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Uminho | 2015

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Olive pomace pretreatments to enhance its valorisation by solid-state fermentation

Dissertação de Mestrado
Mestrado em Bioengenharia

Trabalho efetuado sob a orientação da
Professora Doutora Isabel Belo
e do
Doutor José Manuel Salgado

Outubro de 2015

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Título tese: Olive pomace pretreatments to enhance its valorisation by solid-state fermentation

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Ano de conclusão: 2015

Designação do Mestrado: Mestrado em Bioengenharia

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO QUE A TAL SE COMPROMETE

Universidade do Minho, ___ / ___ / _____

Assinatura _____

AGRADECIMENTOS

Finda mais uma etapa na minha vida e no meu percurso académico, foram muitos os que se cruzaram no meu caminho e aqueles que o fizeram comigo.

Um agradecimento especial ao Doutor José Salgado, pela orientação e dedicação ao longo de todo o projeto, quer no trabalho de laboratório como no esclarecimento de dúvidas e no tempo dispensado na orientação da tese.

Agradeço também à Professora Doutora Isabel Belo, por me ter dado a oportunidade de desenvolver o projeto de mestrado no seu grupo de investigação e ainda pelas suas importantes sugestões.

A todas as minhas colegas de laboratório por toda a ajuda, quer nas dúvidas que me surgiam quer no material que não encontrava.

Uma palavra de carinho para os meus amigos e colegas de mestrado, Sara Baptista, Andreia Fernandes, Rita Silva, Diana Rodrigues e Pedro Soares com quem eu partilhei muitos desabafos, dilemas e impressões sobre o desenvolvimento do projeto e pela constante disponibilidade demonstrada ao longo deste meu percurso.

Um obrigado gigante ao Diogo, a quem eu esgotei a paciência, por ter estado ao meu lado e acima de tudo por me ouvir mesmo não percebendo nada.

Por fim, agradeço àqueles que fazem o caminho comigo desde sempre, a minha família, o meu pilar, o meu apoio. Sem eles trilhar este caminho sozinha seria, não impossível, mas muito difícil.

SUMÁRIO

O azeite é um componente importante da dieta mediterrânea e a sua extração é uma das atividades económicas dominante nas regiões do sul da Europa. Como resultado dos processos de extração, a indústria do azeite gera grandes quantidades de resíduos num curto período de tempo, tornando-se num problema crescente de poluição ambiental.

O sistema de duas fases é um processo recente que permite a produção de azeite com benefícios económicos e ambientais que gera um resíduo semi-sólido.

O bagaço de azeitona húmido, COP é menos eficaz na produção de enzimas do que o bagaço de azeitona esgotado, EOP (COP após secagem e extração do azeite residual).

Neste estudo, foi utilizado o EOP como substrato sólido para a produção de xilanases e celulases por *A. niger*. Para melhorar a produção de enzimas foi avaliado o efeito de um pré-tratamento do bagaço de azeitona por ultrassons. Os resultados mostraram que a sonicação levou a um aumento de 3 vezes da atividade das xilanases e uma diminuição da atividade das celulases, indicando que o tratamento por ultrassons atacou a integridade do material lignocelulósico aumentando a acessibilidade às hemiceluloses, o que induziu a produção de xilanases por fungos.

Foram ainda avaliados outros pré-tratamentos, tais como a hidrólise ácida com ácido diluído ou ultrassons combinado com hidrólise ácida mas não aumentaram as atividades das enzimas produzidas.

O trabalho permitiu concluir que, os açúcares que são libertados no meio reacional durante o pré-tratamento de ultrassons são muito importantes para a produção de enzimas e que os nutrientes suplementados durante o processo de SSF são essenciais ao crescimento de fungos.

O tempo de fermentação foi outro fator com grande importância no perfil de enzimas produzidas por SFF já que tempos de fermentação curtos favorecem a produção de xilanases por SSF e tempos de fermentação maiores favoreceram a produção de celulases.

ABSTRACT

Olive oil is an important component of the Mediterranean diet and its extraction is one of the dominant economic activity in the southern Europe regions. As a result of the processes of extraction, the oil industry generates large amounts of wastes in a short period of time, and these are becoming an increasing problem of environmental pollution.

The two-phase system is a recent process that allows the production of olive oil with economic and environmental benefits and produces a semi-solid waste, termed two-phase olive mill waste or olive pomace.

The crude olive pomace, COP is less effective in production of enzymes that exhausted olive pomace, EOP (COP after drying and residual oil extraction).

In this study, it was used the EOP as solid substrate to produce xylanases and cellulases by *A. niger* which was selected from fungi screening. To improve the enzyme production was evaluated the effect of pre-treatment of olive pomace by ultrasound. The results showed that the sonication led to a 3-fold increase of xylanase activity and a decrease of cellulase activity, indicating that ultrasounds treatment attacked the integrity of lignocellulosic material and increased the accessibility of hemicelluloses which induced the xylanases production by fungi.

Other pretreatments were also tested such as, acid hydrolysis with diluted acid or ultrasound combined with acid hydrolysis, but did not increase the activities of enzymes.

The study leads to the conclusion that the sugars which are released to the filtrate during the ultrasound pretreatment are very important for the enzymes production and the supplemented nutrients during the SSF process are essential for the growth of fungi.

The fermentation time was another factor of great importance in the profile of the enzymes produced by SFF as short fermentation times favour the xylanases production by SSF and longer fermentation times favour cellulases production.

