of OSW, was similar to the results obtained in the liquid hydrolysis. The pre-treated samples reached higher reducing sugars levels than the non-pre-treated OSW regardless of the extract used because most of the reducing sugars were from the SE liquid fraction.

These results showed that with the SE pre-treatment could be used to achieve more reducing sugars in the case of BSG and GP. Similar to the liquid hydrolysis, improving the purification and extraction of the extracts could let achieve higher reducing sugars levels.

7.4 Inhibitory compounds released from the hydrolysis

In the hydrolysis step, the enzymatic activity not only promotes the sugars release, but it is also common to find other compounds derived from the different fractions of the fibres. In general, these compounds are undesired since they could affect the same hydrolysis process, or the further processing of the hydrolysates. Consequently, an evaluation of the inhibitory compounds content in the hydrolysates was performed to observe potential correlations or effects in the different hydrolysates. Commonly, these inhibitory compounds are entrapped in the lignocellulosic structure and are released due to the enzymatic activity, affecting the biological growth and the enzyme activity (Chen and Liu, 2017; Maulana Hidayatullah et al., 2020).

Table 7.6, 7.7 and 7.8 show the different inhibitory compounds analysed and their content in different selected BSG, GP and OSW samples respectively. These samples were selected choosing the non-pre-treated sample and one of the tests of the pre-treated substrate for each lignocellulosic substrate. Then, selecting the Viscozyme samples and the extract that obtained best releasing sugars results, for the solid-state and for the liquid hydrolysis.

As detailed in Table 7.6, acetate and coumaric acid were the main inhibitory compounds presented in BSG samples. In particular, in the not-pre-treated BSG acetate was at least twofold the levels found in the liquid and solid hydrolysates. Results also show that, in general, the content was higher when using viscozyme, and no particular trend was identified in the inhibitory compounds regarding the liquid and solid hydrolysis results. The results are in line with the initial characterization of BSG and their exploded counterparts. The low lignin content is probably one of the factors influencing the low content of lignin-derived inhibitory compounds such as furfuraldehyde, 5-hydroxymethylfurfural (5-HMF), and furfuryl alcohol.

A different scenario was found for GP and their exploded fractions. As Table 7.7 summarizes, apart from acetate, the main components found were furfuraldehyde, 5-hydroxymethylfurfural (5-HMF), and furfuryl alcohol. As occurred with BSG, the highest contents were detected when using Viscozyme. This is a direct consequence of the low selectivity and high efficiency of this enzymatic cocktail, since it is expected that higher enzymatic activity could promote a higher release of these compounds. Results also show that there was a trend to find higher values in the SSEH than in the liquid hydrolysis, and a significant difference among the original GP and the pre-treated ones, being increased the inhibitory content in the second case.

Finally, in the OSW tested samples it was found that vanillin and vanillic acid were the main inhibitory compounds (Table 7.8). There was no particular trend showing differences among the solid and liquid processes, and among the used enzymatic extracts.

Table 7.6 Inhibitory compounds content in some selected BSG samples after the hydrolysis step. Vis: Viscozyme; E1: extract 1; E2: extract 2.

Sample	Furfuraldehyde ($mg \cdot g^{-1}DM$)	5-HMF ($mg \cdot g^{-1}DM$)	Furfuryl alcohol ($mg \cdot g^{-1}DM$)	Acetate ($mg \cdot g^{-1}DM$)	Vanillin ($mg \cdot g^{-1}DM$)	Vanillic acid ($mg \cdot g^{-1}DM$)	Coumaric acid ($mg \cdot g^{-1}DM$)	Syringaldehyde ($mg \cdot g^{-1}DM$)	Syringic acid ($mg \cdot g^{-1}DM$)
Raw BSG	<0.002	<0.003	-	8.32	<0.01	<0.01	<0.00	-	-
BSG liquid Vis	0.016	0.070	<0.01	2.46	<0.01	<0.01	<0.01	<0.00	<0.01
BSG liquid E2	<0.004	0.044	<0.01	2.92	<0.01	<0.01	<0.01	<0.00	<0.01
BSG solid Vis	0.020	0.164	<0.02	2.04	<0.02	<0.02	<0.02	<0.01	<0.02
BSG solid E2	<0.008	<0.008	<0.02	<2.00	<0.02	<0.02	<0.02	<0.01	<0.02
Test 4 liquid Vis	<0.004	<0.004	<0.01	1.28	0.04	0.02	0.04	<0.00	0.01
Test 4 liquid E2	<0.004	<0.004	<0.01	4.80	0.01	0.02	0.03	<0.00	0.01
Test 4 solid Vis	<0.008	<0.008	<0.02	<2.00	0.03	0.03	0.06	<0.01	0.03
Test 4 solid E1	<0.008	<0.008	<0.02	<2.00	<0.02	0.05	0.02	<0.01	<0.02

Table 7.7 Inhibitory compounds content in some selected GP samples after the hydrolysis step. Vis: Viscozyme; E1: extract 1; E2: extract 2.

