Universidade do Minho
Escola de Engenharia

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Bioprocessing of main agro-industrial wastes of Portugal for protein enrichment and lignocellulolytic enzymes production

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Mestrado em Bioengenharia

Trabalho efetuado sob a orientação do

Doutor José Manuel Salgado

e da

Professora Doutora Isabel Belo

Outubro de 2016

DECLARAÇÃO

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RESUMO

Todos os anos, são produzidas grandes quantidades de resíduos agroindustriais e estes são os recursos renováveis mais abundantes na terra. Economicamente, estes resíduos podem ser usados como materiais de baixo custo para a produção de compostos de valor acrescentado, diminuindo os custos de produção. De um ponto de vista ambiental, a reutilização destes resíduos em processos biotecnológicos pode reduzir o seu conteúdo em compostos fenólicos e outros compostos tóxicos que podem deteriorar e degradar o meio ambiente quando descartados na natureza.

Uma possível aplicação para este tipo de resíduos é a sua utilização como alimento animal. Frequentemente, os resíduos agroindustriais são utilizados como alimento animal contudo, muitas vezes estes resíduos apresentam baixo qualidade nutricional, a maior parte tem baixo conteúdo proteico e apresentam uma difícil digestibilidade. Estas desvantagens podem ser evitadas através da fermentação em estado sólido (SSF) destes resíduos.

Durante este estudo foram utilizados resíduos da indústria da produção de azeite, da indústria de produção de cerveja e da indústria de produção de vinho. Estes resíduos foram utilizados como substrato sólido para a produção de enzimas lignocelulolíticas (celulases e xilanases), para degradar parcialmente as fibras dos resíduos e aumentar o conteúdo em proteína dos resíduos agroindustriais em estudo.

Primeiro, foi feita a escolha do fungo a utilizar através da inoculação de três fungos diferentes, *Aspergillus niger*, *A. uvarum* e *A. ibericus* com cada um dos diferentes resíduos e foi avaliada a produção de celulases, xilanases, o aumento da quantidade de proteína e a variação da composição lignocelulósica do sólido. *A. ibericus* mostrou os melhores resultados em todos os resíduos analisados e a SSF permitiu o aumento de proteína em todos os resíduos. Contudo, os compostos lignocelulósicos foram degradados em todos os casos.

Após seleção do fungo, foi realizado um desenho experimental para avaliar os benefícios do uso de culturas mistas de diferentes resíduos em diferentes proporções como substrato sólido para SSF com o *A. ibericus*. O substrato ótimo foi uma mistura de dreche e podas da vinha que aumentou o conteúdo em proteína (16.3 %) e atividades de xilanase (89.33 U/g), celulose (3.46 U/g) e β-glucosidase (21.91 U/g).

No fim, foi realizado um aumento de escala para um reator em tabuleiro com 500 g de substrato sólido. Os resultados obtidos foram semelhantes aos obtidos em Erlenmeyers exceto no caso das

celulases e β-glucosidase em que as atividades foram menores. Através deste estudo foi possível concluir que a SSF com *A. ibericus* é um processo biotecnológico adequado para o aumento da qualidade nutricional dos resíduos agroindustriais e para produzir compostos de valor acrescentado como enzimas com os mesmos baixos custos de produção.

ABSTRACT

Every year, large amounts of agro-industrial wastes are produced which are the most abundant renewable resources on earth. Economically, these residues can be used as low cost materials for the production of value-added compounds by decreasing production costs. From an environmental point of view, the reuse of this wastes in biotechnological processes can reduce its content in phenolic compounds and other toxic compounds which can deteriorate and degrade the environment when disposed in nature.

One possible application for this type of waste is its use as animal feed. Frequently, the agro-industrial wastes are used as animal feed, however many times these wastes have a poor nutritional quality, mainly they have a low protein content and their digestibility is hard. These disadvantages can be avoided by solid-state fermentation (SSF) of the wastes.

This study used wastes from the oil industry, the beer industry, and the wine industry. These residues were used as a solid substrate for the production of lignocellulolytic enzymes (cellulases and xylanases), to degrade partially the fibres of wastes and to increase the crude protein content of agro-industrial wastes in study.

Firstly, it was performed a screening of the filamentous fungi through the inoculation of three different fungi, *A. niger*, *A. uvarum* and *A.ibericus* with each one of the different residues and it was evaluated the production of cellulases, xylanases, the increase of protein and the variation in lignocellulosic composition of solid. *A. ibericus* showed the best results in all wastes evaluated, and the SSF allowed to increase the protein content in all wastes. However, the lignocellulosic compounds were degraded in all cases.

After selection of the fungi, it was performed an experimental design to evaluate the benefits of using mixtures of wastes in different proportions as solid substrate in SSF by *A. ibericus*. The optimum substrate was a mixture of brewery spent grain and vine-shoot trimmings which achieved and increase the protein content (16.3 %), xylanase (89.33 U/g), cellulose (3.46 U/g) and β-glucosidase (21.91 U/g) activities.

At the end, it was performed a scaled up to a tray bioreactor with 500 of solid substrate. The results obtained were similar to flask experiments except to in cellulose and β-glucosidase activities that were lower. Through this study, it was possible to conclude that the SSF by *A. ibericus* is a suitable biotechnology process to increase the nutritional quality of agro-industrial wastes and to produce value-added products as enzymes in the same low-cost process.

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CONTEXT AND MOTIVATION

The global evolution in technology and science, lead to an improvement of standard living conditions and the increase of global population, this is the main cause for the increase of food demand. Traditional ways of food supply consist in using sea and land to obtain the essential nutrients for human survival. Associated with these traditional ways is the problem of wastes disposal which, are mainly generated by agricultural and municipal segments of population (Ajila *et al.*, 2012). The utilization of these wastes has been proposed for animal feed (El Boushy, 1990). Wastes utilization as an alternative source for animal feed need huge attention because its recycle and reduction is a good way to minimize environmental pollution and improve the present living conditions (Ajila *et al.*, 2012). Currently 33 % of croplands are used for livestock feed production (Department, 2012), this is a key factor in deforestation. By using agro-industrial wastes as animal feed, food supply can increase and reduce the environmental impact of these wastes. However, the nutritional quality of these wastes does not meet the standards that are required for animal feed, mainly due to low protein content. The application of biotechnology processes as solid-state fermentation can enrich the agricultural wastes and the fermented solid may be used directly in animal feeds.

On the other hand, the use of enzymes in hydrolysis reactions and their demand in industrial processes is growing. However, their production costs limit their use. Thus, other alternatives of production should be researched to reduce the costs of process. The production of enzymes by solid-state fermentation is a cost-effective technology due to use agro-industrial wastes as substrate, the low energy consumption and production of enzymes in high concentrations that facility their purification. In addition, the protein content of solid waste can be increased and digestibility of wastes could be improved in the same process.

AIMS AND RESEARCH

The main goal of this work was to increase the protein content of brewery, winery and olive mill wastes and the simultaneously production of lignocellulolytic enzymes by solid-state fermentation.

To achieve this main objective, several partial objectives were planned:

- Characterization of main agro-industrial wastes from breweries, wineries and olive mills.

- Selection of suitable fungi to increase the protein content of wastes and to produce lignocellulolytic enzymes.
- Evaluation of mixtures of wastes as substrate in solid-state fermentation (SSF).
- Scale-up of process to tray bioreactor.

1. INTRODUCTION

1.1. Agro-industrial wastes

Presently, there is a growing interest in the exploitation of wastes generated by the food industry. Recently, the European Union (EU) has set the objectives and targets to improve waste management and to turn waste into resource. This is a key to circular economy, which is an alternative to a traditional linear economy, and it searches to use the resources for as long as possible, extract the maximum value from them, and then recover and re-use the wastes. The EU has an ambitious circular economy package, which includes revised legislative proposals on waste. Among which will be planned specific measures to promote re-use and stimulate industrial symbiosis - turning one industry's by-product into another industry's raw material ("Circular Economy Strategy - Closing the loop - An EU action plan for the Circular Economy," 2016). In this way, these measures will contribute to "closing the loop" of product lifecycles and to bring benefits to environment and economy.

Agro-industrial wastes can be classified into different categories, such as crop waste and residues, by-products from agro-food processing industries, among which are the oil, wine and brewery industries.

The reuse of agro-industrial wastes causes economic and environmental benefits. From an environmental point of view, the reuse of this wastes in biotechnology processes can reduce their contain in phenolic compounds and/other compounds of toxic potential, which can deteriorate and degrade the environment when discharged to nature (Mussatto *et al.*, 2012) . Economically, these wastes can be used as solid substrate reducing the cost of process.

The agro-food industries are important in Portuguese economy which represents 4.1 % PIB, 12 % of employment and 8.4% of exports in 2012 (Programa de Desenvolvimento Rural do Continente para 2014-2020). Among the main agro-food industries are the beer, wine and olive oil industries (**Figure 1**). They produce an average of 750,000 t of beer (FAOSTAT, 2012), 7 million hL of wine and 0.7 million hL of olive oil per year (INE, 2015). They are also great producer of solid and liquid wastes which pose important ecological and economic problems, and which imply high handling and disposal costs.

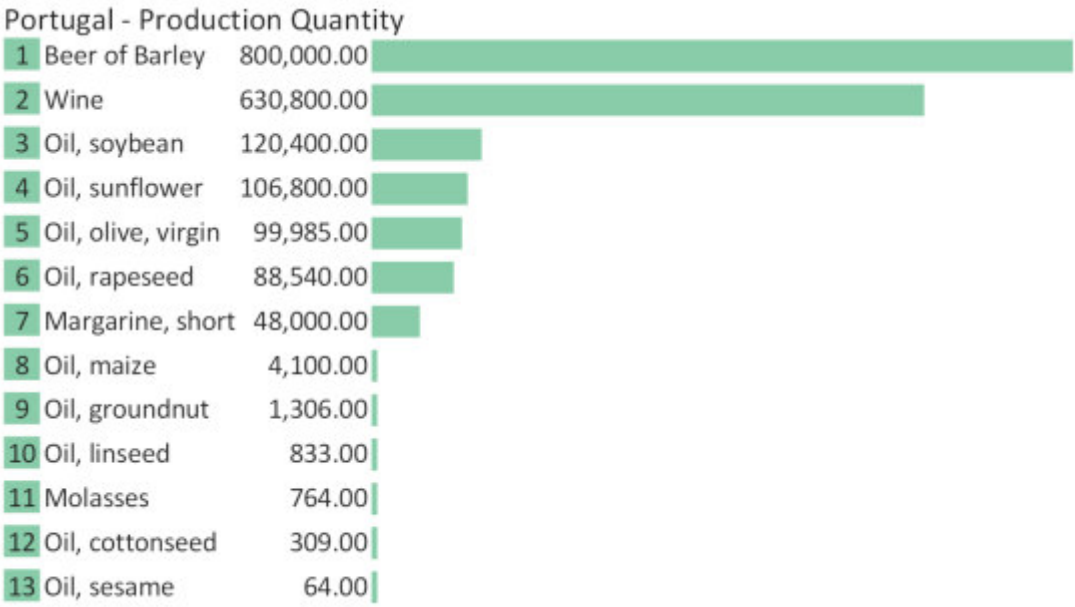

Figure 1- Production, in tonnes, of processed crops in Portugal in 2013 (FAOSTAT, 2015)

1.1.1 Olive oil industry

Mediterranean countries produce more than 98% of the world´s olive oil, which is estimated at over 2.5 million metric tons per year and about 75% is produced in the EU. The largest olive oil producers in the EU are Spain, with 36%, Italy, with 24%, and Greece, with 17%, of the world´s total production. The next largest producer is Portugal, with a production of one order of magnitude lower than the three leading countries. In **Table 1** can be observed the extraction of olive oil in the regions of Portugal in 2015.

Table 1- Production of olive oil (hL) in agricultural regions of Portugal in 2015 (INE, 2016).

Regions	Total	Traditional	Continuous two phases	Continuous three phases	Others
Alentejo	866204	1895	799577	64733	0
Trás-os-Montes	142928	8908	122164	11856	0
Ribatejo e Oeste	64265	10363	28753	16642	8507
Beira Litoral	54596	4284	24902	22043	3367
Beira Interior	51580	11495	23504	16534	47
Algarve	6892	43	3110	3739	0
Entre Douro e Minho	4058	893	2969	196	0
Portugal	**1 190 523**	**37 880**	**1 004 979**	**135 743**	**11 921**

As we all know, the olive oil is obtained by extraction process, during olive oil production there are various steps involved like olive washing, milling/grinding, beating and finally the extraction of the oil (Roig *et al.*, 2006). The extraction of olive oil can be achieved by traditional press (discontinuous process) or centrifugation systems (continuous process), which involves the two-phase and the three-phase systems (Dermeche *et al.*, 2013). In **Figure 2** displays the evolution of extraction systems in olive mills the last years in Portugal. Nowadays, the traditional pressing system is almost extinguished (Alburquerque, 2004). After extraction, this process forms a by-product called olive pomace (solid fraction, also called olive cake) and an emulsion containing the olive oil.

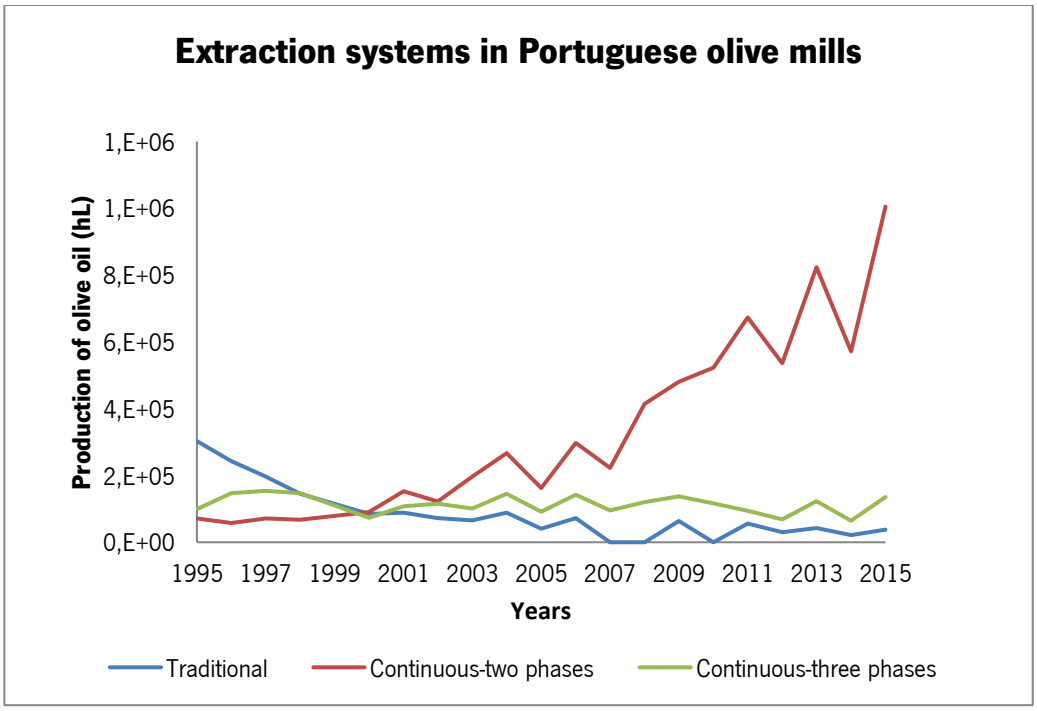

Figure 2- Extraction systems in Portuguese olive mills.

Relatively to the continuous process, the three-phase system was introduced in 1970s to improve extraction yield. From this system they are generated three products: pure olive oil, olive-mill wastewater and a solid by-product called olive cake, olive pomace or *orujo* (Morillo *et al.*, 2009; Antizar-Ladislao *et al.*, 2009).

Then, the two-phase system was introduced in the 1990s in Spain and quickly replaced the three-phase method (Roig *et al.*, 2006), because it reduces the water consumption during the extraction process, thus it also reduces the olive wastewater. This system generates olive oil and a semi-solid waste, known as the two-phase olive-mill waste (TPOMW), wet olive pomace or *alperujo* (Morillo *et al.*, 2009).

Olive mill wastes

By-products originated from the olive trees and olive oil extractions are known as "olive mill wastes". Depending of the process used during the extraction, different by-products can be obtained which show different characteristics and waste management processes. The main by-products obtained from the olive oil industry are the olive leaves (OL), olive pomace (OP) (Molina-Alcaide and Yáñez-Ruiz, 2008), and olive mill wastewater (OMWW) (Dermeche *et al.*, 2013). **Table 2** shows the quantities of by-products generated in each extraction process.

Table 2- Input–output data for the three olive oil production processes. Adapted from Azbar *et al.* (2004).

Production process	Input	Amount of input	Output	Amount of output (kg)
Traditional press process	Olives	1 ton	Oil	~200
	Wash water	0.1-0.12 m^3	Solid waste	~400
	Energy	40-63 kWh	Wastewater	~600
Three-phase process	Olives	1 ton	Oil	200
	Wash water	0.1-0.12 m^3	Solid waste	500-600
	Fresh water for decanter	0.5-1 m^3	Wastewater	1,000-1,200
	Energy	90-117 kWh		
Two-phase process	Olives	1 ton	Oil	200
	Wash water	0.1-0.12 m^3	Solid + waster waste	800-950
	Energy	<90-117 kWh		

OP is a set of olive pulp, skin, stone and water and can be defined with different terms depending on some factors such as composition and oil content (crude or exhausted OP) (Molina-Alcaide and Yáñez-Ruiz, 2008). It is noteworthy that there is a difference between the OP produced by three-phase system and two-phase system, the main difference is in the higher moisture and the lower oil content obtained from the two-phase system (Alburquerque, 2004), resulting on a more efficient and environmentally friendly centrifugation process when compared to the three-phase system (Molina-Alcaide and Yáñez-Ruiz, 2008).