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LIST OF ABBREVIATIONS

AH acid hydrolysis with diluted acid pre-treatment
ANOVA analysis of variance
a_w water activity
BGL β-glucosidases
BSA bovine serum albumin
CAr_n arabinan
CBH cellobiohydrolases
CG_A acetyl groups
CG_n glucan
CMC carboxymethylcellulose
COP crude olive pomace
CP content in polymers
CX_n xylan
DNS dinitrosalicylic acid
EG endo-glucanases
EI enzymatic index
EOP exhausted olive pomace
EU European Union
H Humidity
HPLC High Performance Liquid Chromatography
L:S liquid:solid
MEA malt extract agar
MUM Micoteca da Universidade do Minho
OMW olive mill waste
OMWW olive mill wastewater
P *p-value*
QAH quantitative acid hydrolysis
S:L solid:liquid
SD standard deviation
SmF submerged fermentation
SSF solid state fermentation
TPOMW two-phase olive mill waste
US ultrasound pre-treatment
US + AH ultrasound combined with acid hydrolysis pre-treatment
w/v weight/volume
wt weight

CONTEXT AND MOTIVATION

Olive oil is nearly totally produced in the Mediterranean region. Three quarters of the annual production in the world comes from European Union countries around the Mediterranean Sea. As the demand of olive oil is rapidly increasing worldwide, environmental pollution posed by olive mill wastes is a growing problem especially in the Mediterranean region.

In order to overcome this problem several solutions for the treatment of waste generated by the oil industry have been studied. The olive pomace is a lignocellulosic residue and can be valued biotechnologically. Currently these waste is used for composting, as fuel to obtain energy through combustion, for the extraction the value products, for instance, extraction of pectin or phenol compounds.

Another alternative, little yet explored, is to use one pretreatment of the waste, in order to improve its biodegradability and increase the accessibility of enzymes to materials for the lignocellulolytic enzyme production through solid-state fermentation.

REASERCH AND AIMS

The main goal of this thesis is the study of the effect of ultrasounds pretreatment of olive pomace on cellulases and xylanases production by solid-state fermentation.

In this sense, the secondary objectives were the following:

- Characterization of olive pomace;
- Selection of filamentous fungi;
- Optimization of ultrasounds pretreatment parameters;
- Study the effect of other pretreatment – diluted acid – and compare with ultrasounds pretreatment;
- Characterization of the final residue after solid-state fermentation.

Chapter 1
INTRODUCTION

Olive oil is an important component of the Mediterranean diet and is obtained from olive fruit. It is one of the main agro-industrial activities in southern European regions and is a very important economic activity particularly for countries like Spain, Italy and Greece. Mediterranean countries produce approximately 97.7% of the world's olive oil. In 2014/15 crop year (period from in 1 October to 30 September) global olive oil production was around 2 287 000 t, according to the International Olive Council. The European Union (EU) countries produce around 72% of the global olive oil production, being the main olive oil producers Spain with 61.6%, Italy with 21.1% and Greece with 13.5% of the EU's total production and Portugal produces 3.2% [1]. In 2014, olive oil production in Portugal was 66,000 m^3 that was mainly obtained at Alentejo (46,000 m^3) and North Region (14,000 m^3) (INE, 2014).

1.1. Olive oil extraction processes

The extraction of olive oil involves different processes like olive washing, grinding, beating and the extraction itself, which are the main steps of the whole process. The method used for the extraction influences the amount and the physico-chemical properties of the wastes produced [2].

The olive oil extraction by physical process can be achieved through traditional pressing (discontinuous process) or centrifugation systems (continuous process), three-phase and two-phase systems [2].

Nowadays, the most widely used system is the continuous two-phase in Portugal. About 85% of total olive oil produced in 2014 was extracted by this system (INE, 2014). **Figure 1** shows the evolution of extraction systems used in Portuguese olive mills. 20 years ago, traditional pressing was the most used however along the time this system was replaced by the continuous two-phase. Since 2007 the traditional and three-phase system were barely used. The main cause of this change was the need to reduce the water and energy consumption. In addition, this new system reduce the wastewater produced because do not apply warm water to facilitate the olive oil extraction. In the traditional and three-phase systems a contaminant effluent (olive mill wastewater) is generated which biological treatment is difficult and cause environmental problems, however in two-phase extraction system this effluent is not generated.

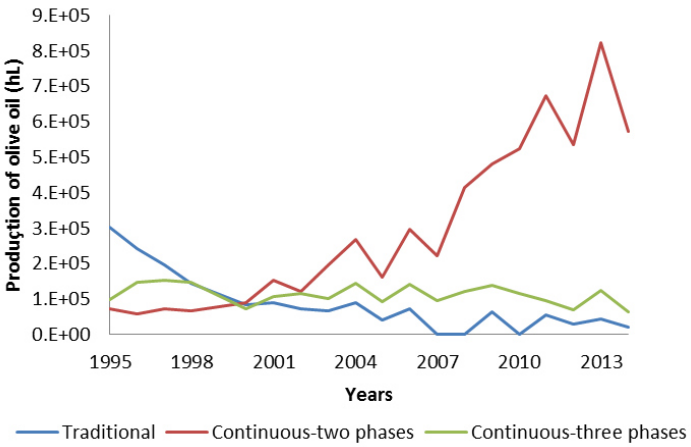

Figure 1 | Extraction systems in Portuguese olive mills between 1995 and 2013.

1.1.1. Traditional pressing

Traditional pressing (**figure 2**) is a relatively old process. However, it is still in use for some small olive mills. After the extraction by pressing, a solid fraction is obtained, pomace (or olive cake), as by-product and an emulsion containing the olive oil that is separated by decantation from the remaining olive mill wastewater (OMWW) [2]. This process offers advantages such as technical simplicity and cheap equipment, produces a small volume of OMWW because it adds a small quantity of water. Process discontinuity and high manpower costs are some disadvantages [3].

1.1.2. Three-phase system

The three-phase oil extraction system (**figure 2**) was introduced in 1970s to improve yield. The products generated are: pure olive oil, OMWW and a solid, pomace (olive cake or *orujo*) [4].

The advantages of this system are better oil quality, complete automation, smaller area needed, however this process presents some disadvantages such as greater water and energy consumption, higher wastewater production and more expensive installations [2].