Sample	Furfuraldehyde ($mg \cdot g^{-1}DM$)	5-HMF ($mg \cdot g^{-1}DM$)	Furfuryl alcohol ($mg \cdot g^{-1}DM$)	Acetate ($mg \cdot g^{-1}DM$)	Vanillin ($mg \cdot g^{-1}DM$)	Vanillic acid ($mg \cdot g^{-1}DM$)	Coumaric acid ($mg \cdot g^{-1}DM$)	Syringaldehyde ($mg \cdot g^{-1}DM$)	Syringic acid ($mg \cdot g^{-1}DM$)
Raw GP	0.019	0.264	-	5.99	<0.01	<0.01	<0.01	-	-
GP liquid Vis	0.112	1.920	<0.01	9.94	<0.01	<0.01	<0.01	<0.00	<0.01
GP liquid E1	<0.004	0.660	<0.01	7.84	<0.01	<0.01	<0.01	<0.00	<0.01
GP solid Vis	<0.010	0.010	<0.03	5.80	0.13	0.14	<0.02	<0.01	<0.03
GP solid E1	0.050	2.050	<0.03	16.55	<0.03	<0.03	<0.02	<0.01	<0.03
Test 5 liquid Vis	0.520	4.080	<0.01	<1.00	0.02	0.02	<0.01	<0.00	<0.01
Test 5 liquid E1	0.090	3.260	0.06	<1.00	0.01	0.01	<0.01	0.01	<0.01
Test 5 solid Vis	0.820	8.100	0.10	<2.50	<0.03	<0.03	<0.02	<0.01	<0.03
Test 5 solid E1	0.320	8.700	0.64	<2.50	<0.03	<0.03	<0.02	<0.01	<0.03

Table 7.8 Inhibitory compounds content in some selected OSW samples after the hydrolysis step. Vis: Viscozyme; E1: extract 1; E2: extract 2.

Sample	Furfuraldehyde ($mg \cdot g^{-1}DM$)	5-HMF ($mg \cdot g^{-1}DM$)	Furfuryl alcohol ($mg \cdot g^{-1}DM$)	Acetate ($mg \cdot g^{-1}DM$)	Vanillin ($mg \cdot g^{-1}DM$)	Vanillic acid ($mg \cdot g^{-1}DM$)	Coumaric acid ($mg \cdot g^{-1}DM$)	Syringaldehyde ($mg \cdot g^{-1}DM$)	Syringic acid ($mg \cdot g^{-1}DM$)
Raw OSW	<0.004	<0.004	-	2.05	0.04	0.06	<0.01	-	-
OSW liquid Vis	<0.004	<0.004	<0.01	2.62	0.09	0.08	<0.01	<0.00	<0.01
OSW liquid E1	<0.004	<0.004	<0.01	4.76	0.06	0.07	<0.01	<0.00	<0.01
OSW solid Vis	<0.008	<0.008	<0.02	5.28	0.10	0.13	<0.02	<0.01	<0.02
OSW solid E2	<0.008	<0.008	<0.02	<2.00	<0.02	0.16	<0.02	<0.01	<0.02
Test 8 liquid Vis	<0.004	0.038	<0.01	1.52	0.08	0.07	<0.01	0.01	<0.01
Test 8 liquid E1	<0.004	0.004	<0.01	6.26	0.07	0.05	<0.01	0.01	<0.01
Test 8 solid Vis	<0.008	0.044	<0.02	2.84	0.08	0.06	<0.02	<0.01	<0.02
Test 8 solid E2	<0.008	<0.008	<0.02	<2.00	<0.02	0.06	<0.02	<0.01	<0.02

7.5 Conclusions

The self-produced extracts resulted competitive to hydrolyse the non-pre-treated samples of BSG and GP in the liquid hydrolysis. Furthermore, the sugars released per unit of xylanase activity with the self-produced extracts were similar to the values obtained by Viscozyme. Using the hydrolysis in liquid phase was more efficient than the solid-state hydrolysis in all the evaluated samples despite the extract used, except for the GP samples, that obtained higher efficiency values. However, the OSW samples did not obtain successful results. Maximum sugars released with the self-produced extracts were obtained with the liquid hydrolysis using non-pre-treated GP (0.45 ± 0.02 g RS·g^{-1}DM) with an efficiency respect Viscozyme of 59%, and non-pre-treated BSG (0.39 ± 0.04 g RS·g^{-1}DM) with an efficiency respect Viscozyme of 69%.