Currently, olive oil industries recover residual olive oil from crude olive pomace (COP). This waste has around 3.5% residual oil in wet basis. In spite of the doughy consistency hinder the drying of COP, the extraction of residual oil is still applied (Rincón *et al.*, 2012). After oil extraction and a drying step, the exhausted olive pomace (EOP) is used in combustion processes (Fernandez-Rodriguez *et al.*, 2014). However, combustion of EOP causes environmental problems due to the emission of toxic compounds in gas form (Niaounakis and Halvadakis, 2006) and it requires high energy consumption to reduce the moisture content. Thus, it is necessary to search new alternatives that could increase the value of these

wastes and reduce their environmental impact. The investment in the revalorization of this waste is a current opportunity, and it may also contribute to the protection of the environment.

The management of OP from two-phase systems is a problem because of its high moisture that hinders its transport. In addition, it causes environmental problems due to the organic load and its chemical composition, which makes it resistant to degradation (Morillo *et al.*, 2009).

Chemical characterization of olive mill wastes

This by-product contains a majority of the water-soluble chemical species present in the olive fruit, a high organic load, high C/N ratio (chemical oxygen demand) and has a pH between 4 and 6. Despite these wastes are formed by large amounts of lipids and polysaccharides, unfortunately they also contain phytotoxic compounds that inhibit microbial growth (Capasso R *et al.*, 1995; Ramos-Cormenzana *et al.*, 1995). Due to the instability of phenolic compounds present in OP, these tend to polymerise during storage forming high-molecular-weight polymers which are difficult to degrade (Morillo *et al.*, 2009).

The OP are considered a lignocellulosic waste due to cellulose, hemicellulose and lignin content (Dermeche *et al.*, 2013). In addition, sugars, proteins, fatty acids like oleic acid, polyalcohol's and polyphenols are also present in this by-product (Karantonis *et al.*, 2007; Molina-Alcaide and Yáñez-Ruiz, 2008).

The composition of COP and exhausted olive pomace (EOP) from the two-phase system are showed in **Table 3**. In this project, it was used the EOP as substrate in solid-state fermentation.

The EOP is obtained after drying and residual olive oil extraction by organic solvents. The drying and extraction of residual oil are physicochemical treatments that can change the composition of olive pomace (Roig *et al.*, 2006). Usually, the COP is centrifuged to extract 40%-50% of the residual oil (Sánchez-Moral and Ruiz-Méndez, 2006), followed by a drying process (400°C-800°C) that reduces moisture from 60-70% to 8% and extraction with solvents (hexane) (Rincón *et al.*, 2012).

The content of cellulose and hemicellulose indicated that these wastes have potential to be used as solid substrate in soli-state fermentation (SSF) and can induce the production of lignocellulolytic enzymes. The content of lipids in EOP is lower than COP due to the residual oil extraction. The content of sugars in EOP can improve the growth of microorganisms (Ertan Inceoğlu *et al.*, 2014). The quantity of phenolic compounds can be important due to they can inhibit the growth of fungi (Medina *et al.*, 2011).

Table 3- Physicochemical composition of COP and EOP. Salgado *et al.* (2016)

Parameter	COP	EOP
Humidity (%)	73.5 ± 0.4	9.9 ± 0.1
Total solids (%)	26.5 ± 0.4	90.1 ± 0.1
Ash (%)	6.6 ± 0.5	3.4 ± 0.2
Lignin (%)	43.2 ± 0.5	41.62 ± 0.04
Hemicellulose (%)	22.3 ± 0.8	24.1 ± 0.2
Cellulose (%)	12.5 ± 0.9	11 ± 2
Lipids (%)	16.65 ± 0.09	4 ± 2
Proteins ($mg/g_{dry\ waste}$)	4 ± 1	2.6 ± 0.3
Reducing Sugars ($mg/g_{dry\ waste}$)	96 ± 6	42 ± 2
Phenols ($mg/g_{dry\ waste}$)	8.4 ± 0.3	8.9 ± 0.2
N (%)	0.6 ± 0.1	1.27 ± 0.07
C (%)	49.7 ± 0.7	46 ± 1
Ca (g/kg)	1.16 ± 0.04	1.8 ± 0.2
K (g/kg)	17 ± 1	14.2 ± 0.7
Mg (mg/kg)	474 ± 22	473 ± 57
Zn (mg/kg)	12 ± 0	10.5 ± 0.7
Cu (mg/kg)	11.5 ± 0.7	11 ± 1
Fe (mg/kg)	42 ± 2	147 ± 33
Mn (mg/kg)	8.6 ± 0.1	10.2 ± 0.4
Cr (g/kg)	<22	<22
Ni (mg/kg)	<22	<22
Pb (mg/kg)	<22	<22
Na (mg/kg)	373 ± 35	92 ± 5

Carbon and nitrogen sources are important components for fungal growth and should be given significant consideration (Rodriguez-Leon *et al.*, 2008).The optimum C/N ratio for SSF is close to 15 (Lemos *et al.*, 2001), so to achieve this value, an additional source of N should be added.

1.1.2 Wine industry

According to the European Union (EU) average production of wine in recent years in Portugal was 7 million hectoliters/per year, being the fifth largest producer of wine in the EU. **Table 4** displays the

quantities of wine that is produced in each agricultural region of Portugal. The wine industry sector in one of the most important in agro-food sector. It represents 10% of business volume and 8% of employment of agro-food sector and 6% of agro-food industries.

Table 4- Wine production in Portugal by agricultural region in 2015 (INE, 2016).

Region	Production (hL)
Portugal	7044677
Ribatejo e Oeste	2286456
Trás-os-Montes	1764681
Alentejo	1163001
Entre Douro e Minho	874735
Beira Litoral	607869
Beira Interior	277902
Madeira	46000
Algarve	13630
Açores	10404

Data collected in Portugal for 2015, suggest that the production of wine in the north region is important. Since, the sum of wine production of Trás-os-Montes, Douro e Minho are 37% of total production in Portugal.

Winery wastes

Winemaking process generates different residues such as remains of plants derived from the de-stemmed grapes (removal of stem), sediments obtained during the clarification process, bagasse and grape pomace from pressing and lees obtained after different decanting steps. Still, wastewaters are also generated from vinification lees and contain grape pulp, skins, seeds and dead yeasts, used during alcoholic fermentation (Devesa-Rey *et al.*, 2011). The residues produced are characterized by high contents of biodegradable compounds and suspended oils (Navarro *et al.*, 2005).

By-products obtained from the winery industry can be divided onto organic wastes (grape pomace, containing seeds, pulp and skins, grape stems and grape leaves), wastewater, emission of greenhouse gases (CO_2 and volatile compounds for example) and inorganic wastes (diatomaceous earth, bentonite clay and perlite) (Oliveira *et al.*, 2013). The main solid products obtained from this industry are

grape leaves (GL), grape stalk (GS), grape pomace or marc (GM) and wine lees (WL) (Bustamante *et al.*, 2008; Mateo and Maicas, 2015). According to the European Council Regulation (EC) 1493/1999, GM and WL must be sent to alcohol distilleries, leading to the production of exhausted grape marc (EG) and a liquid waste (vinasses). Frequently, small wineries manage incorrectly their wastes and they disposal the wastes directly into the environment.

Grape pomace is obtained during the production of must (grape juice) by pressing whole grapes, and due to its composition can be employed in different process, particularly in the extraction of seed oil, polyphenols (Mateo and Maicas, 2015; Teixeira *et al.*, 2014) and to obtain anthocyanic colorants or oils (Thorngate and Singleton, 1994). Grape seeds can be used to obtain catechin polymers (Karleskind, 1992). Wine lees are the residues formed at the bottom of wine barrel, after fermentation, during storage or after authorized treatments and as well as the residue obtained following the filtration or centrifugation of this product. Lees are mainly composed of microorganisms, mainly remains of dead yeasts, and they have been considered for use as a supplement in animal feed.

Grape stalks or stems are residues of the winery industry, applied as a source of astringent compounds, mainly represented by proanthocyanidins (Llobera and Cañellas, 2007). The quantity of stems in processed raw matter can vary between 1.4 % and 7.0 % (Souquet and Moutounet, 2000). Grape stems have a low commercial value and had been used mainly as animal feed or in soil amendment (Teixeira *et al.*, 2014). Grape leaves are the less studied and valorised residue of wine industry (Mateo and Maicas, 2015; Teixeira *et al.*, 2014). This residue, due to its composition led to a considerable interest because it is a promising source of compounds with nutritional properties and biological potential. Grapevine leaves are employed in the production of ingredients and its juice has been recommended as an antiseptic for eyewash (Fernandes *et al.*, 2013; Gurbuz, 2007). Vinasses are wastewaters generated in distillery basely from wine lees and grape pomace and is characterized by a high content of solids composed mainly by dead yeasts, grape pulp, skin and seeds (Devesa-Rey *et al.*, 2011).

Chemical characterization of winery by-products

Grape pomace moisture varies from 50 % to 72 % of total harvest, depending on the grape variety and their ripening strategy. Relatively to insoluble residues, these have a lignin content ranging from 16.8 % to 24.2 % and a very low protein content close to 4 %. The main polymer-type constituent of cell walls are peptic substances, ranging from 37 % to 54 % of cell wall polysaccharides, followed by cellulose

varying from 27 % to 37 % (González-Centeno *et al.*, 2010). When considering the different fractions of grape pomace (seeds and peels), the proportion of seeds ranges from 38 % to 52 % of the dry material (Ghafoor *et al.*, 2009). In relation to the composition of grape seeds, their fibre content is up to 40 %, 16 % of essential oil, 11 % protein and 7 % of complex phenolic compounds like tannins and other substances like sugars and minerals (de Campos *et al.*, 2008). The phenolic compounds are about 60 % to 70 % of total extractable compounds in grape seeds. During the pressing of grapes, only a minor proportion of these compounds are extracted (González-Manzano *et al.*, 2004) and this fact had attracted the industrial interest because they can be a source of natural antioxidants (Bucić-Kojić *et al.*, 2013). It is to be noticed that grape skin is also important because it represents about 65 % of the total material of grape pomace (Mateo and Maicas, 2015) and has been reported that grape skin is a rich source of phenolic compounds, even though the final yield is dependent on the specific vinification process and the extraction method used (Furiga *et al.*, 2009).

Grape stems have a moisture ranging from 55 % to 80 % and this high variability is attributed to the grape variety. They also have alcoholic insoluble residues in the order of 71 % of the dry matter and no differences between red and white varieties have been reported (González-Centeno *et al.*, 2010). In relation to the phenolic composition, their content has been shown in flavan-3-ols, hydroxycinnamic acids, monomeric and oligomeric flavonols and stilbenes (Cao and Ito, 2003; Karvela *et al.*, 2009; Makris *et al.*, 2008).

The content of grape leaves is mainly organic acids, phenolic acids, flavonols, tannis, procyanidins, antocyanins, lipids, enzymes, vitamins, carotenoids, terpenes and reducing or non-reducing sugars (D'arc Felicio *et al.*, 2001; Doshi *et al.*, 2006; Hebash *et al.*, 1991; Xia *et al.*, 2013).

Wine lees are mainly composed of microorganisms, mostly dead yeasts, tartaric acid, inorganic matter and phenolic compounds. The scarce available literature reported the presence of anthocyanins (6-11.7 mg/g x dw (dry weight)) and other phenolics (29.8 mg/g x dw) (Pérez-Serradilla and Luque de Castro, 2011), the concentration of total phenolic compounds in wine lees was described by Tao *et al.* (2014) (50 mg·GAE·g^{-1}·dw).

1.1.3 Brewery industry

World beer consumption exceeds 1700 million hectolitres (hL) every year and, between 1970 and 2004, world beer consumption doubled from around 600 million hL to over 1.2 billion hL (Fao,

2009). In 2013, the production of beer in Portugal reached values between 731 740 x 10^3 litres and 737 613 x 10^3 litres (Ine, 2014) (**Table 5**).

Table 5- Industrial beer production in 2013 (Ine, 2014).

Product	Variable	2013	Unit
Beer made from malt (excluding non-alcoholic beer)	Production	731 740	10^3 L
		737 613	10^3 L
	Sales	643 202	10^3 Euros

According to National statistics relatively to the year of 2011, there were seven factories responsible for the production of beer in Portugal and the total amount produced was of 8,299 thousands hectolitres wherein, 8,166 thousand hL were of beer with alcohol and the rest of beer without alcohol (**Table 6**) (APCV, 2011).

The main products necessary for beer production are barley, corn grit, rice and hops (Westendorf and Wohlt, 2002). As we all know, the major product derived from this industry is beer, although, the generation of beer by-products is unavoidable (McCabe, 1999; Priest and Stewart, 2006; Thomas *et al.*, 2010; Westendorf and Wohlt, 2002).

Table 6- Statistics of the year 2011 relatively to beer production in Portugal (APCV).

National statistics for 2011	Total 2011	Comparative 2010
Unities of production (Factories)	7	7
Total production (thousands hL)	8.299	8.312
Beer with alcohol (thousands hL)	8.166	8.160
Beer without alcohol (thousands hL)	133	152

The brewing process consists initially in mixing the milled barley malt with water in mashing tanks and slowly increase the temperature from 37 ˚C to 78 ˚C, to promote enzymatic hydrolyses of malt constituents (Singh Nee Nigam and Pandey, 2009). During this stage denominated mashing is produced a sweet liquid known as wort and starch that is converted to fermentable sugars (maltose and maltotriose) and non-fermentable sugars (dextrins). In addition, proteins are practically degraded to polypeptides and amino acids (S. I. Mussatto *et al.*, 2006; Singh Nee Nigam and Pandey, 2009). The insoluble, undegraded

part of malted barley grain settles in the bottom of the mashing tank and the sweet wort is filtrated through it. The residual solid fraction obtained is a by-product denominated brewery spent grain (BSG) (S. I. Mussatto *et al.*, 2006). In some cases, due to economic reasons or to produce beer of distinct qualities, 15 to 25 % of the barley malt is replaced by unmalted cereals like corn, rice, wheat, oats, rye or sorghum, denominated adjuncts (Kunze, 1996). At the end of the mashing process, in these cases, the insoluble part of these grains are separated with the undegraded part of malted barley grain (Nigam and Pandey, 2009). The resulting filtered wort is used as fermentation medium in the next step of beer production (Dragone *et al.*, 2002; Linko *et al*, 1998).

The wort is transferred to the brewing kettle where it is boiled for at least one hour with the addition of hops. During this process, the aromatic hop components and the bitter are transferred into the sweet wort. The typical qualities of the beer such as bitter taste, flavour and foam stability are conferred by these substances (Nigam and Pandey, 2009). At the end of the boiling step, the medium is cooled and liquid is extracted and separated from the hop residues for further processing (De Keukeleirc, 2000). The resulting hop residues, denominated as spent hops, are another by-product of the beer production and they are useless and with no further value.

After removal of the precipitated fraction, the cooled hopped wort is placed in a fermentation vessel with yeasts, where the yeast cells will convert the fermentable sugars to ethanol and carbon dioxide. At the end of the fermentation process, yeasts are collected with an excess, because during fermentation, the cell mass increases, and these can be collected from the bottom or even from the top of the fermenter, according to the nature of the yeast used (De Keukeleirc, 2000). After the removal of the yeasts, these can be dried and sold as brewer's yeast and brewers condensed solubles (Westendorf and Wohlt, 2002).

Brewery by-products

In brewery industry are obtained three different by-products, spent grains (BSG), spent hops and surplus yeast. BSG has a now-toxic nature, low cost production and its nutritional value makes it a ready-to-use by-product that is mostly used for animal and human consumption (Mussatto *et al.*, 2006). Spent grains, from a food safety perspective, provide limited risk to the animal consumer and, the level of microbial load following spent grain formation is low, within acceptable limits for food use and would not inhibit the utilization of this by-product as an agribusiness feed source (Pellettieri, n.d.). In 2012, brewers yielded more than 2.7 million tons of spent grain, in the United States of America, as a by-product of

brewery industry and, more than eighty five percent of this by-product was repurposed into agricultural industries rather than landfilled (Hamed *et al.*, 2011; Mussatto *et al.*, 2006).

Chemical characterization

The chemical composition of BSG may suffer variations due to a variety of factors, such as the variety of the barley used in the process, as well as its harvest time and conditions under which it was cultivated, the conditions used in the malting and mashing process and the amount of adjuncts added to the mixture with the barley malt for the wort elaboration (Solange I. Mussatto, 2014). BSG is rich in sugars, proteins and minerals. In **Table 7** is summarized the chemical composition of BSG, reported in different studies, which used barley malt produced in different countries like Brazil (Mussatto and Roberto, 2006), Japan (Kanauchi *et al.*, 2001), Portugal (Carvalheiro *et al*, 2004; Meneses *et al*, 2013; Silva *et al.*, 2004) and Ireland (Waters *et al.*, 2012). **Table 7** shows variations in chemical composition of BSG as a consequence of the factors mentioned above. The variations in the results obtained from the different authors, suggest that the brewering process conditions affect the composition of residual BSG material (Mussatto, 2014).

Table 7- Chemical composition of brewer's spent grain (BSG).

Item	Mussatto and Roberto	Kanauchi *et al.*	Carvalheiro *et al.*	Silva *et al.*	Meneses *et al.*	Waters at al.
Components (gkg^{-1} dry weight basis)						
Cellulose (glucan)	168	254	219	253	217	260
Hemicellulose	284	218	296	419	192	222
Xylan	199	NR	206	NR	136	NR
Arabinan	85	NR	90	NR	56	NR
Lignin	278	119	217	169	194	NR
Acetyl groups	14	NR	11	NR	NR	NR
Proteins	153	240	246	NR	247	221
Ashes	46	24	12	46	42	11
Extractives	58	NR	NR	95	107	NR
Mineral (mg kg^{-1} dry weight basis)						
Silicon	10740	NR	NR	NR	NR	1400
Phosphorus	5186	NR	NR	NR	6000	4600
Calcium	3515	NR	NR	NR	3600	2200
Magnesium	1958	NR	NR	NR	1900	2400
Sulfur	1980	NR	NR	NR	2900	NR
Potassium	258.1	NR	NR	NR	600	700
Sodium	309.3	NR	NR	NR	137.1	100
Iron	193.4	NR	NR	NR	154.9	100
Zinc	178.0	NR	NR	NR	82.1	100
Aluminium	36.0	NR	NR	NR	81.2	NR
Manganese	51.4	NR	NR	NR	40.9	NR
Cobalt	NR	NR	NR	NR	17.8	NR
Copper	18.0	NR	NR	NR	11.4	NR
Strontium	12.7	NR	NR	NR	10.4	NR
Iodine	NR	NR	NR	NR	11.0	NR
Barium	13.6	NR	NR	NR	8.6	NR
Chromium	5.9	NR	NR	NR	<0.5	NR
Molybdenum	NR	NR	NR	NR	1.4	NR
Boron	NR	NR	NR	NR	3.2	NR

NR, not reported.