1.1.3. Two-phase system

Two-phase system (**figure 2**) was introduced in 1990s in Spain, with the objective to produce oil by a more ecological way, once this system drastically reduces the water consumption during the process, and greatly reduces wastewater generation. This system produces olive oil and a semi-solid waste, two-phase olive mill waste (TPOMW) or wet pomace or *alpeorujo* [4].

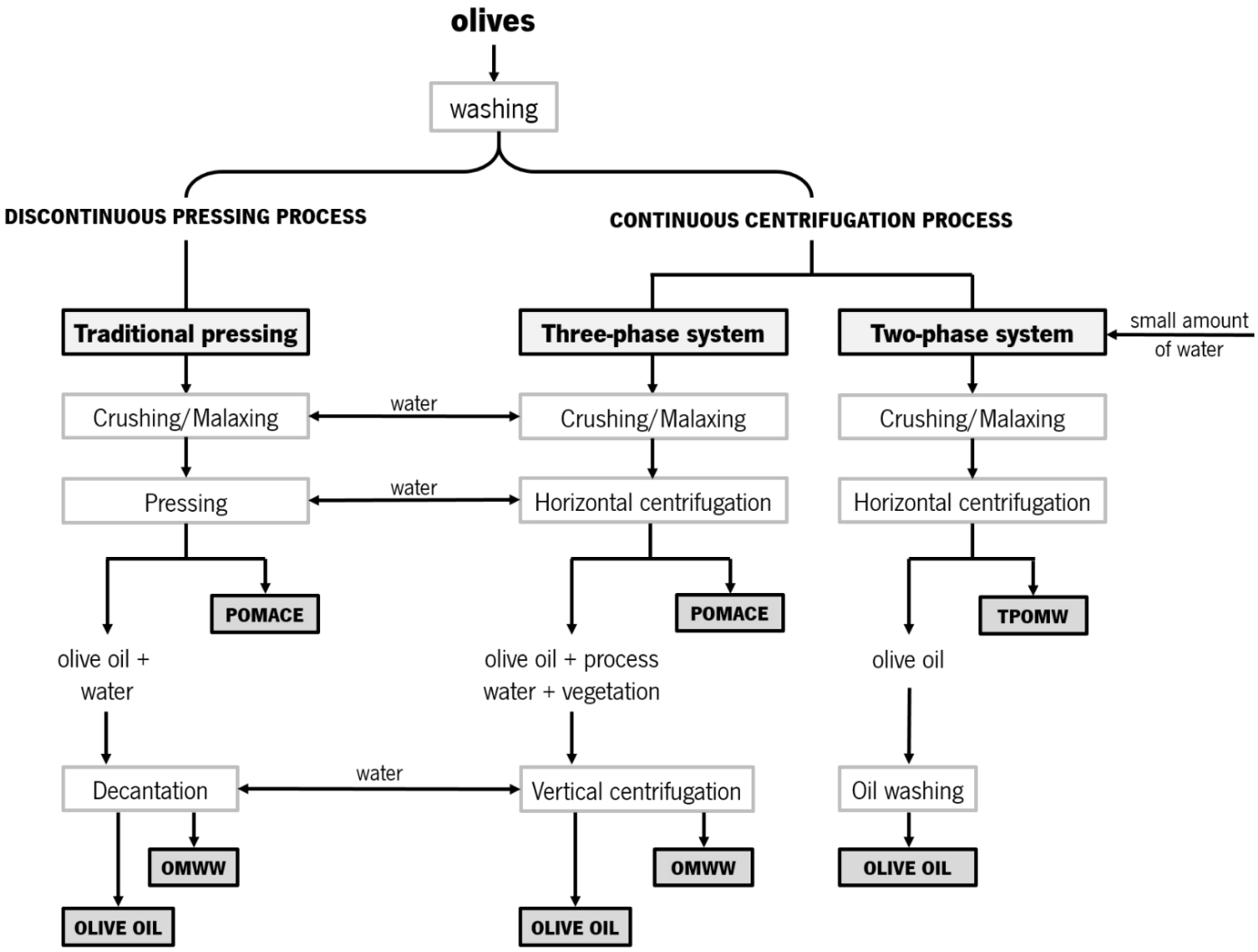

Figure 2 | Processes for olive oil extraction: traditional pressing, three-phase system and two-phase system.

1.2. Olive by-products

The olive oil industry generates large quantities of wastes in short periods of time, and the environmental pollution posed by these is a growing problem. The by-products (wastes) are a solid residue (pomace or TPOMW) from two-phase extraction system and a olive cake and an effluent (OMWW) from three-phases system (**table 1**) – which are ordinarily considered olive mil wastes (OMW)[3]. The olive pomace can be used in a second extraction with organic solvents to extract residual olive oil, then the solid is dried and used in combustion processes.

Different studies have demonstrated that these by-products are harmful to the environment and that cause negative effects on soil microbial populations [5], on aquatic ecosystems [6] and even in air through phenol and sulphur dioxide emissions [7]. The toxicity and antimicrobial activity of the

olive phenols are major contributors to this pollution and hinder the biological treatment of wastes, needed to reduce their pollutant load. Therefore there is an urgent need to find ways of treating this liquid and solid residues from the olive oil industry [3].

Table 1 | Input - output data for the three olive oil production processes. Adapted from Azbar et al. (2004) [8].

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		

1.2.1. Chemical characterization

Among the wastes, pomace is a solid waste obtained from the extraction of olive oil by traditional pressing or three-phase system, and this residue consist in a pieces of skin, pulp, stone and olive kernel with moisture content between 22% - 25% in traditionally pressing and 40% - 45% in three-phase systems [9–11]. The main constituents are cellulose, hemicellulose and lignin; polysaccharides, fatty acids, proteins, lignocellulosic polyalcohols, polyphenols and other pigments are also present [9]. The chemical composition of this waste varies according to the olive species, origin of the olives, culture conditions and extraction process [12]. Pomace of TPOMW from the two-phase systems differs from the other pomace mainly in the amount of humidity (**table 2**).