On the contrary, the hydrolysis efficiency among the pre-treated samples using the self-produced extracts was low, and only Test 5 and 6 obtained acceptable efficiencies with the solid-state hydrolysis (85% and 58% respectively). However, adding the reducing sugars released in the SE pre-treatment to the maximum reducing sugars obtained in the hydrolysis let to achieve higher reducing sugars levels in some pre-treated samples.

Furthermore, the inhibitory compounds in the hydrolysates followed a well-defined trend dependent mostly on the original substrate. No correlation was found among the use of solid or liquid phase in the inhibitory compounds content, and the highest levels found when using Viscozyme were in accordance with the efficiency of this control. Further research on the purification, detoxification and the downstream step of such hydrolysates must be addressed to minimize the potential effects of these compounds.

In general, these results show that the enzymatic extracts obtained through SSF of BSG have the potential to hydrolyse lignocellulosic-derived materials with low lignin content.

Although the release of sugars was significantly lower compared to the commercial control, it was shown that they have similar efficiencies in terms of the supplied enzymatic activity. This allows to think that further purification on the enzymatic extracts could enhance their quality, and better results could be achieved.

Finally, it was shown that these enzymatic extracts were able to perform both in liquid and solid phases, indicating they have the potential to be used in a wide set of applications with different purposes.

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Chapter 8 General conclusions and future work

General conclusions

- BSG could be used as a low-cost raw material for obtaining lignocellulolytic enzymes, PHA, and antioxidants as value-added products resulting from the valorisation of a residue.

- *A. niger* and *T. aurantiacus* were suitable for producing lignocellulolytic enzymes via solid-state fermentation at lab-scale with xylanase activity values between 200-300 $U \cdot g^{-1}DM$ and cellulase activity values between 3.5-8 $FPU \cdot g^{-1}D\text{-}M$.

- The bioreactor configuration at bench-scale that achieve the highest lignocellulolytic activities was PVC reactor, using *A. niger*, with 245.5 ± 21.6 of xylanase activity and 4.5 ± 0.2 of cellulase activity. However, Dewar reactors also arose as a potential system to produce enzymes using the thermophilic strain *T. aurantiacus*, because of their ability to retain the heat produced during the process.

- The enzymatic extracts obtained through SSF of BSG with *A. niger* have the potential to hydrolyse lignocellulosic-derived materials with low lignin content and were able to perform correctly both in liquid and solid phases, indicating they have the potential to be used in a wide set of applications with different purposes. Also, the enzymatic extract were used successfully to obtain fermentable sugars and to produce PHA with the bacterial strains proposed.

- Maximum sugars released with the self-produced extracts at bench scale were obtained with the liquid hydrolysis using non-pre-treated GP (0.45 ± 0.02 g $RS \cdot g^{-1}DM$) with an efficiency respect Viscozyme of 59%, and non-pre-treated BSG (0.39 ± 0.04 g $RS \cdot g^{-1}DM$) with an efficiency respect Viscozyme of 69%.

Future work

- Further research on the purification, detoxification and the downstream step of such hydrolysates have to be addressed to minimize the potential effects of the inhibitory compounds. This purification on the enzymatic extracts could enhance their quality, and better results could be achieved.
- Increasing the scale of the process is primordial to come closer to the reality of the needs of the industry and to assess the possible operational problems that could appear at high loads of solids, in special temperature problems. Also, a bulking agent may be needed at larger scales to avoid the compaction problems of the solid matrix.
- Further research on the application of the *T. aurantiacus* extracts should be done, regarding the characteristics of the thermophile fungal strain, that allows to hydrolyse at higher temperature that could enhance the release of reducing sugars.
- Optimize different parameters of the extraction of the enzymes, as temperature, extraction ratio or solvent of extraction, could improve their performance on the hydrolysis step.
- Futher research at larger scale of the Dewar reactor configuration to study the SSF behaviour at higher quantity of solid material. Also, to implement a humidity control for the tray-type reactor configuration.