Mussatto and Roberto, 2006**;** Kanauchi *et al.*, 2001; Carvalheiro *et al.*, 2004; Silva *et al.*, 2004; Meneses *et al.*, 2013; Waters *et al.*, 2012

1.2. Solid-state fermentation

Solid-state fermentation (SSF) process has gained interest in biotechnological industries due to its possible applications for the production of value added products such as enzymes, organic acids, poly unsaturated fatty acids, single cell protein, antibiotics, biopesticides, biofuel and aroma production (Bhargav *et al.*, 2008; Singhania *et al*, 2009). This process can use products or by-products of agriculture, forestry or food processing as substrate and adds value to them, making it possible, to use this products in areas of interest, and address the waste disposal issues (Horita *et al.*, 2015; Lio and Wang, 2012).

SSF is a fermentation process in which microorganisms grow on solid materials without the presence of free liquid (Cannel and Mooyoung, 1980). It is to notice that the substrate must contain enough moisture to support the growth and metabolism of the microorganism (Pandey, 1992).

Usually, the source of nutrients comes from within the particle used as substrate but in some cases, the nutrients necessary to this process come from an external source. A polymer gives the solid structure to the particle and it may or may not be degraded by the microorganism during the fermentation process. There are also, some cases that is necessary to use an artificial or inert support with a nutrient solution absorbed within the matrix (Horita *et al.*, 2015).

When compared to submerged fermentation (SmF), SSF appears to be an attractive alternative that can provide substantial advantages to SmF (Udo Hölker and Lenz, 2005). The main differences between these two processes are the microbial growth and product formation because, in SSF, this occurs at or near the surface of the solid substrate particle resulting in a low moisture content (Pandey *et al.*, 1999). There are many advantages in the use of SSF for bioprocessing and production of various value-added products compared to SmF like for example, when SSF is used the products have higher yield, need less energy, produces less wastewater with less risk of bacterial contamination, the process is less prone to problems with substrate inhibition and also, the fermentation time may be shorter and the degradation of enzymes by undesirable proteases is minimized (Ali and Zulkali, 2011; U. Hölker *et al.*, 2004; Udo Hölker and Lenz, 2005). This process also requires a small volume of fermentation leading to low operating costs, has lower probability of contamination due to low moisture, has an easy separation process and oxygen is typically freely available at the surface of particles. However, this process also has some disadvantages such as difficulties in monitoring some parameters of the process (pH, moisture content, oxygen and biomass concentration) due to the solid nature of the substrate, difficulties in temperature control and ventilation systems (Krishna C., 2008).

1.2.1. Microorganisms

Microorganisms, in SSF, grow under conditions closer to their natural habitats and so, they are more capable of producing certain enzymes and metabolites which, usually, they don't produce or produce with a low yield in SmF (Jecu, 2000).

Filamentous fungi are the microorganisms better adapted to SSF due to the fact that they have the ability of growing on solid substrate and can produce several extracellular enzymes (Vishwanatha *et al.*, 2010).

The filamentous fungus *Aspergillus* strain of the *Nigri* section are considered of wide economic importance due to the production of metabolites like enzymes and are used in the fermentation industry to produce hydrolytic enzymes, such as amylases or lipases (Graminha *et al.*, 2008; Pelizer *et al.*, 2007; Perrone *et al.*, 2008; Sharma *et al.*, 2001).

Aspergillus niger is a species widely used in SSF process and is one of the best extracellular enzyme producer (e.g. lipases and cellulases) (Dos Santos *et al.*, 2012; Falony *et al.*, 2006; Hosseinpour *et al.*, 2012; Pothiraj *et al*, 2006).

A. ibericus is a new species isolated from wine grapes (Serra *et al.*, 2006) and do not produce any relevant mycotoxins so, this species is safe to use for biotechnological applications. Studies shown that this species was able to produce lipases in SmF using OMWW as substrate (Abrunhosa *et al.*, 2013).

A. uvarum is described within *Aspergillus* section and is isolated from grape berries in Mediteranean area (Perrone *et al.*, 2008). It was found to be related to *A. japonicas* based on morphological data (Perrone *et al.*, 2008). No studies have been made in relation to enzymes production by SSF with *A. uvarum*, unlike *A. niger*. In relation to *A. japonicas*, this was isolated from olive mill wastes and demonstrated a lipolytic and cellulotic activity (Gopinath *et al.*, 2005).

Filamentous fungi of the *Trichoderma* genus are notable due to their high enzymatic productivity (Florencio *et al.*, 2012).

1.2.2. Enzymes

The production of enzymes is one of the most important applications of SSF and the agro-industrial substrates are considered the best substrates for the enzymes production. The enzymes produced by microorganisms can be cellulases, amylases, pectinases, proteases, lipases, xylanases, ligninases, among others. These enzymes have great commercial value and play a very important role in

the food, textile areas, paper, pharmaceutical. There are important factors that can affect the yield of enzymatic production like the type of strain, culture conditions, nature of the substrate and availability of nutrients (Nishio *et al.*, 1979).

Cellulases

Cellulases are enzymatic complexes that can degrade lignocellulosic residues, are one of the largest industrial enzyme worldwide, and can be used for production of ethanol, single-cell protein, for treatment of waste papers, for fruit juice extraction, cotton processing, animal feed additives and bleaching of pulp (Krishna C., 2008; Singhania *et al.*, 2010). The advantage of using lignocellulosic wastes like substrate, allows the reduction of costs in the production of cellulases, making the process more cheap (Xia and Cen, 1999).

These enzymes catalyse the hydrolysis of the β-1,4-glucosidic linkages of cellulose (Pérez *et al.*, 2002) and produce glucose, cellobiose and cello-oligosaccharides as primary products. The joint action of the enzymes, endo-glucanases (EG), cellobiohydrolases (CBH) and β-glucosidases (BGL) are used for hydrolysing cellulose. The rate-limiting step is the capacity of EG to reach for amorphous regions within the crystalline matrix and create new ends with which EG can act (Malherbe and Cloete, 2002). So, EG produces cuts in the cellulose polymer exposing reducing and non-reducing ends, CBH acts upon these reducing and non-reducing ends to liberate cellobiose and cello-oligosaccharides units, and BGL cleaves the cellobiose to liberate glucose (Singhania *et al.*, 2010).

From among the fungal strains that produce cellulases stand out *Aspergillus*, *Trichoderma*, *Penicillium*, and *Fusarium* genera (Brijwani *et al.*, 2010).

It has been found that strains of *Trichoderma* are poor in BGL, but can accumulate high activities of exo- and endo-glucanase (respectively CBH and EG) (Dueñas *et al.*, 1995). However, the strains of *Aspergillus* are high in BGL activity (Grajek, 1987).

Xylanases

Xylanases (endo-1,4-β-D-xylanases) are included in hemicellulase system and have been used for hydrolyse internal bonds in xylan chain from lignocellulosic materials, products such as brans and straws of different cereals, corn, hull and cobs, sugarcane and cassava bagasse, various saw dusts and different fruit processing and oil processing residues. Moreover, xylanases have immense potential for

increasing the production of several valuable products like xylitol and ethanol in a most economical way (Kapoor *et al.*, 2001).

The production of these enzymes requires substrates in very high concentration. Xylanases are produced mainly by *Trichoderma* spp. and *Aspergillus* spp. (Archana and Satyanarayana, 1997; Haltrich *et al.*, 1996).

The enzymes production was achieved successfully by *Aspergillus niger*, *Aspergillus fischeri* using wheat straw and wheat bran as main substrates (Ghanem *et al.*, 2000; Senthilkumar *et al.*, 2005). Ghanem *et al.* (2000) produced xylanases using *Aspergillus terreus* on wheat straw medium.

It was also observed that the addition of nitrogen source as supplement is an important step for xylanases production (Ghanem *et al.*, 2000).

Other cellular models can be used for xylanases production using SSF such as *Aureobusidium pullulans*, (Dorta and Arcas, 1998), *Thermomyces lanuginosus* (Christopher *et al.*, 2005), *Humicola insolens* (Dorta and Arcas, 1998) and *Melanocarpus albomyces* (Jain *et al.*, 1998).

1.3. Agro-industrial wastes for animal feed using solid-state fermentation

The FAO (2011) calculated an increase in cereal prices of 43 % from 2008 to 2011 for animal feed, this increase has driven the attention of ruminant nutritionists towards alternative sources of feedstuffs (Molina-Alcaide *et al.*, 2010; Salem, 2010), in order to reduce the production costs. By-products from agriculture represent a good alternative not only for the reduction of feeding costs, but also to reduce environmental problems associated with the accumulation of certain by-products (Vasta *et al.*, 2008).

Even though, by-products from agro-industries represent a good and alternative source for animal feed production, they present some limitations like high fibre content which results in lower digestibility (Levic *et al.*, 2010), antinutritional factors such as phenolic compounds that inhibit the ruminal symbionts (Martin and Akin, 1988; Mole *et al.*, 1993), and low protein, vitamins and minerals content (Hadar *et al.*, 1992).

SSF has been used as pre-treatment to improve the nutritive value of agriculture by-products (Ajila *et al.*, 2012). The growth of fungi in these by-products causes an increase in protein enriched and feed additives. The agro-industrial wastes have low protein content (0.5-6 %) and fermented waste can improve it to 10-15% (Shojaosadati *et al.*, 1999). In addition, other compounds can be increase as total lipids and fatty acids. Another advantage of SSF treatment is the increase of biodegradability of agro-industrial

wastes by ruminant animals and reduces the concentration of antinutritional factors such as phytic acid, polyphenols and tannins (Elyas *et al.*, 2002; Manmdebvu *et al.*, 1999).

The wastes from olive oil industries have already been evaluated as animal feed without SSF treatment. The olive cake can be used as supplementation in multi-nutrient blocks however cannot be used only as feed. Shabtay *et al.* (2009) studied the growth of *Pleurostus* species on olive mill solid waste, however these fungi degraded the lipids and reduced the metabolyzable energy of the olive mill waste.

Winery wastes also can be used as animal feed. Ishisida *et al.*, (2015), observed that winery sediment and grape pomace could alter nitrogen metabolism and/or act as new antioxidants for ruminants. Grape pomace is used in dry season when pastures are scarce, but its use is limited due to its low nutritive value (Sanchez *et al.*, 2002). Some strains of *Pleurotus* improve the nutritive value of these wastes and decrease their phenolic compounds (Sanchez *et al.*, 2002).

Brewery wastes were probed as feed in broiler chickens, ducks and carps (Carías and Millán, 1996; Dong and Ogle, 2004; Kaur and Saxena, 2004).

Some studies showed that the use of SSF increase the nutritional content of the by-products, for desirable values, to use them as animal feed (Hadar *et al.*, 1992; Iluyemi *et al.*, 2006; Jahromi *et al.*, 2010; Lio and Wang, 2012; Sanchez *et al.*, 2002; Tengerdy and Szakacs, 2003).

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Agro-industrial wastes

During this work, they were used four wastes: brewery spent grain (BSG) obtained from the brewery industry, exhausted olive pomace (EOP) collected from olive oil industry, exhausted grape mark (EGM) and vine-shoot trimmings (VST) from the winery industry. These residues were all obtained from industries in the north of Portugal during the season 2015/2016.

BSG was collected after mixing hot water and malt; EOP is obtained after recovery of residual olive oil from crude olive pomace (COP). VST are the mixture of leaves, stems and other compounds obtained from the pruning. EGP is the grape pomace after distilling of the residual alcohol. These residues were dried at 65 ˚C during 24 hours and stored at room temperature.

Enzymatic extracts obtained during this study were maintained at -20 ˚C for preservation prior to analysis. The fermented solids were dried at 65°C during 24 hours and stored in a dry place.

2.1.2. Reagents

Table 8- List of reagents used during the study.

Reagents	Company
3,5-dinitrosalicylic acid	Acros Organics
4-nitrophenol	Sigma
Agar	MERCK KGaA
AZO-CM-CELLULOSE (4M)	Megazyme
AZO-WHEAT ARABINOXYLAN	Megazyme
Boric acid	MERCK KGaA
BSA	SIGMA Diagnostics
Caffeic acid	Fluka
Chloridric acid	Fluka
Coomassie Blue	Thermo SCIENTIFIC
Folin-Ciocalteau reagent	AppliChem Panreac ITW Companies
Glacial acetic acid	Acros Organics
Glucose	Fisher Chemical
Hexane	Fisher Chemical
Malta extract	Fluka
Methanol	Fisher Chemical
Peptone	Chem Lab NV
Potassium hydroxide	AppliChem Panreac ITW Companies
Potassium Sodium Tartrate	MERCK KGaA
Sodium acetate trihydrate	SIGMA
Sodium carbonate	Fluka
Sodium carbonate	Fluka
Sodium chloride	AppliChem Panreac ITW Companies
Sodium hydroxide	Fisher Chemical
Sulfuric acid	PanReac AppliChem
Triton X-100	Acros Organics
Tween-80	Fisher Cientific
Urea	VWR Chemicals ProLab

2.1.3. Microorganisms

During this study were used different species of *Aspergillus*: *A. niger*, *A. uvarum* and *A. ibericus*.

To obtain inoculum for solid-state fermentation (SSF), the selected fungi were cultived on malt extract agar (MEA, 20 g/L malt extract, 1 g/L peptone, 20 g/L glucose and 20 g/L agar) slants, and incubated at 25 ˚C for 6 days. During the experimental period, strains were preserved at 4 ˚C and cultured monthly on fresh MEA slants.

2.2. Physical-chemical characterization of the residues

Residues were analysed for the physicochemical characterization, according to the procedures described in the next sections.

2.2.1. Humidity determination

A known quantity of waste (close to 0.5 g) was added to a metallic crucible with a known weight (without humidity). The crucible with the waste was placed in a hot air oven for 24 h at 105 ℃, to ensure that the waste was dried. After 24 h, the crucible was cooled in a desiccator containing silica gel and its weight was determined. This procedure was performed in triplicate. The Humidity (H) percentage was calculated according to **equation 1**:

$$\boldsymbol{H}\ (\%) = \frac{WCHS - WCDS}{WCHS - WC} * 100 \qquad \textbf{(eq. 1)}$$

WCHS is the weight of the crucible with humid waste in grams, WCDS is the weight of crucible with dry waste in grams and WC is the weight of the crucible in grams.

2.2.2. Ash determination

A known quantity of waste (close to 0.5 g) with known humidity was added to a porcelain crucible (previously exposed to a temperature of 575 ºC during 30 minutes in a muffle). The porcelain crucible with the waste was placed in a hot air oven at 105 ºC for about 24 h and after it was placed in the muffle for 2 h (until constant weight). After cooling in a desiccator containing silica gel, the container was

weighed. This procedure was made in triplicate. The ash percentage was calculated according to **equation 2**:

$$\boldsymbol{Ash}\ (\%) = \frac{WCA - WC}{(WCHS - WC) * (1 - H)} * 100 \qquad \textbf{(eq. 2)}$$

WCA is the weight of porcelain crucible with ash in grams, WC is the weight of porcelain crucible in grams, WCHS is the weight of porcelain crucible with humid waste in grams and H is the humidity in grams of water / grams of humid waste.

2.2.3. Nitrogen determination

Total nitrogen of the solid was determined using Kjeldhal method. This method is used in the measurement of protein content of biological materials, as so in the determination of nitrogen in inorganic materials, solids or liquids.

Kjeldahl method can be divided in two steps, first a digestion and then a titration. In the first step is performed a digestion of the sample by heating with concentrated sulfuric acid in the presence of a catalyzer (selenium or red Hg, the second one more efficient than the first but also with more ambient implications). This step is responsible for the reduction of organic nitrogen to ammonia, which is recovered in solution in the form of ammonium sulfate:

$$Norganic + H_2SO_4 + cataliser \rightarrow CO_2 + H_2O + (NH_4)_2SO_4$$

After digestion, the ammonium is displaced by a strong base in excess. It is used aqueous NaOH at 400 g/L. Stoichiometrically:

$$(NH_4)_2SO_4 + 2NaOH \rightarrow 2NH_3 + Na_2SO_4 + 2H_2O$$

The resultant solution, with NH_3 is distillated with vapor that drags with it the NH_3 being this recovered in a solution of boric acid:

$$NH_3 + H_3BO_3 \rightarrow NH_4^+ + H_2BO_3^-$$

The borate of the acid solution is titrated (second step) with sulfuric acid to quantify the quantity of ammonium according to the reaction:

$$2H_2BO_3^- + 2H^+ + SO_4^{2-} + 2NH_4^+ \rightarrow 2H_3BO_3 + (NH_3)_2SO_3 + H_2O$$

The Kjeldahl method protocol consists in turn on the thermoblock (tecator system 1007/6) which should achieve the 400 °C. In each digestion tube is placed 0.5 g of the sample and then it is added 10 mL of H_2SO_4 concentrated and tablet of catalyzer selenium (Tecator S/3.5). The solution is carefully mixed and placed on the thermoblock previously heated to 400°C. After digestion is performed the titration.

2.2.4. Lignocellulosic characterization

The structural components of lignocellulosic wastes (BSG, EOP, EGP and VST) such as cellulose, hemicellulose and klason lignin were characterized by quantitative acid hydrolysis (QAH) in a two-stage acid treatment. The first stage with 72 % wt H_2SO_4 at 30 ºC for 1 h and the second stage after dilution of 4 % wt H_2SO_4 at 121 ºC for 1 h.

A sample (close to 0.5 g) was weighted into a test tube, 5 mL of 72 % H_2SO_4 was added to the tube and they were placed in a water bath at 30 ℃ during 1 hour with agitation (first stage). After this period, the test tubes content was transferred for flasks and, the waste that stayed attached to the walls was dragged with distilled water. Distilled water was added to dilute the solution up at 4% (w/w) H_2SO_4. The flasks were closed and introduced in the autoclave during 1 hour at 121 ºC (second stage). Posteriorly, the flasks were cooled and was determined the originated losses during the second stage by weighting the flasks. The entire content of each flask was filtered through a Gooch crucibles with known weight. The Gooch crucibles with insoluble fraction were placed on an oven at 105 °C. After 24 h the crucibles were cooled on a desiccator containing silica gel and then weighted. This determination was performed in duplicate.