TPOMW is characterized by pH values slightly acidic, high moisture content (65% a 75%) and very high content of organic matter (mainly lignin, cellulose and hemicellulose). Other important organic components are fats, proteins, water-soluble carbohydrates and a small fraction of hydrosoluble phenolic substances. This by-product is rich in K, containing an intermediate level

of N (mainly organic) and poor in P and others micronutrients (Fe, Cu, Mn, and Zn) [10], that are described in **table 2**. This waste is difficult to manage because its pollutant loads is more concentrated [3]. TPOMW is currently the main waste produced by the olive mill industry.

Table 2 | Chemical composition of TPOMW given by eight authors and reported in Roig *et al.* (2006) [2].

Parameter	Mean	Range
Humidity (%)	62.16	49.6 – 71.4
pH (H_2O)	5.48	4.9 – 6.8
Organic Matter (%)	90.66	60.3 – 98.5
C/N	44.99	29.3 – 59.7
P (g/kg)	0.97	0.3 – 1.5
K (g/kg)	18.73	6.3 – 29
Ca (g/kg)	5.08	2.3 – 12
Mg (g/kg)	1.03	0.5 – 1.7
Na (g/kg)	0.67	0.2 – 1
Fe (mg/kg)	1 107.80	526 – 2 600
Cu (mg/kg)	41.20	13 – 138
Mn (mg/kg)	25.80	13 – 67
Zn (mg/kg)	19.60	10.01 – 27
Lignin (%)*	38.82	19.8 – 47.5
Hemicellulose (%)*	29.70	15.3 – 38.7
Cellulose (%)*	23.47	17.3 – 33.7
Lipids (%)*	11.01	3.76 – 18
Protein (%)*	6.95	6.7 – 7.2
Carbohydrates (%)*	12.32	9.6 – 19.3
Phenols (%)*	1.36	0.5 – 2.4

*(% w/w) of total organic matter

OMWW is also a serious waste produced in olive mills due its chemical composition and its high organic load [4]. The composition is not constant – both qualitatively and quantitatively, and varies according to climate conditions, olive species, cultivation practices, the olive oil extraction process, the olive storage time [3]. OMWW has a red-to-black colored, an acidic pH and of high

conductivity. Further to the high percentage of water (83% - 92%), has an high organic load, mainly sugars, lipids, protein, polysaccharides and minerals, K, Mg, Ca, Cl, F. Also contains phenolic compounds that inhibit microbial growth (antimicrobial activity) [13,14] and the germination and vegetative growth of plants [15], therefore are the main determinants of phytotoxic and antimicrobial actions of OMWW [4]. OMWW are particularly difficult to degrade because the phenols tend to polymerize during storage into condensed high molecular weight polymers [16].

1.2.2. Lignocellulosic materials

The processes that use lignocellulosic wastes (olive pomace) as raw material, can minimize the lack of food, fix problems of waste, decrease dependence on fossil fuels and mitigate the effects on climate and the environment [17]. The utilization of fermentable sugars, like glucose, from lignocellulosic material is the most plentiful renewable resource in the Earth [18].

Lignocellulose is mainly constituent of plant cell walls. It consists of three types of polymers, cellulose, hemicelluloses, lignin, and a smaller amount of pectin and extractives (soluble non-structural materials as non-structural sugars, chlorophyll, nitrogenous material, and waxes) [19–21]. The composition and percentages of these constituents in the plants can vary depending of the species, and the ratios between various polymers within a single plant vary with age, stage of growth and other conditions [20].

These polymers are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross-linkages, therefore these binding of the polymers hinders their biodegradation by fungi and bacteria [20].

Cellulose and hemicelluloses are macromolecules from different sugars and lignin is an aromatic polymer synthesized from phenylpropanoid precursors [20].

Cellulose is a linear polymer composed of D-glucose subunits linked by β-1, 4-glycosidic bonds forming cellobiose molecules; and form long chains (elemental fibrils) linked together by hydrogen bonds and van der Waals forces. Microfibrils (formed by elemental fibrils) group together to constitute the cellulose fiber. Hemicelluloses and lignin cover microfibrils. Cellulose can appear in crystalline form, crystalline cellulose, and a small percentage of non-organized cellulose chains, form amorphous cellulose [20].

Hemicelluloses are polysaccharides that consists of D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O- methyl-glucuronic, D-galacturonic and D-glucuronic acids and sugars are linked together by β-1, 4- and occasionally β-1, 3-glycosidic bonds [20].

Lignin is an amorphous heteropolymer and non-water soluble; confers structural support, impermeability, and resistance against microbial attack and oxidative stress. This polymers composed of phenylpropane units are joined together by different types of linkages [20].

1.2.3. Phenol Compounds

Olive oil contains phenolic substances with antioxidant properties and all of these compounds are potent inhibitor of free radical generation. This substances prevent human diseases and are associated with lower incidences of atherosclerosis, certain cancers, and cardiovascular and neurodegenerative diseases. Beyond to antioxidant activity, phenolic compounds also have anti-inflammatory, antiatherogenic and anti-proliferative properties. Phenol compounds are responsible for the brightly colored pigments of many vegetables and fruits, are responsible for protecting plants from disease and ultraviolet light, also help prevent damage to seeds until they germinate and are quantitatively and qualitatively abundant in olive oil by-products.

Olive oil wastes are an excellent source of natural antioxidants. Phenols compounds, which are considered to be the main antioxidant compounds in OMW, are able to donate a hydrogen atom to the lipid radical formed during the propagation phase of liquid oxidation [22].