The filtrate was analysed by High Performance Liquid Chromatography (HPLC) system for measure sugars (glucose, xylose and arabinose) and acetic acid. Using a Jasco830-IR intelligent refractive-index detector and a Varian MetaCarb 87H column. The column was eluted with 0.005 M H_2SO_4

and the flux was 0.7 mL/min at 60 °C. Calibration curves were constructed with glucose, xylose, arabinose and acetic acid standard solutions between 0.1 g/L e 10 g/L.

With the data of sugars concentrations (glucose, xylose, arabinose and acetic acid) was calculated the content in polymers (CP). The CP, glucan (CGn), xylan (CXn), arabinan (CArn), and acetyl groups (CGA) were calculated according to **equation 3** and expressed as grams of polymer per 100 grams of dry waste.

$$\boldsymbol{CP}\ (\%) = F * SCF * \frac{[S]}{\rho} * \frac{W+WHS*H}{WHS*(1-H)} * 100 \qquad \textbf{(eq.3)}$$

Wherein F is a factor which corrects degradation of sugars (1.04 for CGn, 1.088 for CXn/CArn and 1.00 for CGA), SCF is a stoichiometric correction factor to take account the increase in molecular weight during hydrolysis (162/180 for CGn, 132/150 for CXn/CArn and 43/60 for CGA), S is the monomer concentration in g/L, ρ is the density of the analysed dissolution in g/L (as the samples were diluted in water for HPLC analysis the value is about 1000 g/L), W is the weight of added water in grams and corrected to take account the losses during second stage of QHA, WHS is the total weight in grams of humid waste and H is the humidity in grams of water/grams of humid waste.

Cellulose (grams of cellulose per 100 grams of dry waste) and hemicellulose (grams of hemicellulose per 100 grams of dry waste) content were determined according to **equation 4** and **equation 5**, respectively.

$$\boldsymbol{Cellulose}\ (\%) = \mathrm{CG_n} \qquad \textbf{(eq.4)}$$

$$\boldsymbol{Hemicellulose}\ (\%) = CX_n + CAr_n + CG_A \qquad \textbf{(eq.5)}$$

The increase weight of the Gooch container matches to Klason lignin, thus the content of lignin (grams of lignin per 100 grams of dry waste) was calculated according to **equation 6.**

$$\boldsymbol{Klason\ lignin}\ (\%) = \frac{WCDS-WC}{WCHS*(1-H)} * 100 \qquad \textbf{(eq.6)}$$

Wherein WCDS is the weight of Gooch crucible with dry sample in grams, WC is the weight of Gooch crucible in grams, WCHS is the weight of Gooch crucible with humid initial sample who underwent

to the QHA in grams and H is the humidity in grams of water/grams of humid waste. The value of ashes was removed from lignin content.

Natural detergent fiber (NDF) was calculated as sum of cellulose, hemicellulose and lignin, acid detergent fiber (ADF) was calculated as sum of cellulose and lignin.

2.2.5. Reducing sugars determination

Free reducing sugars were measured by the 3,5-Dinitrosalicylic acid (DNS) method (Miller, 1959). To each test tube was added 0.1mL of DNS reagent and 0.1 mL of sample (0.1 mL of distilled water to perform the blank) in duplicate. Then, the test tubes were placed in a bath at 100 ºC for 5 minutes. After cooling, it was added 1 mL of distilled water in order to stop the reaction. The absorbance was measured at 540 nm. Calibration curve was constructed with glucose standard solutions between 0 g/L and 4 g/L.

2.2.6. Total phenols determination

Total phenols were measured by the Folin-Ciocalteu method (Commission Regulation (EEC) No. 2676/90).

To each test tube was added 100 µL of sample or 100 µL of distilled water (to perform the blank), 2 mL of Na_2CO_3 at 15 %, 500 µL of Folin-Ciocalteu reagent and 7.4 mL of distilled water in duplicate.

The test tubes were placed on a bath at 50 °C during 5 minutes. After cooling at room temperature, the tubes were vortexed during 30 seconds proximally. After stabilization, the absorbance was read at 700 nm. Calibration curve was constructed with standard solutions of caffeic acid with concentrations between 0 g/L and 2 g/L.

2.2.7. Protein determination

Proteins were measured by the Bradford method (Marion Bradford, 1976). 10 µL of sample and 300 µL of Coomassie Blue reagent was added (in duplicate) to each well of microplate. For the blank analysis, it was added 10 µL of distilled water. The microplate was stirred and left for stabilization during 10 minutes at room temperature. Absorbance was read at 595 nm. Calibration curve was constructed with standard solutions of bovine serum albumin (BSA) with concentrations between 0 g/L and 1 g/L.

2.2.8. Analysis of enzyme activities

Cellulase and xylanase activities

The activity of cellulases (endo-1, 4-β-Glucanase) was determined with the enzymatic kit Azo-CM-Cellulose S-ACMCL 094/12 (Megazyme International, Ireland). Enzymatic extracts were diluted with 0.1 M sodium acetate buffer (pH 4.6) to final volume of 0.5 mL and mixed with 0.5 mL substrate solution (CM-Cellulose 4M). Before analysis, the solutions were pre-equilibrated at 40 °C. The blank was performed with water and substrate solution. Then, the mixture was stirred and incubated for 40 °C and 10 minutes. To end the reaction, substrate was precipitated by the addition of 2.5 mL of precipitant solution (40 g/L $C_2H_3NaO_2.3H_2O$, 4 g/L $Zn(O_2CCH_3)_2$ in ethanol solution at 76%, pH 5) with vigorous stirring for 10 seconds on a vortex mixer. The reaction tubes were stabilized 10 minutes at room temperature. The tubes content was stirred again and centrifuged at 1,000 g for 10 minutes. The supernatant solution absorbance was measured at 590 nm and it was determined the enzyme activity by reference to a standard curve.

One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of glucose reducing sugar equivalents from CM-Cellulose in 1 min at 40 °C and pH 4.6. The values of cellulases activity were expressed in units per gram of dry substrate (U/g).

The activity of xylanases (endo-1, 4-β-xylanases) was determined with the enzymatic kit Azo wheat arabinoxylan S-AWAXL 05/14 (Megazyme International, Ireland).

The procedure to determine of xylanases activity was the same as for the determination of cellulases activity but the buffer solution was 0.1 M sodium acetate buffer (pH 4.5), substrate solution was wheat arabinoxylan and precipitation solution was ethanol (≈95%).

One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of xylose reducing sugar equivalents from wheat arabinoxylan in 1 min at 40 °C and pH 4.5. The values of xylanases activity were expressed in units per gram of dry substrate (U/g).

An alternative method for enzymes activity determination was performed. In the case of cellulases the substrate was carboxymethylcellulose (CMC) and with xylanases, the substrate was from beechwood xylan.

The procedure for determination of cellulases activity was to add 250 μL of cellulase substrate (CMC 1% in 0.1 M sodium acetate buffer, pH 4.6) to test tubes and then, 250 μL of diluted sample in buffer. The test tubes were placed on a bath at 50 °C for 30 minutes. After 30 minutes it was added 500

µL of DNS and then the test tubes were placed on a bath at 100 °C for 5 minutes. Finally, they were added 5 mL of distilled water and the absorbance was read at 540 nm. The blank was performed with sodium acetate buffer.

A calibration curve was constructed with glucose standard solutions in buffer between 0 g/L and 2 g/L.

The procedure to determine the xylanases activity was the same as for the determination of cellulases activity but the duration of the reaction was of 15 minutes instead of 30 minutes and the substrate solution was beechwood xylan (2%).

β-glucosidase activity

β-glucosidase determination was performed using p-Nitrophenyl-β-D-glucopyranoside (PNG) as substrate. The reaction was carried out adding 100 µL of PNG to 1.5 mL amber glass vials and then 100 µL of sample with suitable dilution in cellulase buffer (above described). The reaction was at 50 °C during 15 minutes. After 15 minutes, it was added 600 µL of Na_2CO_3 (1M) and 1700 µL of distilled water. Finally, the absorbance was read at 400 nm.

A calibration curve was made with 4-nitrofenol with concentrations between 0 g/L and 100 g/L. One International unit (IU) of enzyme activity was defined as the quantity of enzyme required to liberate 1 µmol of p-nitrophenol per minute under standard assay conditions. The values were expressed in units per gram of dry substrate (U/g).

2.2.9. Quantification of ergosterol

The first step was to weigh 1 g of sample in a tube (in duplicate) and to add 20 mL of potassium hydroxide (10%) solution in methanol. The solution was stirred for 20 minutes. After, 10 mL of the solution were removed to another cup and was heated at 55-60 °C for 20 minutes. During the heating the cups must be sealed. After cooling, the solutions were placed on test tubes and they were added 3 mL of distilled water and 2 mL of hexane. The test tubes were vortexed during 1 minute obtaining two phases. The upper phase (hexane) was removed to an amber glass vial. The extraction with hexane was performed two more times to each sample.

The amber glass vials with samples were placed in contact with a nitrogen source in order to evaporate the hexane. After the evaporation, 2 mL of methanol were added and the samples were transferred to 1.5 mL amber glass vials to be analyzed by HPLC.

The ergosterol content in methanol solution was determined using an HPLC Varian equipped by autosampler (Basic-Marathon, Spark), isocratic pump (Varian 9002) and UV detector (Varian ProStar). The column was a reverse phase-C18 (Waters Spherisorb ODS2, 4.6 x 250 mm). The mobile phase was methanol at a flow rate of 1 mL/min and detection was made at 282 nm. The ergosterol was identified by comparing retention times with ergosterol standard (from Sigma) and it was quantified by calibration curve.

2.4. Solid-state fermentation

2.4.1. Screening of filamentous fungi by SSF

BSG, EOP, EGP and VST were used as substrate in SSF experiments. The fungi used during SSF with the different substrates were *A. uvarum*, *A. niger* and *A. ibericus.*

SSF process was carried out in 500 mL Erlenmeyer with 10 g of dry substrate. Moisture level was adjusted to 75 % (wet basis) with urea solution in water. Urea was added to adjust the ratio C/N to 15. When BSG was used as substrate, urea was not added because the C/N ratio was already near 15. Erlenmeyers with solid medium were sterilized at 121 ˚C for 15 minutes.

For inoculation, the selected fungi were suspended in a sterile solution (1 g/L peptone and 0.1 g/L Tween 80). The inoculum spore concentration was adjusted to 10^6 spores/mL using a Neubauer counting chamber. Each Erlenmeyer was inoculated with 2 mL of spore suspension and incubated at 30 ˚C for 6 days. The extraction of enzymes was performed at the end of each experiment with a solution of 10 g/L NaCl and 5 g/L Triton X-100 at room temperature with agitation (150 r.p.m.) for 1 h. Following, extract was filtered through a net and the liquid fraction was centrifuge at 6000 r.p.m. for 10 minutes. The pellet was added to the filtrated solid and weighted and used for determination of lignocellulosic composition, total nitrogen, ashes and moisture; the liquid was recovered and enzymatic activity, soluble protein, phenolic compounds and reducing sugars were quantified.

2.4.2. Experimental design

To evaluate the effect of mixture of agro-industrial wastes, a simplex-centroid design it was performed an experimental design (Simplex-centroid design) was carried out. This design consists of mixture runs characterized by all one factor, all combination of two factors at equal levels and all combinations of three factors at equal levels. In addition, a center point with equal amounts of all wastes is studied. Thus, this design allows to test four agro-industrial wastes as substrate and to evaluate the interaction effects among them in SSF. The fungus selected in **2.4.1.** section was used in this study and the four residues were combined in different proportions as seen in **Table 9**.

All experiments were performed in duplicate and in randomized run order. In runs with EGM, EOP and VTS urea was added as source to fit the ration C/N close to 15. The variables dependents studied were xylanase, cellulose, β-glucosidases activities and the increase of N.

Table 9- Residues mixture, obtained from Simplex-centroid design

					Dried solid			
	A	B	C	D	BSG (g)	EGM (g)	EOP (g)	VST (g)
1	1	0	0	0	10	0	0	0
2	0	1	0	0	0	10	0	0
3	0	0	1	0	0	0	10	0
4	0	0	0	1	0	0	0	10
5	0.5	0.5	0	0	5	5	0	0
6	0.5	0	0.5	0	5	0	5	0
7	0.5	0	0	0.5	5	0	0	5
8	0	0.5	0.5	0	0	5	5	0
9	0	0.5	0	0.5	0	5	0	5
10	0	0	0.5	0.5	0	0	5	5
11	0.33	0.33	0.33	0.00	3.33	3.33	3.33	0.00
12	0.33	0.33	0.00	0.33	3.33	3.33	0.00	3.33
13	0.33	0.00	0.33	0.33	3.33	0.00	3.33	3.33
14	0.00	0.33	0.33	0.33	0.00	3.33	3.33	3.33
15	0.25	0.25	0.25	0.25	2.50	2.50	2.50	2.50

BSG- brewery spent grain; EGM- exhausted grape marke; EOP- exhausted olive pomace; VST- vine-shoot trimmings

All experiments were performed in duplicate and in randomized run order. In runs with EGM, EOP and VTS urea was added as source to fit the ration C/N close to 15. The variables dependents studied were xylanase, cellulose, β-Glucosidases activities and the increase of N.

A multiple regression model (special cubic type), given by **Equation 7 (Eq 7)**, was used to fit the experimental results.

$$\boldsymbol{y} = b_1 \cdot x_1 + b_2 \cdot x_2 b_3 \cdot x_3 + b_4 \cdot x_4 + b_{12} \cdot x_1 \cdot x_2 + b_{13} \cdot x_1 \cdot x_3 + b_{14} \cdot x_1 \cdot x_4 + b_{23} \cdot x_2 \cdot x_3 + b_{24} \cdot x_2 \cdot x_4 + b_{34} \cdot x_3 \cdot x_4 + \\ + b_{123} \cdot x_1 \cdot x_2 \cdot x_3 + b_{124} \cdot x_1 \cdot x_2 \cdot x_4 + b_{134} \cdot x_1 \cdot x_3 \cdot x_4 + b_{234} \cdot x_2 \cdot x_3 \cdot x_4 \quad \textbf{(Eq 7)}$$

where *y* represents the response variable, *b* denotes the coefficients of regression and *x* denotes the independent variables. The experimental data were evaluated using Statistica 5.0 software. Each dependent variable was optimized individually using an application of commercial software (Solver, Microsoft Excel 2007, Redmon, WA, USA). To maximize several dependent variables together, an optimization of multiple response was carried out with Statgraphics plus 5.1 software.

The inoculation process, incubation and enzymes extraction were performed as described in section **2.4.1**. After enzymes extraction, the samples were all analyzed to determine the enzymes activities, total proteins, reducing sugars, total phenols in the extract and total nitrogen, cellulose, hemicellulose and lignin content in fermented solid.

2.4.3. Study of kinetic enzymes production

In order to study the kinetic of enzymes production, the evolution of reducing sugars, proteins, phenols, nitrogen and ergosterol, the experiments were observed over time. During this process was used the optimal operational conditions obtained from experimental design. Erlenmeyers with the same conditions were used and in each time one Erlenmeyer was taken for analysis. The SSF, inoculation and extraction of enzymes was carried out in the same conditions as in section **2.4.1**. The incubation was at 30 ˚C during seventeen days.

2.4.4. Tray bioreactor

The final step of this study was a scale up of the best conditions, obtained from the screening of fungus, optimization of substrate composition to achieve the maximum enzymes production and increase the protein content in fermented solid.

The scale up was performed using a tray bioreactor where it was added 500 g of solid with 0.6 g BSG/ g solid and 0.4 g VST/ g solid. The moisture was adjusted with urea solution (0.028 g urea/g solid) to adjust the moisture to 75% (wet basis). For inoculation, it was used 50 mL of sterilized spore suspension, adjusted to a concentration of 10^6 spores/mL using a Neubauer counting chamber. The incubation conditions and enzyme extraction process were the same as described in section **2.4.1.** After incubation, the samples were analyzed like it was described in section **2.4.2.**.

2.3. Statistical analysis

Results are presented as the mean ± standard deviation (SD) of at least two replicates. The analysis were carried out using Microsoft Office Excel software.

3. RESULTS AND DISCUSSION

3.1. Physical-chemical characterization of the residues

The solid wastes used during this study were first characterized to assess their potential as solid substrate in SFF. The physical and chemical characteristics of BST, EOP, EGM and VST were compared with others values obtained in literature. It was analysed the content of moisture, ash, total solids, nitrogen, some minerals and organic constituents such as hemicellulose, cellulose and lignin. In addition, it was measured the concentration of proteins, reducing sugars and phenols in the extract.

An important parameter is the cellulose and hemicellulose content because they can induce the production of enzymes such as cellulases and xylanases (Leite *et al.* , 2016), which are capable of degrading cellulose and hemicellulose respectively, contributing to a better digestibility by the ruminant. The ratio C/N is important in growth of fungi, it is estimated that the optimal ratio is among 10-20 depending of fungus strain (Gao *et al.*, 2007). Another important parameter is the phenols, sugars and protein content in the extract. These compounds are easily available for fungi and can induce or inhibit the growth of fungi in solid-state fermentation (SSF). Phenolic compounds must be taken in consideration because they can inhibit the growth of fungi in SSF (Medina *et al.*, 2011). However, the simple sugars can improve the initial growth of fungi (Ertan Inceoğlu *et al.*, 2014). On the other hand, a high concentration of simple sugar can actuate as repress of microbial enzyme synthesis (Ertan Inceoğlu *et al.*, 2014).

3.1.1. Brewery wastes

It is possible to observe on **Table 10** that every parameter is according to the values obtained on literature. The most significant difference is in cellulose content and in the case of soluble proteins and reducing sugars. These differences may occur due to the fact that BSG can be a mixture of different cereals with different composition which can influence the content of cellulose. The content of total sugars can be influenced by the storage conditions. The storage of wet BSG, after removing of the mash tank, at room temperature can allow the growth of microorganisms, which can degrade the simple sugars rapidly. In this work the BSG was storage at 4°C after removing the mash tank. Then, it was dried and held in moisture free conditions. Thus, the proliferation of microorganism and the consumption of total sugars

were avoided. The soluble protein is relatively low when compared to the literature and this is an intrinsic characteristic of the residue in study and as said before can be a mixture of cereals which can contribute to a smaller value when compared to the literature, another explanation is relatively to the malting process, during this process the total protein is concentrated and in this case, this process could not be as efficient as it should be (Waldron, 2009).

In comparison to winery and olive mill wastes, the BSG had an important quantity of nitrogen and crude protein (216.69 g/kg). For this reason, the BSG is more suitable for animal feed. The ratio C/N was between 10-20, thus it is suitable for growth of filamentous fungi by SSF (Gao *et al.*, 2007). In this case, the addition of supplement of nitrogen for fungi growth is not necessary reducing the cost of process.

In terms of micronutrients, most of them are near the values obtained on the literature and the one with more discrepancy is the Na which as a lower value than literature.

Table 10- Composition of BSG and comparison with literature.

Parameter	BSG*	Literature
Humidity (% w/w)	73.5 ± 0.4	72.5 - 82.6
Total solids (% w/w)	26.5 ± 0.4	17.4 - 27.46
Ash (g/kg)	12.75 ± 0.05	11 – 46
Lignin (g/kg)	153.68 ± 17.58	119 - 278
Hemicellulose (g/kg)	339.96 ± 5.05	192 -419
Cellulose (g/kg)	362.16 ± 10.24	168 - 260
Crude Protein	217 ± 21	153 – 247
***Soluble protein (g/kg)**	1.07 ± 0.33	23.1
***Reducing Sugars (g/kg)**	132.53 ± 0.41	6.5
***Phenols (g/kg)**	2.39 ± 0.04	n.d.
N (g/kg)	34.67 ± 3.36	24.4 - 43
C (g/kg)	471.4 ± 0.01	457 - 523
C/N	12.23	11-9 - 12.1
Ca (g/kg)	2.45 ± 0.06	0.5 – 3.6
K (g/kg)	0.72 ± 0.04	0.26 – 0.7
Mg (g/kg)	1.81 ± 0.06	1.9 – 2.4
Zn (mg/kg)	154 ± 4.24	82 – 200
Cu (mg/kg)	12.5 ± 0.7	11.4 – 18
Fe (mg/kg)	138.5 ± 4.95	155 – 280
Mn (mg/kg)	40.5 ± 0.71	41 – 51
Cr (g/kg)	<22	n.d.
Ni (mg/kg)	<22	n.d.
Pb (mg/kg)	<22	n.d.
Na (mg/kg)	56 ± 1.4	137 - 309

All values are expressed per kg of dry material; n.d.: not determined.

BSG- Brewery spent grain

*Value in aqueous extract

Canedo *et al.*, 2016; Carvalheiro *et al.*, 2004; Dos Santos *et al.*, 2012; Kanauchi *et al.*, 2001; Meneses *et al.*, 2013; S. I. Mussatto *et al.*, 2006; Silva *et al.*, 2004; Waters *et al.*, 2012; Xiros and Christakopoulos, 2012

3.1.2. Olivemill wastes

Table 11 shows the characterization of EOP and the values observed in olive pomace by other authors. The main difference was the lipids content, this is because other authors characterized the olive pomace without extraction of residual olive oil, but in this work the residual olive oil was removed from olive pomace remaining low concentration of lipids on the waste.

Nitrogen and carbon levels are also below the mean values observed in literature, although, carbon levels are very high when compared to the nitrogen levels which causes a high C/N ratio of 50.1 which is very distant from the optimum range.

It is remarkable the higher content of phenolic compounds in EOP comparing to winery and brewery wastes. In this sense, it was reported the phytotoxic and antimicrobial effect of olive mill wastes due to the phenol content and other compounds as organic and fatty acids (Alburquerque, 2004).

Table 11- Composition of EOP and comparison with literature.

Parameters	EOP*	Literature
Humidity (% w/w)	9.66± 0.12	5.4 - 9.9
Total solids (% w/w)	90.34 ± 0.12	90.1 – 94.6
Ash (g/kg)	33.6 ± 1.68	48 - 66
Lignin (g/kg)	550 ± 15	416.2
Hemicellulose (g/kg)	289.16 ± 0.54	241
Cellulose (g/kg)	128.77 ± 2.42	110
Crude Protein	57.51 ± 3.58	79.4
***Soluble protein (g/kg)**	0.54 ± 0.08	2.6
***Reducing Sugars (g/kg)**	33.07 ± 1.59	42 - 174.5
***Phenols (g/kg)**	7.07 ± 0.07	8.9 - 16.42
N (g/kg)	9.2 ± 0.57	11 - 24
C (g/kg)	460.7 ± 12.9	481 – 516
C/N	50.1	21.5 – 43.7
Ca (g/kg)	1.8 ± 0.2	0.57 - 6.2
K (g/kg)	14.2 ± 0.7	6.9 - 21.2
Mg (g/kg)	0. 47 ± 0.01	0.51 - 1.6
Zn (mg/kg)	10.5 ± 0.7	7.9 - 31.5
Cu (mg/kg)	11 ± 1	9.4 - 14.41
Fe (mg/kg)	147 ± 33	566.37 - 589
Mn (mg/kg)	10.2 ± 0.4	16.45 - 20
Cr (g/kg)	<22	4.31 – 10.8
Ni (mg/kg)	<22	2.38 – 5.8
Pb (mg/kg)	<22	0.53 – 1.4
Na (mg/kg)	92 ± 5	255 - 300

All values are expressed per kg of dry material; n.d.: not determined.

EOP: exhausted olive pomace

*Value in aqueous extract

Camposeo and Vivaldi, 2011; Leite *et al.*, 2016; López-Piñeiro *et al.*, 2007; Reina *et al.*, 2016; Russo *et al.*, 2015

3.1.3. Winery wastes

Table 12 represents the chemical characterization of EGM and VST. EGM presents very low humidity when compared to the values from the literature and this was due to the waste was dried after distillation process, in the other hand VST is slightly below the mean values.

Table 12- Composition of winery wastes and comparison with literature.

Parameter	EGM *	EGM in literature	VTS*	VTS in literature
Humidity (% w/w)	10.9 ± 0.1	59.3	6.33 ± 0.53	7.85 – 8.3
Total solids (% w/w)	89.1 ± 0.1	40.7	93.67 ± 0.1	91.7 - 92.15
Ash (g/kg)	90.95 ± 5.58	81	35.87 ± 4.32	30.4 – 37.8
Lignin (g/kg)	666.26 ± 5.09	609	340.85 ± 5.54	192.7 – 271
Hemicellulose (g/kg)	101.78 ± 3.81	75	237.79 ± 3.81	178.3 – 260
Cellulose (g/kg)	144.49 ± 2.11	124	423.96 ± 5.25	378.3 – 436.4
Crude Protein	134.74 ± 3.97	112.5 – 171.9	37.9 ± 0.2	35.12
***Soluble protein (g/kg)**	0.54 ± 0.08	1.3	0.54 ± 0.08	1.27
***Reducing Sugars (g/kg)**	4.1 ± 0.07	3	4.1 ± 0.07	55.35
***Phenols (g/kg)**	1.72 ± 0.01	0.19	1.72 ± 0.01	1.25
N (g/kg)	21.56 ± 0.64	18 -27.5	6.06 ± 0.03	5.62
C (g/kg)	482.4 ± 16.1	233 - 533	462.6 ± 2.2	453.6
C/N	22.38	8.5 – 29.6	74.85	80.71
Ca (g/kg)	3.04 ± 0.19	0.1 -25.8	2.02 ± 0.12	0.27
K (g/kg)	7.14 ± 0.22	5.6 -19.7	4.76 ± 0.15	1.54
Mg (g/kg)	0.14 ± 0.01	0.3 -2	0.09 ± 0.01	0.32
Zn (mg/kg)	9 ± 2.55	12 - 28	6 ± 1.7	2.2
Cu (mg/kg)	50.1 ± 8.9	15 – 99.84	33.4 ± 5.9	1.06
Fe (mg/kg)	1255.5 ± 69.16	161 - 842	817 ± 46	1.36
Mn (mg/kg)	43.44 ± 4.16	4-28	28.96 ± 2.77	2.58
Cr (mg/kg)	47.4 ±3.82	<0.2 – 21.1	31.6 ±1.7	n.d.
Ni (mg/kg)	45.9 ± 3.8	<0.2 – 16.3	30.6 ±2.55	n.d.
Pb (mg/kg)	<22	<0.4 -133.6	<22	n.d.
Na (mg/kg)	6110.1 ± 485.78	165 -1601	4073.4 ± 323.85	n.d.

All values are expressed per kg of dry material; n.d.: not determined.

EGM: exhausted grape marc; VTS: Vinetrimming shoots

*Value in aqueous extract; Minerals of VTS in aqueous extracts

Bustamante *et al.*, 2008; Jiménez *et al.*, 2006; Jiménez *et al.*, 2007; Moldes *et al.*, 2007; Paradelo *et al.*, 2013; Salgado *et al.*, 2014; Sánchez-Gómez *et al.*, 2014; Toscano *et al.*, 2013

In relation to ashes content, EGM as also a higher content of ashes than the ones observed in the literature which is explainable by a high content of Fe, Mn, Cr, Ni and Na. On the case of VST the ashes content is in the range of literature values. Although in mineral composition, it is possible to observe high values of Ca, K, Zn, Cu, Fe and Mn. Some variations in mineral contents such as Cu, Mn, Zn, Cd, Cr and Pb could be due to the phytosanitary treatments of plants with pesticides (Kristl *et al.,* 2003).

The EGM showed the higher content in lignin of studied wastes. The high lignin content can hinder the digestion of polysaccharides within rumen (Buswell and Eriksson, 1994). Thus, the direct use of EGM as animal feed is not suitable. Mixture with other agro-industrial wastes or biotechnological treatments could improve its digestibility.

3.2. Evaluaton of agro-industrial wastes as substrate in SSF by filamentous fungi

During this section, BSG, EOP, EGM and VST were inoculated with three different filamentous fungi such as *A. uvarum* MUM 08.01, *A. ibericus* MUM 03.49 and *A. niger* 01UAs183. The goal was the selection of fungus that produces higher cellulose and xylanase activities and increase the protein content of agro-industrial wastes.

In a previous work, Leite *et al.* (2016) studied the lignocellulytic activities on agar plates of several fungi. *A niger* CECT 2915 showed the high cellulose and xylanase activities, however the use of this fungus was already studied. Thus, others fungi from MUM collection (Micoteca of Universty of Minho) less studied and with potential to produce lignocellulolytic activities were evaluated.

3.2.1. Evaluation of cellulases and xylanases production

Figure 3 shows the activities of cellulases and xylanases achieved after SSF. Values obtained after the analysis of controls shown that there is none activity of cellulases or xylanases. So, after SSF there is always an increase in cellulases and xylanases activity.

Respect to cellulose activity, it is possible to see that *A. ibericus* was the fungus which contributes to a higher cellulose activity using BSG as solid substrate. *A. niger* was the fungus which produced cellulases with higher activity in EGM and VTS. In the case of EOP, there was not statistically significant differences (p-value < 0.05) in cellulases activity between *A. ibericus* and *A. niger*. *A. uvarum* was the

fungi with lower cellulase activity for every residue. Only in the case of EOP, the cellulose activity was similar to the achieved activity by *A. uvarum* and *A. niger*.

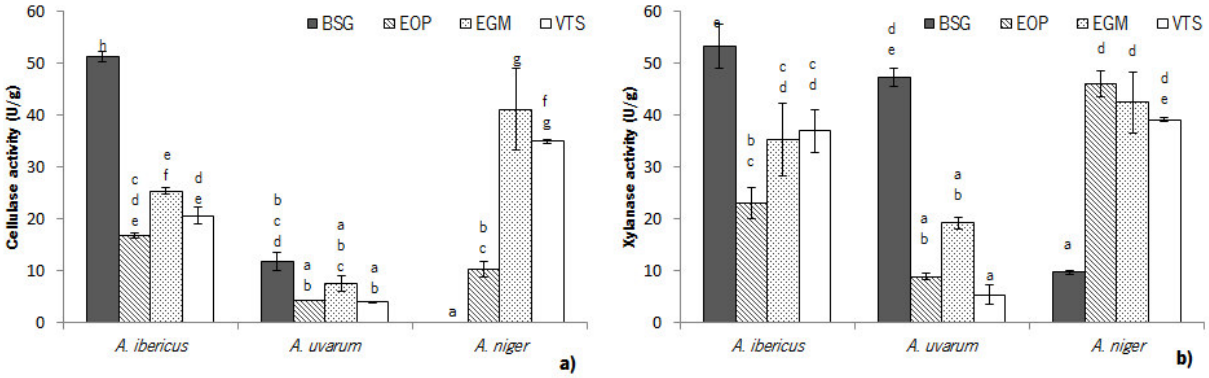

Figure 3- Cellulases (a) and xylanases (b) production during SSF. These results represent the average of two independent experiments and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different. BSG- brewery spent grain; EOP- exhausted olive pomace; EGM- exhausted grape marke; VTS- vine shoot trimmings.

On xylanases case, the three fungi showed high xylanase production. As in the case of cellulases, xylanases activity is also low using BSG and *A. niger*. The BSG was not supplemented by additional nitrogen source, this could have affected the growth of *A. niger*. As it was mentioned before, the ratio C/N is an important parameter that can be different for each microorganism (Gao *et al.*, 2007). In this study, the ratio C/N was fixed in 15 for all wastes, however different enzyme production by fungi was observed. The maximum xylanase activity was achieved by *A. ibericus* and *A. uvarum* using BSG as solid substrate, it was not observed statistically significant differences (p-value < 0.05) among them. The others wastes studied showed similar xylanase activities when they were inoculated by *A. niger.*

Previous studies show that *A. niger* 01UAs101, *A. ibericus* MUM 03.49 and *A. uvarum* achieved similar cellulose and xylanase activities using as substrate mixture of crude olive pomace, EGM and VTS (Salgado *et al.*, 2014b). The use of BSG as solid substrate was barely studied, Sim *et al.*, (1990) evaluated the BSG as solid substrate for cellulose production using a typical cellulases producer as *Trichoderma reesei* and they achieved a 287 U/g cellulose. This value was higher than the achieved in this study (51.20 U/g cellulose), however the quantity of solid and bed height that they used was lower (5 g) than the present study. The bed height is an important parameter that can affect the enzyme production (Casciatori *et al.*, 2014).

The use of EOP as solid substrate for cellulose and xylanase production was evaluated by Leite *et al.*, (2016). They observed a maximum enzyme production using *A. niger* CECT 2915 achieving about 25 U/g and 35 U/g of xylanase and cellulose, respectively. Salgado *et al.* (2015) evaluated the mixture

of EGM and crude olive pomace as solid substrate and they achieved 32 U/g of cellulose activity and 10.5 U/g of xylanase activity using *A. uvarum* as fungus.

Cellulose, hemicellulose and lignin present in agro-industrial wastes can induce the production of lignocellulolytic enzymes (Chmelova *et al.*, 2011) so, it was expectable that the residues with higher content of lignocellulosic compounds should produce more enzymes which contributes to a higher enzyme activity. BSG is the residue with higher content in cellulose which can contribute to produce cellulases in more quantity and this was observed in the case of *A. ibericus* and *A. uvarum*.

In general, all wastes showed that are suitable substrates for cellulose and xylanase production. Thus, the mixture of wastes was evaluated in an optimization stage using an experimental design that allows to observe the effect of mixture of wastes on dependent variables. The *A. ibericus* was selected to carry out this stage, since it showed the best cellulose and xylanase production.

3.2.2. Increase of protein content after SSF

The protein content was determined in solid substrates before fermentation, in control (solid substrate after addition of urea and sterilization) and in fermented solids by the three fungi studied. As can be observed in **Figure 4**, the protein content of four fermented wastes was increased with respect to not fermented and control.

As it was observed in characterization of wastes, BSG is the waste with higher initial protein content, however the protein content in other wastes is very low, particularly in EOP and VTS. The addition of urea and sterilization process (control) barely modified the protein content, only in winery wastes were observed significant differences, increasing the content of protein.

Although BSG had an important content in protein, the fermentation with three fungi increased further the protein. There was not significant difference between three fungi, the increase of protein was about 25 % respect to BSG not fermented (weight loss was taken into account). Canedo *et al.* (2016)

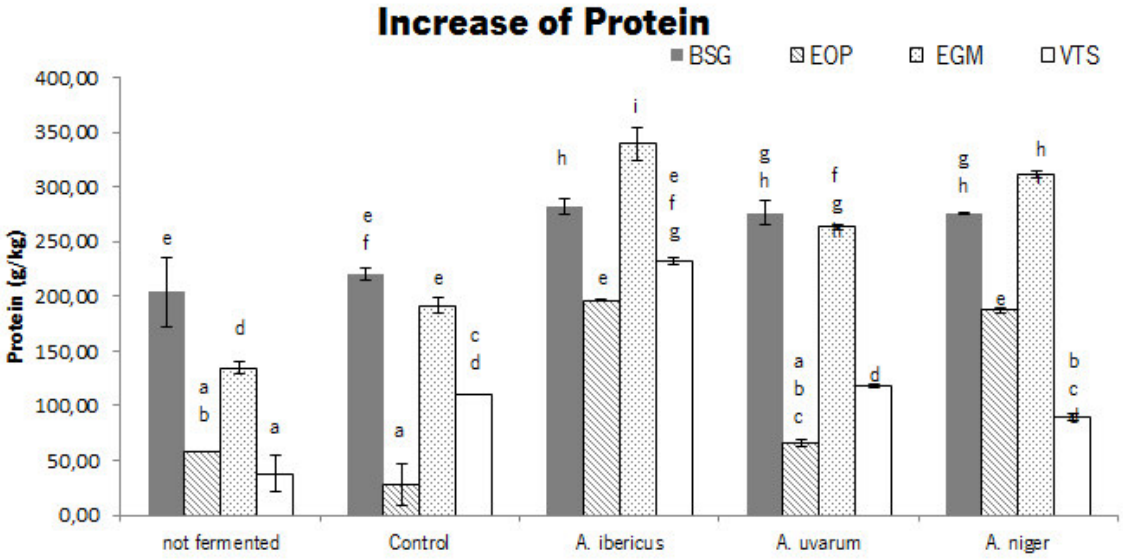

Figure 4- Protein content of the initial substrate, control and residues after SSF with *A. ibericus*, *A. uvarum* and *A. niger*. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different. BSG- brewery spent grain; EOP- exhausted olive pomace; EGM- exhausted grape marke; VTS- vine shoot trimmings.

evaluated the protein increase in BSG fermented by *Rhizopus oligosporus*, without nitrogen supplementation the protein content was increased at 31.5% after SSF.