The phenolic profile of OMW is variable and complex because the occurrence of specific phenolic compounds depends on the olive variety, climatic conditions, storage time, the treatments applied to extract the oil from the olives and to treat the olive mill wastes, and its mode of culture also significantly influence the qualitative and quantitative phenolic content of the residues [3,4].

The main phenolic compounds detected in residues were phenolic acids, flavonoids and secoiridoids [3]. There are also others compounds namely, hydrocarbons, tocopherols, sterols and triterpenoids [23–26].

The recuperation of phenolic compounds by olive by-products can be done by different techniques such solvent extraction, enzymatic preparation, membrane separation, centrifugation and chromatographic procedures [3].

1.3. Valorisation of olive mill wastes

Olive oil extraction produces a dark-colored wastewater and a solid residue containing nutrients that can be further bioprocessed for disposal [3].

The wastes from olive oil industry are a serious environmental problem, due to its heavy load of lipids, organic acids and phenolic compounds. The reutilization of olive mill wastes are of

great interest since, due to legislation and environmental reasons; the industry is increasingly being forced to find an alternative use for its residual matter. There are two main approaches for the re-used of waste, the extraction of valuable phytochemical compounds with beneficial properties for the food, cosmetic and pharmaceutical industries and the other focuses on the bioconversion of olive mill by-products without environmental impacts [3].

Efficient biotechnological treatments were developed as anaerobic fermentation which can produce different types of value-added products (fuels, organic acids, enzymes, biopolymers) and biological remediation of olive by-products that have been reported as environmental-friendly process [3].

Since early 1990, the two-phase system was introduced as a new method more environmentally friendly and resulted in the production of a new semi-solid waste, TPOMW. This new method of extraction of oil has more advantages comparatively to the three-phase system. Therefore a new waste, with singular physico-chemical properties, is generated in large quantities. TPOMW is a solid waste with a strong odour and a doughy texture, and this makes its transport, storage and handling hard. There are great difficulties for its revalorization due to the high moisture content (65%) and carbohydrate concentration and the high energy consumption required for drying. This residue also has become a serious problem for olive mills, because its management requires specific facilities [2].

1.3.1. Physical-chemical processes

1.3.1.1. Drying and second extraction of oil

TPOMW can be dried and further subjected to second extraction of oil with solvents. This waste tends to stick to the furnace walls blocking the gaseous stream and causing an risk of explosion due to high humidity and sugars present – it cannot be piled and must be kept in large ponds [27]. The moisture also affects the drying process because demands a lot of energy that substantially increases costs. The problems associated with this process of recovery have led to investigation of other alternative technologies [2].

After the second extraction, TPOMW is usually used as fuel to obtain electric or thermal energy through combustion [28,29]. This method is currently used in most of the olive mills because the residue has a high calorific power (400 kcal/kg). However, most of the energy obtained by

combustion is used for dryness of the fresh TPOMW and therefore the total energy recovery is low [8].

1.3.1.2. Extraction of valuable products

TPOMW is suggested as a low-cost source of interest compounds.

This residue has been proposed for the extraction of pectins. These are natural hydrocolloids used as stabilizers agents, emulsifiers and gelling in the food industry and are currently obtained from citrus peel and apple pomace. One intensive search has been made for other sources of pectins using waste as raw materials. Cardoso *et al.* (2003) [30] studied the economic feasibility of TPOMW for the extraction of pectins and obtained positive results.

This residue is also a potential source of phenols that has an extensive range of biological activities. The OMW are rich in polyphenols and contains about 98% of the olive fruit phenols [31]. Various authors have been proved the antimicrobial, cardio-protective, antioxidant, antihypertensive and anticarcinogenic activities of these compounds and can be applied in food, cosmetic and pharmaceutical industries [2].

1.3.2. Biotechnological processes

1.3.2.1. Composting

Composting is a technology that utilizing solid wastes and produces a fertilizer from such wastes. Removes the phytotoxicity of the residues within a few weeks and allows the subsequent enrichment of croplands with compost nutrients that were originally taken up by olive tree cultivation. Composting of solid wastes requires the proper adjustment of temperature, pH, moisture, nutrients and oxygenation, allowing the adequate development of the microbial populations [32]. Optimal conditions for an ideal composting process are a C/N between 20 and 40 of the composting material, moisture content of 50% to 65%, an adequate oxygen supply, a small particle size and enough void space through which air can flow [33]. This recycling process is gaining interest as a sustainable strategy to reuse this residue for agricultural purposes [34–37]. Compost, rich in organic matter and free of phytotoxicity, can thus be obtained [34] after 60 days of composting, these exhibited a microbial stability and a clear absence of phytotoxicity [4].

The major problem of this process from olive by-products is odour emission and the drainage water that has to be treated. To minimize this problem biofilters are used to treat the released gas from composting piles, which increases the total costs of the technology [4].

This process may be an appropriate low-cost strategy for the recycling of solid wastes with a complete detoxification of starting materials, representing an alternative to combustion [2].

1.3.2.2. Anaerobic digestion

The biogas (a mixture of CH_4 and CO_2) and partially stabilized organic matter that can be obtained through anaerobic digestion. Biogas can be used to obtain energy and organic matter and can be applied as soil conditioner. The high level of phenolic compounds and biotoxicity present as a limiting factor [38].

The main limitation of this process is the inhibition of methanogenic bacteria by the phenolic substances and the organic acids present in residue [39]. Pretreatment stage is necessary to remove undesirable compounds [8]. Filidei *et al.* (2003) [40] proposed sedimentation–filtration pretreatment prior to anaerobic digestion as a useful way of solid wastes disposal.

Anaerobic digestion presents advantages such as low sludge generation, less energy requirement for operation and methane production [8].

Biomass gasification is a new physicochemical method that transforms solid biomass into synthetic gas (''syngas''), a mixture of CO and H_2. Synthetic gas is used to obtain important chemical products such as NH_3 or CH_3OH and for preparation of synthetic fuel [2].