The protein was increased in EOP after SSF. The increase was higher than fermented BSG, the fungi that achieved a maximum increase were *A. ibericus* and *A. niger* increasing 3.1 and 2.7 times the protein content, respectively. The SSF allowed to increase protein to similar values of initial BSG, which is considered a nutritional waste to use as animal feed. Brozzoli *et al.*, (2010) studied the increase of protein in stoned olive pomace by *Pleurotus* species. In this case, the olive pomace was mixed with other conventional feedstuffs as wheat bran, barley grans, etc. In the best conditions, the protein was increased 1.3 times.

The EGM showed the higher protein increase after SSF, *A. ibericus* and *A. niger* were also the fungi that further increased the protein content. Among all wastes, the solid that achieved the maximum protein content was EGM fermented by *A. ibericus* achieving a 34% of protein, although not significant differences were observed with *A. niger*. Thus, the SSF was a suitable process to increase the nutritional value of wastes with low protein content. The bioconversion of EGM to nutrien-rich feed was studied by Jin *et al.*, (2016), they enhanced the protein content up to 26% after SSF. The increase of protein in VTS was not studied, only Salgado *et al.*, (2015) evaluated the increase of protein of a mixture of EGM, olive pomace and VTS. They got an increase of protein from 8.5% to 17.08% after SSF by *A. uvarum*.

In general all fungi increased the protein content, however *A. ibericus* MUM 03.49 showed the maximum increase in each waste studied. Thus, *A. ibericus* was selected for optimization of solid substrate composition to maximize the protein increase mixing the different wastes.

3.2.3. Variation of sugars, protein and phenol compounds in the extract after SSF

The effect of fungus growth on sugars, phenolic compounds and proteins content was evaluated. These compounds were analysed in the enzymatic extract after SSF. **Figure 5** represented the proteins content in the extract obtained from the initial substrate, the control and the different residues after SSF with the three fungi. As can be seen on **Figure 5**, the control has higher protein content than the initial substrate because of urea which acts as nitrogen source. In BSG, it was not added urea and there was not significant difference between initial substrate and control.

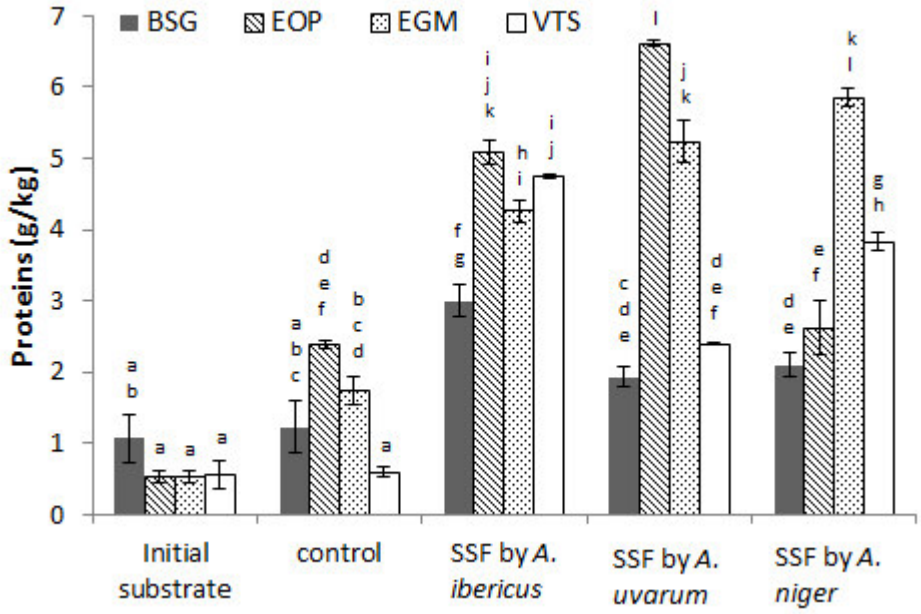

Figure 5- Content of soluble protein in the initial substrate, control and residues after SSF with *A. ibericus*, *A. uvarum* and *A. niger*. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different. BSG- brewery spent grain; EOP- exhausted olive pomace; EGM- exhausted grape marke; VTS- vine shoot trimmings.

After SSF, every residue presented an increase in protein content in relation to the initial substrate, although only EOP fermented with *A. niger* and BSG fermented with *A. uvarum* are the only ones without statistically significant differences in relation to the control. The increase of protein can be linked to the enzyme production during SSF.

In **Figure 6** is represented the free sugars available in the control, initial substrate and solid wastes after SSF. It is difficult observe the kinetic profiles of reducing sugars generation and consumption during SSF. The fungus consumes the reducing sugars and then it produces enzymes that can hydrolyse the lignocellulosic wastes to more reducing sugars, which can be again consumed by the fungus. It was observed a reduction of the concentration of free sugars in the fermentations with *A. ibericus* and *A. uvarum*, with the exception of EGM that has a concentration of free sugars similar to the control. This data can indicate the use of free sugars in the medium for the growth of the fungi. On the other hand, the fermentation where it was used the fungus *A. niger*, the results show that there was not significant difference (p-value < 0.05), between BSG and EOP. In the case of EGM, the value obtained after fermentation was higher than the control. When it was used VST, it is the only case that is possible to observe a decrease in concentration of free sugars.

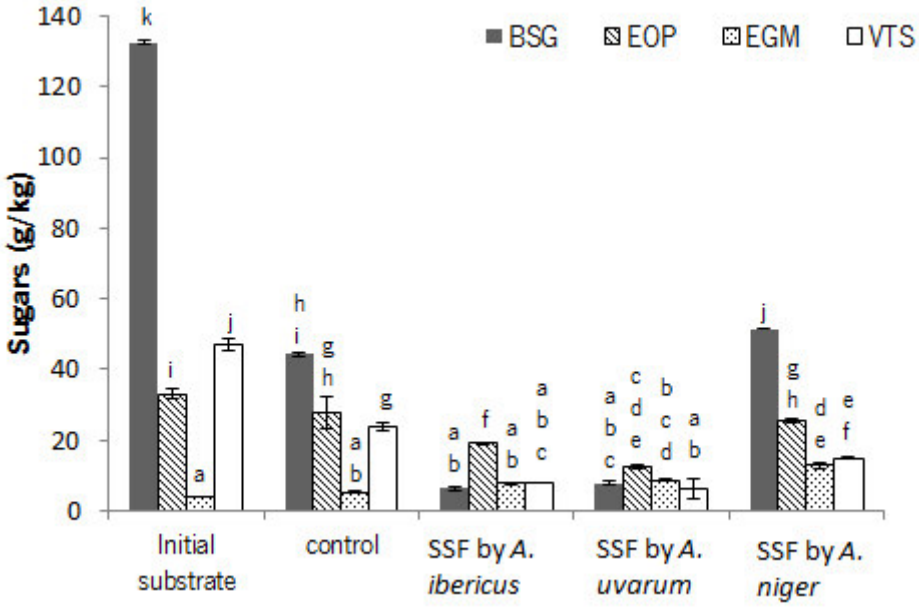

Figure 6- Content of soluble free sugars in the initial substrate, control and residues after SSF with *A. ibericus*, *A. uvarum* and *A. niger*. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different. BSG- brewery spent grain; EOP- exhausted olive pomace; EGM- exhausted grape marke; VTS- vine shoot trimmings.

Figure 7 represents the results of total phenols determined in the initial substrate, control and the four residues after SSF. In these results, it is possible to observe that the concentration of total phenols after SSF was very similar to the values obtained in the control, no significant differences were observed (p-value < 0.05), with the exception of EOP where it is possible to observe a clear decrease of total phenols concentration. It was expected that the phenolic compounds could decrease after SSF because the fungi have the ability to degrade phenolic compounds presents in wastes (Salgado *et al.*, 2016).

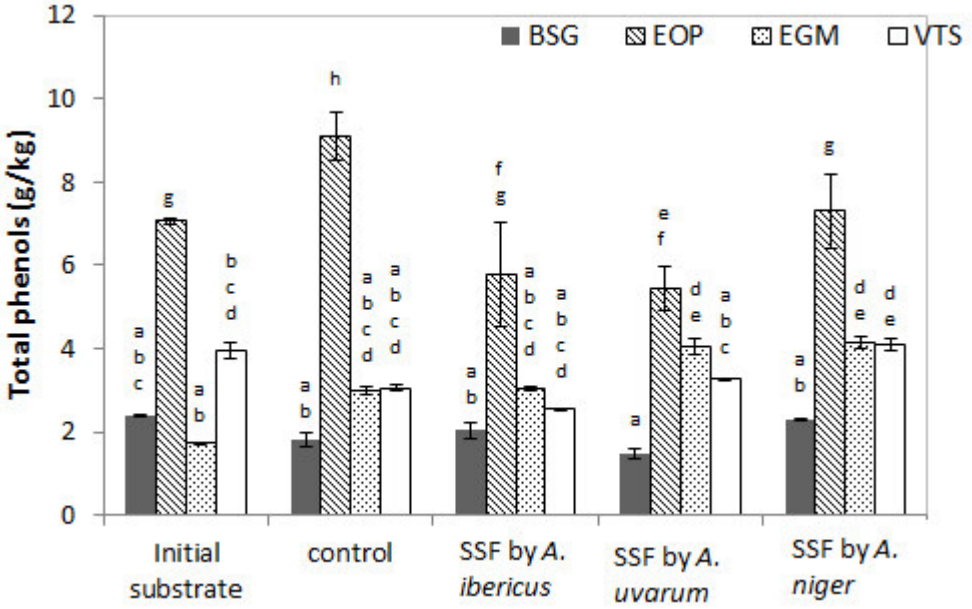

Figure 7- Content of soluble phenolic compounds in the initial substrate, control and residues after SSF with *A. ibericus*, *A. uvarum* and *A. niger*. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different. BSG- brewery spent grain; EOP- exhausted olive pomace; EGM- exhausted grape marke; VTS- vine shoot trimmings.

3.2.4. Effect of fungi on lignocellulosic composition of wastes

The lignocellulosic composition of the different wastes was evaluated after SSF with the different fungi, in order to determine the reduction of cellulose, hemicellulose, and lignin content.

In **Figure 8** is represented lignocellulosic composition of initial waste, control and each fermented waste by different fungi. All lignocellulosic components were reduced when they were submitted to SSF. A small reduction was observed in control which could due to the effect of sterilization processes. The SSF caused a decrease in the initial dry matter, this decrease was similar in all SSF from 6.9 to 14% of initial dry solid waste.

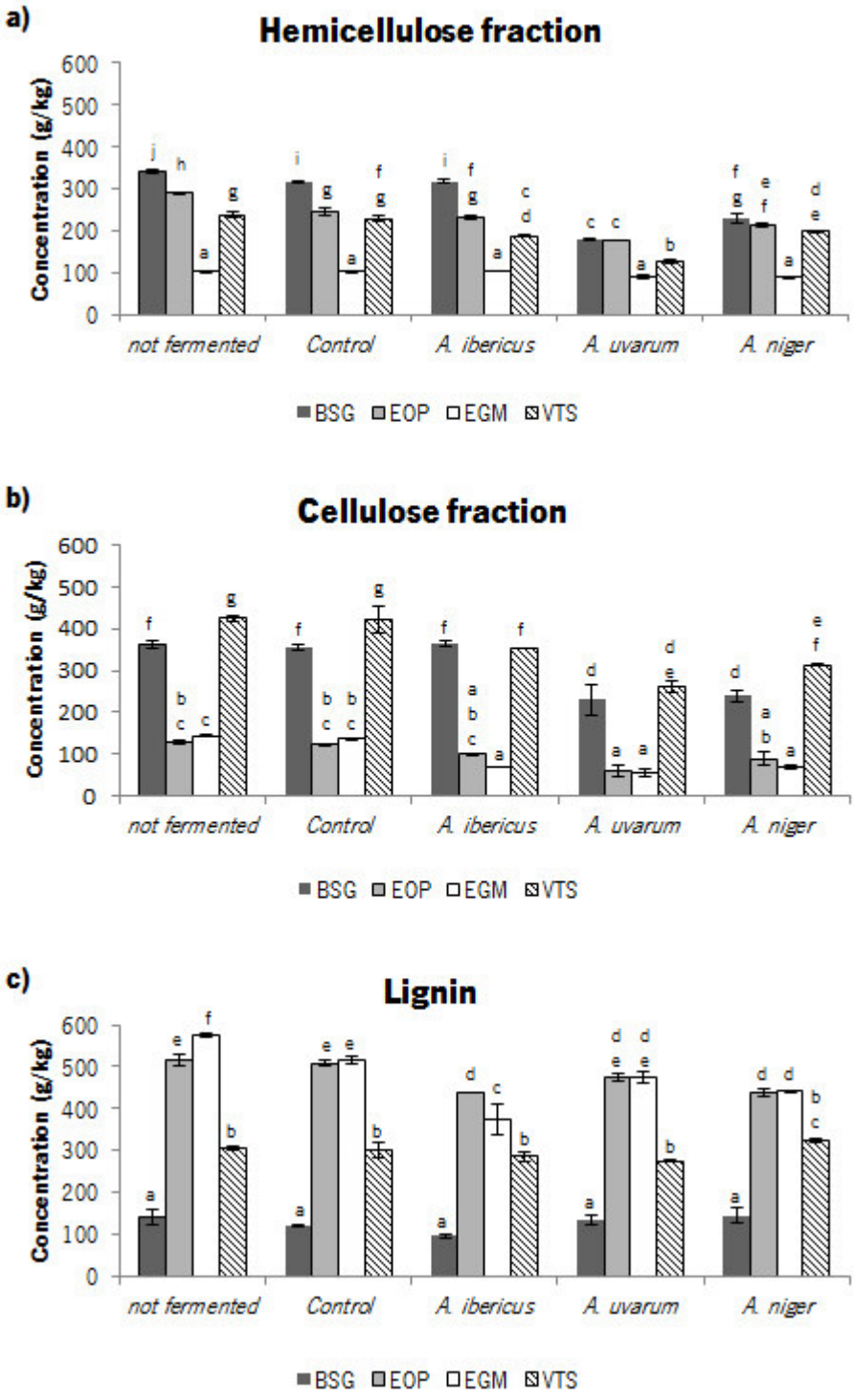

Figure 8- Lignocellulosic composition of different residues with the different fungi. a) hemicellulose fraction, b) cellulose fraction, c) lignin. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different. BSG- brewery spent grain; EOP- exhausted olive pomace; EGM- exhausted grape marke; VTS- vine shoot trimmings.

The maximum reduction of hemicellulose was calculated taking into account the loss of dry matter. *A. uvarum* showed the maximum reduction in BSG, EOP and VTS, 54.2, 46.7 and 53 % of

reduction, respectively. The results of hemicellulose reduction were not correlated with xylanase activities, the maximum xylanase activity was achieved by *A. ibericus*, however this fungus did not achieve the maximum reduction of hemicellulose fraction.

Shinners *et al.*, (2002) evaluated the reduction of hemicellulose of canola stable and they observed a reduction of 25% by *Cyathus olla* in SSF. In other study, *A. niger* reduced the hemicellulose content 36% (2012) and *Rhizopus* sp. reduced 25% after 10 days of SSF of cactus pear (Santos *et al.*, 2015). Tuyen *et al.*, (2012) studied 11 fungi and observed a hemicellulose reduction from 7.5 to 53%. The maximum xylanase production with less hemicellulose degradation was evaluated, BSG and EGM fermented by *A. ibericus* showed the best ratio xylanase activity:reduction of hemicellulose (3.1). In this way, the fermented waste keeps the hemicellulose fraction to be used as animal feed or to use in biorefinery industries.

The cellulose content was reduced in all fermentations. *A. ibericus* showed the less cellulose degradation in all wastes except to in EGM that was similar in the three fungi. The best ratio cellulase activity:reduction of cellulose was achieved by *A. ibericus* in BSG (4.9). The reduction of cellulose in SSF was observed in other fungi, *Trametes versicolor* reduced the cellulose a 44.8% of wheat straw after 49 days of SSF (Tuyen *et al.*, 2012). *A. niger* achieved a similar reduction of cellulose content (40%) in SSF of cactus pear after 10 days (Santos *et al.*, 2015).

The reduction of lignin content can improve the rumen fermentability of lignocellulosic wastes (Tuyen *et al.*, 2012). In **Figure 8 c)** can be observed that the content of lignin decreased after SSF. The reduction of lignin content (taking into account the loss of dry matter) was higher when *A. ibericus* was inoculated for all wastes. The maximum reduction of lignin was achieved in SSF of EGM (43%) followed by SSF of BSG (40%). Santos et al., (2015) observed a decrease of lignin of 28% after SSF by *A. niger* and 18% by *Rhizopus sp.* Tuyen *et al.*, (2012) demonstrated a high correlation between reduction of lignin and hemicellulose reduction (r = 0.96). In the present study, this correlation was evaluated showing a r = 0.68. Thus, the degradation of hemicellulose was accompanied by delignification of waste. This correlation could be due to the fungi can actuate in ether and ester bonds that linked covalently the lignin and hemicellulose. Other reason can be the delignification of waste can improve the accessibility of xylanases to hemicellulose, increasing their degradation (Tuyen *et al.*, 2012).

Figure 9 show the NDF ash free (sum of hemicellulose, cellulose and lignin) and ADF (sum of cellulose and lignin) in not fermented, control and fermented wastes. These parameters are linked to hemicellulose, cellulose and lignin content. Thus, it can also see a decrease in NDF and ADF after SSF.

For BSG, maximum reduction of NDF was achieved by *A. uvarum* (41.6 %) and *A. niger* (43.6%). For EOP, the degradation was similar for three fungi about 27.5%.

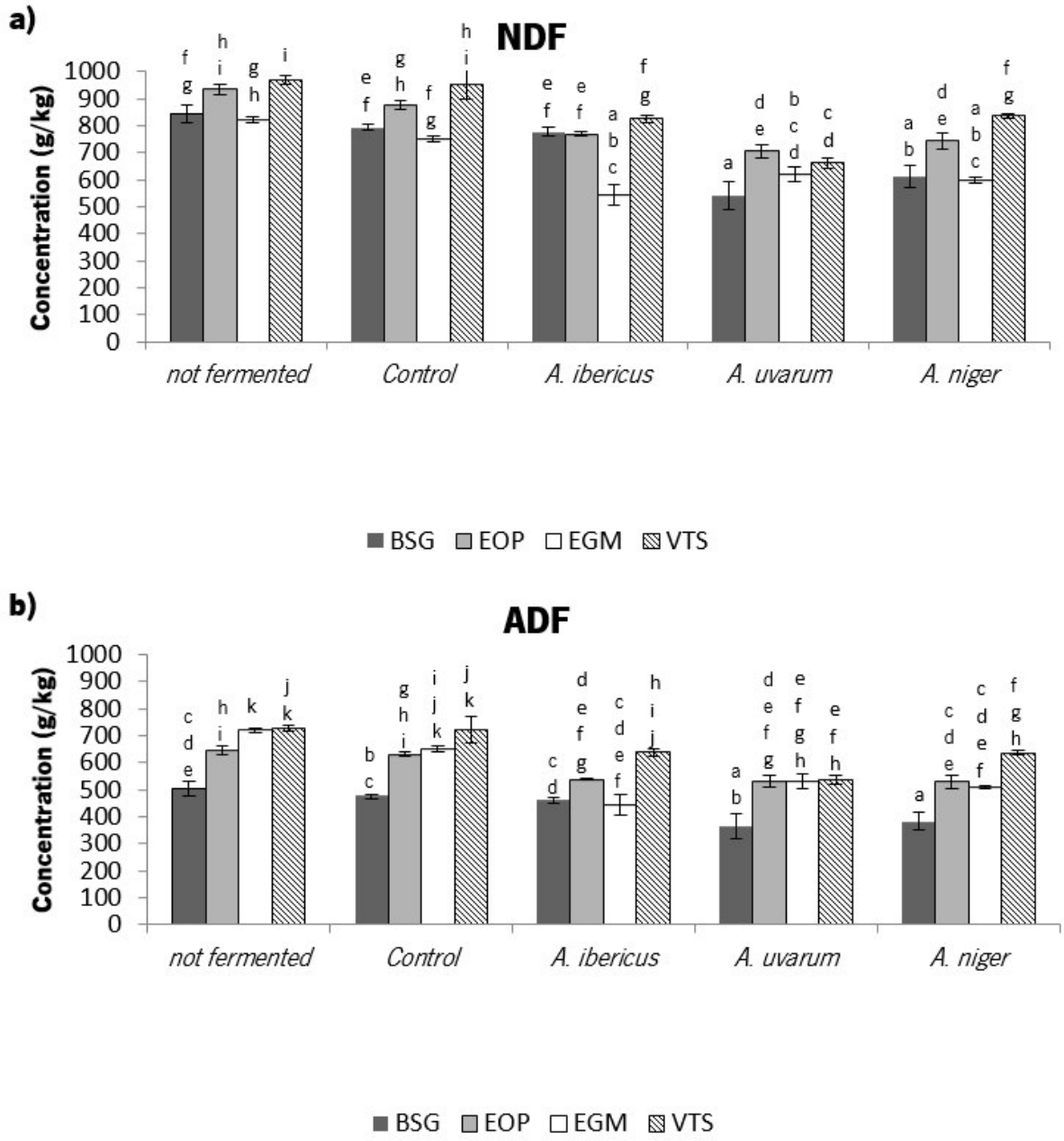

Figure 9- NDF and ADF of the different residues with fungi, in control and not fermented substrates. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different. BSG- brewery spent grain; EOP- exhausted olive pomace; EGM- exhausted grape marke; VTS- vine shoot trimmings.

For EGM, the maximum degradation was achieved by *A. ibericus* (45.9 %) and in fermentation of VTS *A. uvarum* (26.6 %) and *A. ibericus* (39.6 %) got the maximum degradation. The reduction of ADF was similar to NDF reduction.

After screening stage, *A. ibericus* was selected for optimization of substrate composition. This fungus was selected because it achieved the maximum increase in protein content, the maximum xylanase and cellulose production and the degradation of cellulose and hemicellulose was lower.

3.3. Evaluation and optimization of agro-industrial wastes mixtures as substrate in SSF

The simplex-centroid design is an effective technique of response surface methodology widely employed in the optimization of culture media and in the evaluation of relationships existing between various parameters with a minimum number of experiments (Arous *et al.*, 2016) . The design supports a model containing both linear and interaction terms. Through this statistical model it was obtained **Table 13** that shows the dependent variables studied in order to observe if the mixture of different wastes is better than a fermentation with only one residue. In the case of xylanase and cellulase activities, two analysis methods were used with two substrates and two temperatures (40 and 50 °C) different. In this way, the specificity of enzymes for different substrates can be evaluated.

The variation in enzymes activities and increase of N by *A. ibericus* can be observed in a triangular-dimensional contours diagrams (**Figures 9 a) – f)**). The three wastes with higher effect were represented in each figure, the other waste was fixed to 0. In the case of xylanases, the activities of the two analysis methods were optimized.

Figure 9 a) shows the contour plot of xylanase activity (xylan beechwood), it can be observed an optimum region in the area near to apex BSG. Thus, the BSG has a clear positive effect on xylanase activities. The same effect is showed in **Figure 9 b)** in which was analyzed the xylanase activity using Azo-wheat arabinoxylan as substrate. The maximum production of xylanases using xylan beechwood was obtained with the combination of BSG, EOP and VST which corresponds to the assay number 13. On the other hand, the maximum obtained using AZO-wheat arabinoxylan was achived using only BSG and VST as described on assay 6. Data from experimental design allowed to calculate the coefficients of regression to build polynomial equations which describes the correlation between xylanase activities and substrate composition:

Table 13-. Activities of each dependent variable and their theoretical value obtained using Statistica 5.0 software.

Assay	BSG (g)	EGM (g)	VTS (g)	EOP (g)	Xylanase activity at 40 ºC (U/g)		Xylanase activity at 50 ºC (U/g)		Cellulase activity at 40 ºC (U/g)		Cellulase activity at 50 ºC (U/g)		β-glucosidase (U/g)		Increase of N (%)	
					Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	10.00	0.00	0.00	0.00	72.95	72.84	63.28	63.11	47.96	47.85	51.20	51.45	1.67	1.64	0.88	0.89
2	0.00	10.00	0.00	0.00	42.77	42.66	35.18	35.02	53.46	53.34	25.40	25.66	6.00	5.97	0.99	1.00
3	0.00	0.00	10.00	0.00	47.58	47.47	36.95	36.79	41.11	41.00	20.62	20.87	1.76	1.73	1.38	1.39
4	0.00	0.00	0.00	10.00	44.17	44.06	23.03	22.87	25.46	25.34	16.90	17.15	9.10	9.07	-0.04	0.01
5	5.00	5.00	0.00	0.00	73.56	74.45	85.63	86.93	33.54	34.43	75.87	73.86	13.27	13.53	1.18	1.12
6	5.00	0.00	5.00	0.00	84.92	85.81	87.19	88.48	63.57	64.46	74.43	72.42	21.80	22.06	2.66	2.61
7	5.00	0.00	0.00	5.00	90.15	91.04	90.40	91.69	38.83	39.72	75.96	73.95	18.75	19.00	1.81	1.76
8	0.00	5.00	5.00	0.00	45.75	46.64	22.72	24.02	53.18	54.07	87.73	85.71	13.38	13.64	0.90	0.84
9	0.00	5.00	0.00	5.00	34.20	35.09	14.56	15.85	23.08	23.97	65.78	63.77	6.99	7.25	0.66	0.60
10	0.00	0.00	5.00	5.00	37.14	38.03	4.84	6.14	29.51	30.40	7.72	5.71	8.23	8.49	-0.58	-0.05
11	3.33	3.33	3.33	0.00	60.04	57.04	77.78	73.40	48.62	45.61	24.66	31.46	14.39	13.52	0.90	1.07
12	3.33	3.33	0.00	3.33	85.91	82.91	64.56	60.18	32.97	29.96	28.46	35.26	15.42	14.54	0.50	0.67
13	3.33	0.00	3.33	3.33	93.82	90.82	66.70	62.31	54.85	51.84	25.63	32.43	22.37	21.50	0.34	0.51
14	0.00	3.33	3.33	3.33	31.92	28.92	10.57	6.19	72.60	69.60	17.27	24.07	12.34	11.47	0.17	0.34
15	2.50	2.5	2.50	2.50	59.28	66.40	38.19	48.58	47.73	54.85	22.97	6.86	13.46	15.53	0.71	0.29

BSG- brewery spent grain; EGM- exhausted grape marke; VTS- vine shoot trimings; EOP- exhausted olive pomace

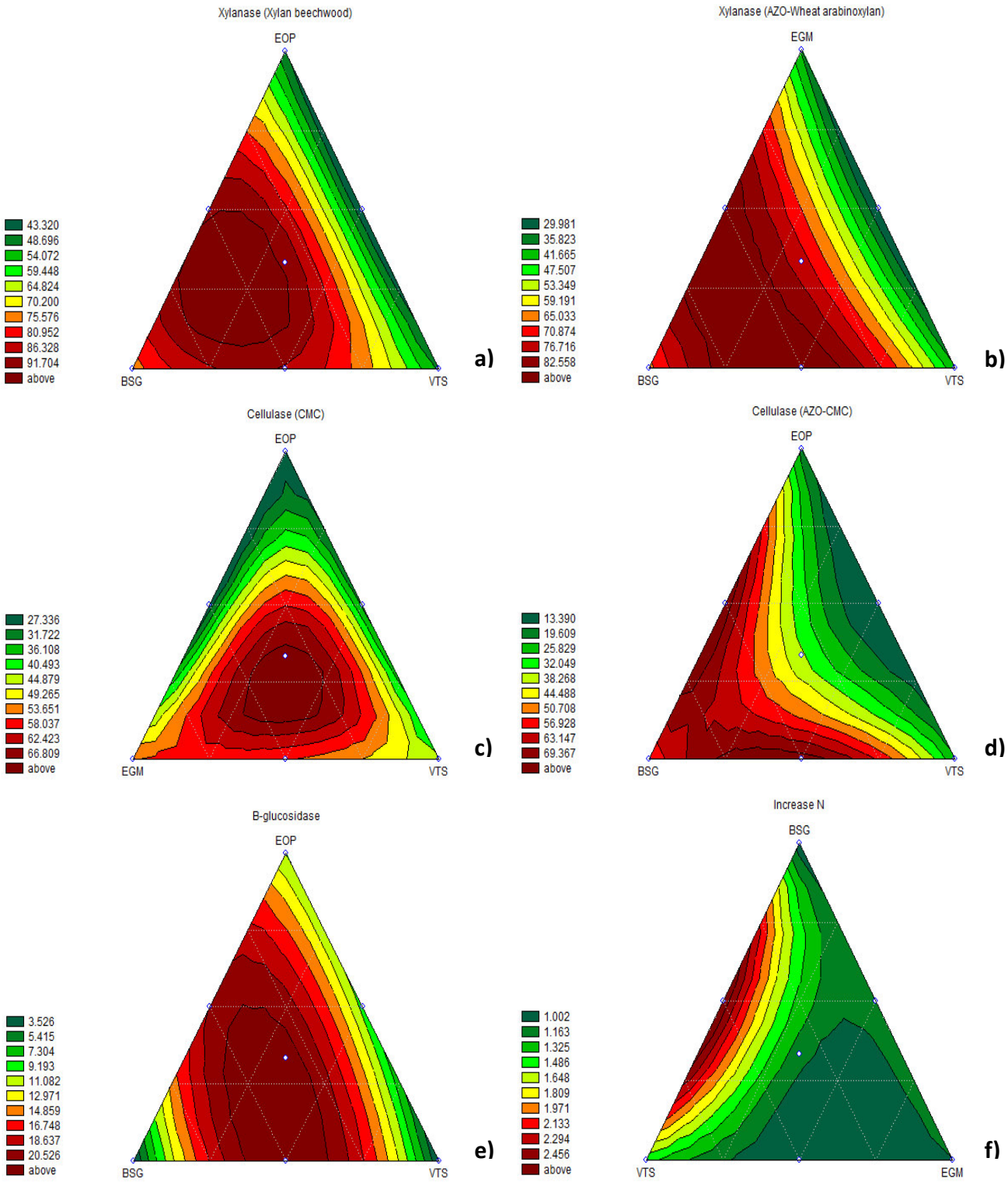

Figure 10- Contour diagram for (a) xylanase at 50 °C (xylan beechwood); (b) xylanase at 40 °C (AZO-wheat arabinoxylan); (c) cellulose at 50 °C (CMC); (d) cellulose at 40 °C (AZO-CMC); (e) β-glucosidase; (f) Increase of N. BSG- breweryspent grain; EGM- exhausted grape marke; EOP- exhausted olive pomace; VTS- vine shoot trimmings.

$$y(xylanases\ beechwood) = 72,84.x1 + 42,66.x2 + 47,47.x3 + 44,06.x4 + 66,79.x1.x2 + 102,63.x1.x3 + 130,36.x1.x4 + 6,28.x2.x3 - 33,09.x2.x4 - 30,95.x3.x4 - 453,81.x1.x2.x3 + 310,20.x1.x2.x4 + 366,54.x1.x3.x4 - 253,79.x2.x3.x4 \quad \textbf{(eq. 8)}$$

$$y(xylanases\ Azo - wheat\ arabinoxylan) = 63,11.x1 + 35,02.x2 + 36,79.x3 + 22,87.x4 + 151,44.x1.x2 + 154,13.x1.x3 + 194,81.x1.x4 + -47,53.x2.x3 - 52,37.x2.x4 - 94,76.x3.x4 - 6,68.x1.x2.x3 - 345,72.x1.x2.x4 - 185,00.x1.x3.x4 - 101,04.x2.x3.x4 \quad \textbf{(eq. 9)}$$

The value of regression coefficients indicates the positive or negative effect of each independent variable (waste) on xylanase production. The optimal conditions that conduced to maximum xylanase activity were calculated with Solver tool. These optimal conditions were showed in **Figure 9 a)** and **9 b)**. In both cases, it was observed a positive effect of mixture of wastes than using wastes separately.

In the case of cellulases, the maximum production of cellulases was obtained using carboxymethyl cellulose (CMC) as substrate. **Figure 9 c)** showed a clear optimum region in the centre of triangle that indicates the mixture of EGM, VTS and EOP led to maximum cellulose activity. The coefficients of regression are showed in **eq. 10**:

$$y(celullases\ CMC) = 47,85.x1 + 53,34.x2 + 41,00.x3 + 25,34.x4 - 64,66.x1.x2 + 80,13.x1.x3 + 12,50.x1.x4 + 27,58.x2.x3 - 61,49.x2.x4 - 11,08.x3.x4 - 177,37.x1.x2.x3 + 11,07.x1.x2.x4 + 127,47.x1.x3.x4 + 937,2.x2.x3.x4 \quad \textbf{(eq. 10)}$$

The other substrate used to determine cellulases was AZO-CMC. This analysis method is more specific to determine endocellulase activities, these enzymes can hydrolyze β-1, 4-glucosidic bonds internally in the cellulose chains, whereas cellobiohydrolases act at the end of the chains. The products of these enzymes are disaccharides or oligosaccharides which are hydrolyzed by β - glucosidases (Salgado *et al.*, 2014). In cellulose activity analysed with AZO-CMC substrate, the maximum activity was achieved using EGM and BSG. The coefficients of regression are showed in **eq. 11**:

$$y(cellulases\ Azo - CMC) = 51,45.x1 + 25,66.x2 + 20,87.x3 + 17,15.x4 + 141,20.x1.x2 + 145,03.x1.x3 + 158,58.x1.x4 + 249,78.x2.x3 + 169,47.x2.x4 - 53,22.x3.x4 - 1640,48.x1.x2.x3 - 1303,94.x1.x2.x4 - 680,84.x1.x3.x4 - 1021,33.x2.x3.x4 \quad \textbf{(eq. 11)}$$

In the analyses of enzymes, it was also studied the production of β-glucosidase. This enzyme along with endoglucanases and cellobiohydrolases (exoglucanases) are the cellulose-hydrolysing enzymes (cellulases), that are capable of degrade cellulose (Kumar *et al.*, 2008). The maximum production of β-glucosidase was obtained using BSG, VST and EOP which corresponds to assay number 13. The contour plot (**Figure 9 e)**) shows a clear optimum zone in the centre of triangle, thus maximum activity was achieved mixing BSG, EOP and VTS. The coefficients of regression are shown in **eq. 12**:

$$y(\beta - glucosidase) = 1,64.x1 + 5,97.x2 + 1,73.x3 + 9,07.x4 + 38,91.x1.x2 + 81,51.x1.x3 + 54,60.x1.x4 + 39,17.x2.x3 - 1,08.x2.x4 + 12,38.x3.x4 - 197,77.x1.x2.x3 - 34,86.x1.x2.x4 + 23,04.x1.x3.x4 + 7,32.x2.x3.x4 \quad \textbf{(eq. 12)}$$

Nitrogen was also evaluated in this design, the content in nitrogen can indicate the total amount of crude protein available in a sample using a multiplication factor that in the case of these residues was 6,25 and it has been used in several studies (Salgado *et al.* , 2015). The mixture of BSG and VTS led to a maximum increase of N (**Figure 9 f)**). Thus, the SSF of both wastes increase their protein content (16.3 %). The coefficients of regression are shown in **eq. 13**:

$$y(nitrogen) = 0,89.x1 + 1,00.x2 + 1,39.x3 + 0,01.x4 + 0,71.x1.x2 + 5,89.x1.x3 + 5,23.x1.x4 + -1,40.x2.x3 + 0,40.x2.x4 - 3,00.x3.x4 - 16,09.x1.x2.x3 - 17,89.x1.x2.x4 - 31,06.x1.x3.x4 - 0,23.x2.x3.x4) \quad \textbf{(eq. 13)}$$

Table 14 describes the optimal conditions for each dependent variable and statistical parameters of the models. The Fisher test (F) can show better fit of the model, the higher value of F demonstrated a good fit. The determination coefficients (R^2) were from 0.95-0.98, which demonstrated that a satisfactory adjustment of the model, and indicating that 95-98 % of the variability in the responses could be explained by the model. As it would be expectable, the conditions that maximizes one dependent variable may not be the same that maximizes another dependent variable, for example, the maximum

production of xilanases using beechwood as substrate was obtained on assay 13 but in the case of nitrogen, the maximum production was obtained in the assay number 6.