1.4. Pretreatments of lignocellulosic materials

The purpose of the pretreatment is to prepare lignocellulosic materials for enzymatic degradation. Pretreatment enhance the biodigestibility of the residues and increase the accessibility of the enzymes to the materials. Without an initial pretreatment, enzymatic hydrolysis of the lignocellulosic materials into fermentable sugars is not as effective due to the high stability of these compounds to enzymatic attack. The efficiency of this process is mainly influenced by the nature and composition of lignocellulosic biomass [41]. The main factors that can affect the rate of biological degradation of lignocelluloses by enzymes can be its accessible surface area and protection by hemicelluloses and lignin, the crystallinity of cellulose, degree of cellulose polymerization, and degree of acetylation of hemicelluloses [42]. The successful pretreatment should, 1) minimize loss of hemicelluloses and cellulose; 2) maximize the enzymatic convertibility; 3) maximize the recovery

of valuable by-products, e.g. lignin; 4) not require the addition of chemicals toxic to the enzymes or fermenting microorganisms; 5) minimize the use of energy, capital equipment and chemicals; and 6) be scaled up to industrial size [43].

The presence of lignin in lignocelluloses leads to a protective barrier that prevents plant cell destruction by fungi and bacteria. The cellulose and hemicellulose must be broken down into their corresponding monomers (sugars), so that microorganisms can utilize them [44] (**figure 3**).

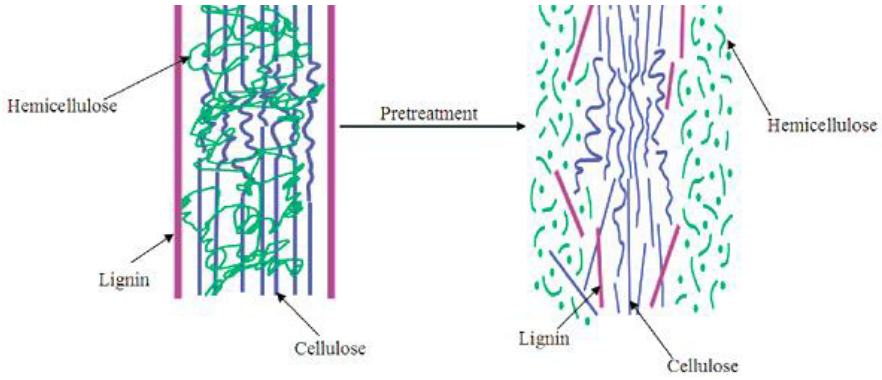

Figure 3 | Schematic representation of the effect of pretreatment in conversion of carbohydrates polymers into fermentable sugars [44].

Different strategies are used to increase the enzymatic susceptibility and have been introduced several pretreatments of lignocellulosic materials. The methods can be classified into physical, physical-chemical, chemical and biological pretreatments [41]. The pretreatments increase accessibility of the fungi to the cellulose, which can actuate as inductor of cellulases production by fungi.

1.4.1. Ultrasound

The physical pretreatment can increase size of pores and accessible surface area, and decrease degrees of polymerization of cellulose and crystallinity. There are different types of physical processes that can be applied to lignocellulosic waste materials as milling (e.g. two-roll milling, ball milling, colloid milling, hammer milling and vibro energy milling) and irradiation (e.g. by gamma rays, microwaves or electron beam) and these can be used to improve the biodegradability or enzymatic hydrolysis of these residues [41].

Ultrasound pretreatment (US) causes a cavitation bubbles formation in the liquid phase [45], the bubbles grow and then violently collapse when they reach a critical size. Cavitational collapse

produces turbulence, intense local heating and high pressure at the liquid-gas interface, high shearing phenomena in the liquid phase and formation of radicals [46,47]. It was also proven that the degradation of excess sludge is more efficient when using low frequencies: mechanical effects facilitate particles solubilisation [45].

Compared with some other physico-chemical pretreatment methods, microwave irradiation seems to have less energy consumption. When were pre-treated lignocellulosic materials with microwave irradiation or microwave-assisted pretreatment in the presence of water, their enzymatic susceptibility increased [48]. The combination of radiation and other methods (e.g., acid treatment) can accelerate the enzymatic hydrolysis [49,50]. The irradiation also increased the enzymatic degradation of cellulose to glucose. However, pre-irradiation in air is more effective than an acid solution [50]. The waste pre-treated using irradiation resulted in double yield of glucose from the hydrolysis compared with the untreated waste [51].

1.4.2. Acid

The acid pretreatment can operate either under a low acid concentration (dilute-acid pretreatment) and high temperature or under a high acid concentration (concentrated-acid pretreatment) and low temperature [41].

Concentrated acids such as HCl and H_2SO_4 have been used to treat the lignocellulosic materials. These concentrated acids are powerful agents for cellulose hydrolysis, however concentrated acids are toxic, corrosive, dangerous and therefore corrosion resistant reactors is necessary, making the pretreatment very expensive. The concentrated acid should also be recovered in order to make the process economically viable [52,53].

Dilute acid hydrolysis also has been successfully developed for pretreatment of lignocellulosic materials. Is used H_2SO_4 at concentrations usually below 4% wt, and has been of the most interest in such studies as it is effective and inexpensive [44]. Dilute acid pretreatment can achieve high reaction rates and significantly enhance cellulose hydrolysis. Dilute acid effectively removes and recovers most of the hemicelluloses as dissolved sugars [44]. When added H_2SO_4, the hemicelluloses are removed and this increases the digestibility of the cellulose in the solid waste [54] and cellulose can actuate as inductor for cellulases production by filamentous fungi. In the treatment with dilute acid is favourable to apply high temperature to the hydrolysis of cellulose [55]. The most used and tested approaches typically employ dilute H_2SO_4, however, HNO_3 [56], HCl [57,58], and H_3PO_4 [57] have also been tested. A number of plant materials has been examined particularly, legume

by-products; corn (cobs, husks and stover); hardwood bark from poplar, aspen, and sweet gum; reed canary grass and mixed hardwood (0% maple and 90% birch) [44].