Table 14- Optimum parameters for each dependent variable and statistical parameters.

	Enzyme activities (U/g)					
	Xylanase at 40 °C	Xylanase at 50 °C	Cellulase at 40 °C	Cellulase at 50 °C	B-glucosidase	Increase of N (%)
BSG (g/g)	0.6	0.53	0	0	0.46	0.5
EGM (g/g)	0	0	0.5	0.38	0	0
VTS (g/g)	0	0.2	0.5	0.38	0.38	0.5
EOP (g/g)	0.4	0.27	0	0.24	0.16	0
OV (U/g)	93.77	98.36	85.71	71.77	22.53	2.61
R^2	0.97689	0.98031	0.95045	0.96016	0.96678	0.95113
R^2 adj	0.95532	0.96194	0.90421	0.92298	0.93579	0.90553
F-ratio	45.3	53.36	20.55	25.83	31.19	20.86
P-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

BSG- brewery spent grain; EGM- exhausted grape marke; VTS- vine shoot trimings; EOP- exhausted grape pomace; OV- optimum value; R2- coefficient of correlation of a linear regression; R2- adjusted coefficient of determination

So, in order to select a unique optimal substrate composition that maximize every dependent variable, it was performed an optimization of multiple response using the software Statgraphics plus 5.1. The mixture of BSG and VST was the optimal substrate that maximizes all dependent variables. The theoretical maximum activities for every variable are identified on **Table 15**. If it compares the maximum enzymes production optimizing each dependent variable by separate (**Table 14**) and optimizing all at once (**Table 15**), it can be observed that there were not great differences. Thus, the optimum condition selected can achieved high enzymes activities and a high increase in protein content of fermented waste.

Table 15- Optimization of multiple response.

	Enzyme activities (U/g)					
	Xylanase at 50 °C	Xylanase at 40 °C	Cellulase at 50 °C	Cellulase at 40 °C	β-glucosidase	Increase N %
Optimum Value	89.33	86.39	73.46	64.6	21.91	2.58
Experimental	84.92±1.52	87.19±1.72	63.57±3.67	74.43±3.09	21.81 ± 0.91	2.66±0.13
BSG- 0.54; EGM- 0.00; EGM- 0.00; VST- 0.46						

In others works, it was also observed the positive effect of mixture of wastes to improve enzymes. Salgado *et al.* (2014) observed a positive effect on lipase production by *A. ibericus* mixing crude olive pomace and EGM. The increase of production of lignocellulolytic enzymes was also demonstrated with the mixture of crude olive pomace, VTS and EGM (Salgado *et al.*, 2015). The use of mixture wastes to increase their protein content by SSF was barely studied. Aggelopoulos *et al.*, (2013) evaluated the mixture of discarded oranges and BSG in SSF and they observed an increase in protein content. Rajesh *et al.* (2010) used a mixture of olive cakes and vegetable wastes in SSF by *A. niger*, they increased the protein content from 20.6 % to 28.3 % after 9 days.

3.4. Study of kinetic of SSF with optimal medium

After identification of the optimum fungus and mixture of residues that maximizes the production of all dependent variables, it was performed a study of the SSF kinetic to evaluate the evolution of fungus growth, enzyme production and increase of protein over time.

In this stage, it was performed eleven fermentations that were inoculated in the same day and incubated with different periods of time. In addition, it was also determined the content of ergosterol that is a component of the fungi cellular membrane (Seitz *et al.*, 1977).

In **Figure 11** are identified the enzymes activities, ergosterol and protein content during the SSF. Through the analysis of the figure, it is possible to see an increase of ergosterol during the first five days of fermentation, where it gets its maximum value, after day five of fermentation there are slightly variances but the concentration of ergosterol stays proximally the same. Ergosterol analysis is an indirect method for determination of fungi biomass, since ergosterol is a component of cellular membrane. The variances after the five day of fermentation in the ergosterol concentration may be caused by sporulation of the fungus (Desgranges *et al.*, 1991). With the increase of ergosterol, there is a simultaneous decrease of

sugars because the fungus consumed the free sugars available. By the analysis of **Figure 11** it can also be observed that during the first day of fermentation, when the concentration of ergosterol was low, there was not any kind of enzymes being produced or their concentration was very low. The nitrogen content was increased (35.1 %) after 7 days of SSF, thus the protein content was increased from 130.3 to 176 g/kg. Total phenols barely change during fermentation. The sugars were consumed at start of SSF (3 days), however a residual quantity of sugars remained constant until the end of SSF. This could be due to release sugars from cellulose and hemicellulose fraction, resulting from enzymatic activity (Kumar *et al.*, 2008; Pérez *et al.*, 2002).

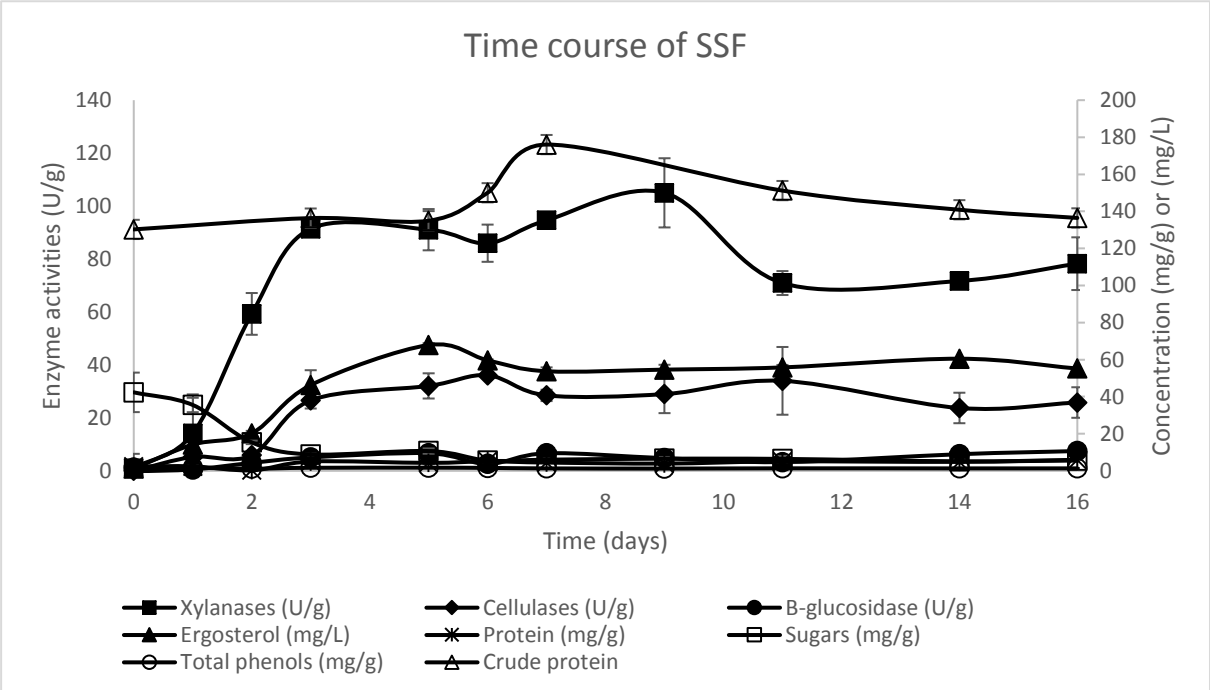

Figure 11- Variation of dependent variables in study during the course of SSF.

It is noteworthy that in the first days of fermentation, they were produced xylanases enzymes. However, the cellulose production started after two days of fermentation. This effect was observed by others authors as Leite *et al.* (2016). Usually, short fermentation times are needed for xylanases production by SSF. Longer fermentation times favoured endocellulases production; this may be due to the need of a prior action of the xylanases to expose the cellulose fibres, which will induce cellulases production (Salgado *et al.*, 2013).

3.4.1. Effect on the lignocellulosic compounds

During the course of fermentation, the lignocellulosic compounds where also evaluated. **Figure 12** shows the concentrations of lignin, hemicellulose and cellulose during the course of the fermentation. It is possible to see a clear decrease of the lignocellulosic compounds during the course of two weeks. The decrease is not very accentuated although it was expected because, as said before, it is difficult for the enzymes to degrade the lignocellulosic compounds without any pre-treatment, in order to see a great decrease of this compounds. Despite the fact that there were not used any kind of pre-treatments to wastes, it is possible to see a decrease in the cellulose and lignin content of proximally 24 %, on the other hand, in the case of hemicellulose, the decrease was not so accentuated but it was a decrease of 19 %.

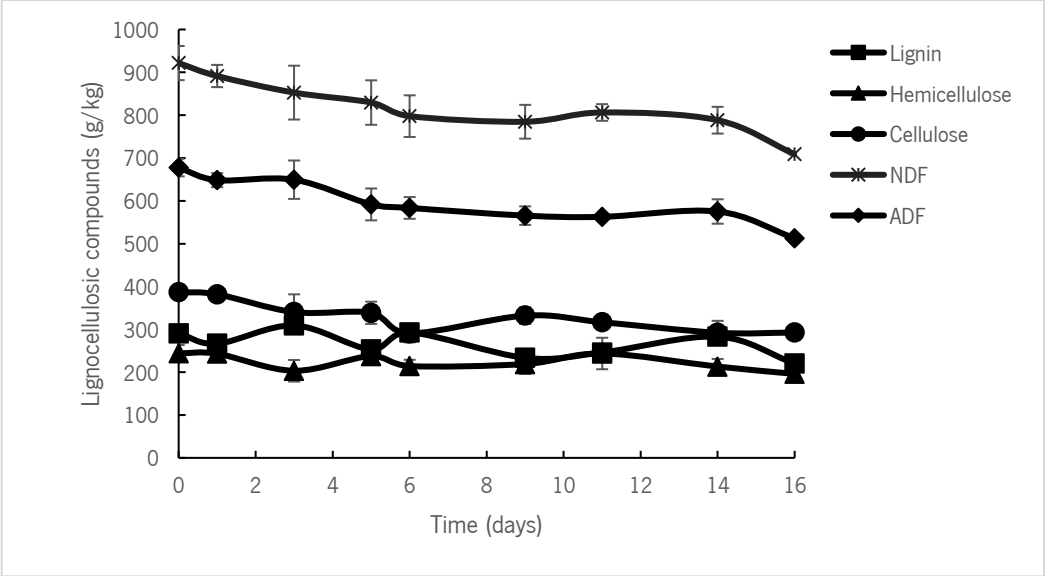

Figure 12- Effect on the lignocellulosic compounds during the course of fermentation.

It is possible to see a relation between the decrease of hemicellulose and an increase of xylanases production. NDF and ADF are parameters that indicate the digestibility of a product. NDF is more closely associated with intake than digestibility, while ADF does not represent all the insoluble fibre that can provide scratch factor in the rumen, although it is unusually better correlated with digestibility than NDF. NDF represents the total insoluble matrix fibre which is better related to rumination, fill, passage, and feed intake and thus is more for ration balancing. Furthermore, intake is more relevant to animal production than digestibility (Van Soest, 1994). In **Figure 12** it is observed a decrease of this parameters, although the values obtained are very high when compared to literature that presents percentages of NDF near 30 and 40 (Beauchemin and Buchanan-Smith, 1991), while the minimum percentage obtained for NDF in these results is 78,46 %. Despites the high value obtained for NDF, the reduction of this parameter

since day one until the end of SSF on day sixteen is about 23 % from the initial value. In the case of ADF the reduction since the beginning of fermentation is of 24 %. Despite the high values of NDF and ADF, there are significant reduction even without any kind of pre-treatment.

3.5. Scale-up of SSF to tray bioreactors

The final part of this work consisted in performing a scale up of the SSF. The optimum substrate obtained through the simplex-centroid design (assay 6) was tested in a tray type bioreactor with a final weight of 500 g of dried waste. In **Table 16** is identified the activity of cellulases, xylanases, β-glucosidase and the increase of N observed predicted by the model, the results obtained experimentally in flasks and performing the tray bioreactor.

Table 16- Comparison of theoretical values of optimum conditions from model, experimental values in optimum conditions and the activity obtained using tray bioreactor.

	Cellulases at 40 °C	Xylanases at 40 °C	Cellulases at 50 °C	Xylanases at 50 °C	β-glucosidase	Increase of N %
Theoret.	72.42	88.48	64.46	85.82	22.06	2.61
Experim.	74.43 ± 3.09	87.19 ± 1.72	63.57 ± 3.67	84.92 ± 1.52	21.81 ± 0.91	2.66 ± 0.13
Tray bioreactor	18.02 ± 8.26	85.98 ± 7.70	3.16 ± 3.69	78.31 ± 39.29	2.63 ± 0.14	2.40 ± 0.46

Through analysis of **Table 16** it is possible to see that the values of xylanases activity using tray bioreactor were very close to the values obtained in a small scale and are also close to the values obtained in literature research. The same happens in the case of nitrogen, the value obtained using tray bioreactor is slightly lower than the experimental and theoretical but it is not a big difference. Relatively to the cellulases and β-glucosidase content, it is possible to see a big variance in relation to the theoretical and to a small-scale experiments. In the case of cellulases using AZO-CMC (40 °C) substrate, the difference was of about 50 %. On the case of cellulases using CMC (50 °C), the value obtained using tray bioreactor is 95 % of the experimental value and in the case of β-glucosidase the difference was of 90 %.

In **Table 17** shows the concentration of proteins, sugars and total phenols using tray bioreactor and the concentration obtained in a small scale, when it was performed the simplex-centroid design.

Table 17- Concentration of proteins, reducing sugars and phenols in control, in flask experiments and achieved using tray bioreactor with the optimum combination of residues.

	Proteins (mg/g)	Sugars (mg/g)	Phenols (mg/g)
Control	2.32 ± 0.14	42.35 ± 10.66	1.78 ± 0.11
Flask experiments	3.54 ± 0.08	5.97 ± 0.34	2.21 ± 0.29
Tray bioreactor	5.10 ± 0.37	5.56 ± 0.12	1.58 ± 0.32

By comparison of the concentration obtained in the different scales, it is possible to see that the values obtained using the tray bioreactor are not so different from the ones obtained using the simplex-centroid design. In the case of sugars the values are similar, the concentration obtained using tray bioreactor is slightly low. In the case of proteins the concentration obtained using the tray bioreactor is even higher (44 % higher) than the one obtained during the design which is good and goes in accord to previous studies performed in tray bioreactor where it was observed an increase of soluble protein (M. D. Oliveira *et al.*, 2010). In relation to the phenolic composition, it is possible to see that the concentration of phenols has diminished, this decrease is on the order of 28 %.

4. CONCLUSIONS AND FUTURE PRESPECTIVES

This work searched to improve the nutritional quality of main agro-industrial wastes of Portugal and produce value-added products in a low-cost biotechnology process.

The solid-state fermentation (SSF) has demonstrated be a suitable biotechnology process to increase the protein content in winery, olive mill and brewery wastes. In addition, other products of industrial interest as lignocellulolytic enzymes can be produced in the same process.

• Brewery spent grain (BSG), exhausted olive pomace (EOP), exhausted grape marc (EGM) and vine-shoot trimmings (VTS) showed suitable physicochemical characteristics to be used as substrate in SSF. The initial content of N in EOP, EGM and VTS was lower which led to high ratio C/N. Thus, these wastes were supplemented with a N source (urea).

• The three fungi studied (*A. niger, A. ibericus* and *A. uvarum*) were able to grow in the four agro-industrial wastes studied. The content of protein in VTS, EOP and EGM was increased to a optimum value to be used as animal feed. *A. ibericus* achieved the maximum enzymes production (cellulases and xylanases). The lignocellulosic compounds were reduced after SSF, the decrease of lignin and hemicellulose content was correlated and *A. ibericus* showed a low degradation of lignocellulosic compounds.

• The mixture of wastes improved the lignocellulolytic enzymes production and protein content in comparison to the use of solids separately. The simplex-centroid design allowed to select the optimum substrate to maximize the enzyme production and the protein content. The multiple response optimization selected only one combination of wastes that led to a maximum of all dependent variables studied. The optimum waste combination was BSG (0.54 g/g) and VTS (0.46 g/g).

• The fungi showed growth during the first 5 days of SSF, after the biomass remained constant. The xylanases were produced in the first days of SSF, the maximum activity was achieved after 3 days, then the cellulases was increased until achieve a maximum production in the 5th day. The high protein content was achieved after 7 days of fermentation. Thus, 7 days is the suitable time to carry out SSF and to maximized enzymes production and protein increase.

• The scale-up of process in tray bioreactor (500 g) was performed successfully, the production of xylanases and the protein increase achieved similar values to flask experiments. However, the cellulase and β-glucosidase production were lower.

In future research, it would be interesting to analyse other parameters such as the digestibility of agro-industrial wastes fermented by SSF. In addition, fungi can release antioxidant phenolic compounds from lignocellulosic materials, so that it would be interesting determine the antioxidant effect of fermented wastes.

Others future studies could be focused on the test of the fermented wastes in animals (fish, ruminants), and to evaluate the addition of lignocellulosic enzymes to animal feed as supplement. It would be also interesting to perform this process in other bioreactors such as packed-bed and rotating drum bioreactors.

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