Dilute acid pretreatment can improve considerably the hydrolysis of cellulose but their cost is usually higher than those of physicochemical pretreatments. In these pretreatment is also necessary the pH neutralization for the downstream enzymatic hydrolysis or fermentation processes [44].

Cara *et al.* (2008) [59] studied the production of fermentable sugars from olive-tree biomass by dilute acid pretreatment and more scarification of the pre-treated solid residues.

There are studies that have demonstrated that materials that are subjected to acid hydrolysis may be more difficult to ferment due to the presence of toxic substances [60].

Acid pretreatment results in high pressures, costly materials of construction, neutralization and conditioning of hydrolysate prior to biological steps, non-productive binding of enzymes to lignin and slow cellulose digestion by enzymes [61]. At a low concentration of acid (e.g. 0.1% - 1% H_2SO_4) and elevated temperature (e.g. 140 °C – 190 °C), the dilute-acid pretreatment can obtain high reaction rates and significantly better cellulose hydrolysis. With this process, almost 100% hemicelluloses removal is possible, and the pretreatment is not effective in dissolving lignin, but it can increases the cellulose's susceptibility to enzymatic hydrolysis and disrupt lignin [42,62]. Dilute-acid pretreatment can be performed either in a relatively long retention time (e.g. 30 min – 90 min) at lower temperatures (e.g. 120 °C) or in short retention time (e.g. 5 min) at high temperature (e.g. 180 °C) [41].

1.4.3. Alkaline

The alkaline pretreatment applies an alkaline solution such as NaOH, NH_3 or $Ca(OH)_2$ (lime) to remove the lignin and a part of the hemicellulose, and also increase the accessibility of enzyme to the cellulose. This alkaline pretreatment removes or modifies the lignin by fracturing the ester bonds that form cross-links between xylan and lignin, thus increasing the porosity of the biomass [63]. However, the process is very complicated, involving several reactive and not reactive phenomena, for example, peeling-off reactions (referred to as formation of alkali-stable in end-groups), dissolution of non-degraded polysaccharides, decomposition of dissolved polysaccharides dissolved and hydrolysis of glycosidic bonds and acetyl groups [64]. Thus, the efficiency of the NaOH pretreatment is very dependent process conditions, like temperature, NaOH concentration, and treatment time, as well as the inherent characteristics of lignocellulose used [65–67].

The processes of pretreatment with NaOH can be performed with high concentrations or low concentrations (in terms of NaOH). In processes of low NaOH concentration (0.5% – 4%), high temperature and pressure are used, NaOH recycling does not occur, and this mechanism is a reactive destruction of lignocelluloses. NaOH at high-temperature disintegrate the lignin and hemicellulose and removes them from the solid phase [18].

Is reported in the literature, for example, when soybean straw was soaked in ammonia liquor (10%) for 24 h at room temperature, the hemicellulose decreased by 41.45% and lignin by 30.16% [68]. Also, alkaline pretreatment was shown to be more effective on agricultural residues than on wood materials [41].

Vaccarino *et al.* (1987) [69] studied the effects of Na_2CO_3, NaOH and SO_2 pretreatments on the enzymatic digestibility of grape marc, and the better degrading effects were obtained by pretreatment with 1% NaOH solution at 120 °C. Zhao *et al.* (2008) [70] also reported that pretreatment with NaOH could obtain a higher enzymatic conversion ratio of cellulose compared with H_2SO_4 pretreatment.

Alkaline treatment appears to be the most effective method in breaking the ester bonds between lignin, hemicellulose and cellulose, and avoiding fragmentation of the hemicellulose polymers, when compared with acid or oxidative reagents pretreatments [71].

1.5. Solid-state fermentation

The solid-state fermentation (SSF) has gained interest in biotechnology 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 [72,73]. These processes use agro-industrial residues as the substrates, and provides an alternate way of value-addition to these otherwise under- or non-utilized residues [73]. Thus, these approaches arise when it is imperative to use a solid waste to avoid environmental impacts that may be caused by direct elimination [74] and in addition, the costs of process are reduced.

The SSF is defined as a fermentation process that occurs in the absence or near-absence of free water in which it is applied a natural or inert substrate used as solid support. The substrate must have enough moisture to support the growth and metabolism of the microorganism [75–77].

Normally the source of nutrients comes from within the particle, however there are cases in which the nutrients come from an external source. A polymer gives the solid particle structure, and

it may or may not be degraded by the microorganism during fermentation. There are also cases in which the artificial or inert carriers are used with a nutrient solution absorbed into the matrix [74].

The SSF also appears as an attractive alternative to submerged fermentation (SmF). In SSF the microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents [78]. There are several potential advantages for bioprocessing and production of various value-added products compared to submerged fermentation, because products has higher yield, lower energy requirements and produces less wastewater with less risk of bacterial contamination [79]. Also requires a small volume of fermentation mash or reactor volume, leading in lower capital operating costs, lower probability of contamination due to low moisture, easy product separation, simple technology and oxygen is typically freely available at the surface of the particles.

The SSF also has some disadvantages such as difficulties in monitoring the process parameters (pH, the moisture content, oxygen, biomass concentration) due to the solid nature of the substrate, difficulties in temperature control and ventilation systems, the impossibility of using microorganisms that grow at low humidity levels, the need for large volumes of inoculum and the possibility of contamination by unwanted fungi.

1.5.1. Microorganisms

In SSF, the microorganisms grow in conditions that more closely resemble their natural habitats, in order to be able to produce certain metabolites and enzymes which are normally not produced or are produced with low yield in SmF [80].

The ability of microorganisms to grow on the solid substrate depends on the requirements of their water activity (a_w), the capacity of adhesion and penetration into the substrate and the ability to assimilate mixtures of different polysaccharides. In SSF, the low moisture within the substrate limits the growth and metabolism of microorganisms when compared to SmF. a_w of substrates have determinant influence on microbial activity, can determine the type of microorganisms that can grow in SSF. a_w of the medium has been attributed as a fundamental parameter for mass transfer of water across the microbial cells [81]. Moreover, reduced water activity causes lower mass transfer and little water availability for microorganisms [82]. Operation at low moisture levels, provides a selective environment for the growth of mycelial organisms, even avoiding sterilization steps.

The filamentous fungi are microorganisms better adapted to the SSF by their physiological, biochemical and enzymological properties, and are particularly interesting due to their high production of extracellular enzymes [83].

The hyphae generated by these fungi have the capacity to penetrate solid substrates and still have the advantage over unicellular microorganism colonization of the substrate and utilization of nutrients available. The filamentous fungi also have the ability to grow at low a_w and at high osmotic pressure conditions (high concentrations of nutrients) making these microorganisms efficient and competitive for bioconversion of solid substrates microorganisms. There are still some SSF involving bacteria and yeasts.

Filamentous fungi have the propensity to grow adhered to surfaces. Not yet been thoroughly studied the influence of this type of growth on fungal physiology, particularly when related to productivity [84]. Under natural conditions, fungal contact with surfaces is required for nutrient uptake, hyphal apical growth and enzyme secretion. These microorganisms can grow in the absence of free water [85], and considering that submerged free floating fungal growth is not natural, growth on and within solid substrates is fundamentally related to cell adhesion. Growth morphology of filamentous fungi is an important factor related to productivity of various industrial processes [86].

The filamentous fungus *Aspergillus* of the section Nigri are considered of wide economic importance due to production of metabolites such as enzymes [87–89]. Apart from their economic importance, some black aspergilli are also ochratoxin-producing organisms which contaminate several agricultural products, including grape-derived products, cocoa and coffee [90–92]. These fungi are one of the more difficult groups concerning identification and classification, and several taxonomic schemes have been proposed. New molecular approaches have shown that there is a high biodiversity, but that species are difficult to recognize based solely on their phenotypic characters [93].

Among these fungi, *A. niger* is known as one of the best extracellular enzyme producers (e.g. lipases, cellulases) [94–97].

Serra *et al.* (2006) [98] described a new species belonging to section Niger, called *A. ibericus*. This species do not produce any relevant mycotoxins, therefore are safe for biotechnological applications to produce many metabolites with commercial value.

The fungus *A. foetidus* is reported in several studies as a source of enzymes with biotechnological interest. Shah *et al.* (2005) [99] and Chapla *et al.* (2010) [100] used lignocellulosic wastes for production of xylanases using solid-state fermentation. Kumar *et al.* (2012) [101] produced

pectinase from Mango Peel in solid-state and submerged fermentation. Mukherjee et al (2004) [102] used tannin rich substrates for the production of tannases and gallic acid.

A. uvarum sp. is described within *Aspergillus* section Nigri by Perrone *et al.* (2008) [103] and this isolated not produces ochratoxin A.

Also filamentous fungi of the *Trichoderma* genus are especially notable for their high enzymatic productivity [104].

T. reesei is one of the main industrial source of cellulases and hemicellulases due to its ability to secrete high quantities of hydrolytic enzymes [105], and are also used as biological agents to control plant pathogens in agriculture [106,107]. Is the most widely employed fungus for the production of cellulolytic enzymes and has been extensively studied [108]. These fungi has been widely investigated for cellulases production from various materials such as wheat bran [109], wheat straw [110] and wood [111].

1.5.2. 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 [112].

1.5.2.1. 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 [82,86]. The advantage of using lignocellulosic wastes like substrate, allows the reduction of costs in the production of cellulases, making the process more cheap [113].

These enzymes catalyse the hydrolysis of the β-1,4-glucosidic linkages of cellulose [20] 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 [114]. 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 [86].

From among the fungal strains that produce cellulases stand out *Aspergillus*, *Trichoderma*, *Penicillium*, and *Fusarium* genera [115].

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) [116]. However, the strains of *Aspergillus* are high in BGL activity [117].

1.5.2.2. 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 [118].

The production of these enzymes requires substrates in very high concentration. Xylanases are produced mainly by *Trichoderma* spp. and *Aspergillus* spp. [119,120].

The enzymes production was achieved successfully by *Aspergillus niger*, *Aspergillus fischeri* using wheat straw and wheat bran as main substrates [121,122]. Ghanem *et al.* (2000) [122] 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 [122].

Other cellular models can be used for xylanases production using SSF such as *Aureobusidium pullulans*, [123], *Thermomyces lanuginosus* [124], *Humicola insolens* [125] and *Melanocarpus albomyces* [126].

Chapter 2
MATERIALS AND METHODS

2.1. Raw material

Two types of olive pomace (crude olive pomace, COP and exhausted olive pomace, EOP) were collected from olive oil industry of north of Portugal in season 2013/2014. COP is a semi-solid waste of the two-phase system that was recovered after olive oil extraction. EOP is obtained after recovery of residual olive oil from COP and dried to use in combustion processes. The pomaces samples were stored, respectively, at –20 °C and at room temperature and dry conditions.

2.2. Physical-chemical characterization of olive pomace

Both olive pomaces were analysed for the physical-chemical characterization according to the procedures described in the next sections.

2.2.1. Humidity determination

A known quantity of waste, about 1 g, was added in a container with a known weight (previously dried in hot air oven until constant weight). The vessel with the waste was placed in the hot air oven at 105 °C for about 24 h (until constant weight). After cooling in desiccator containing silica gel it was weighed. This determination was performed in triplicate. Humidity (H) percentage (grams of water per grams of humid waste) was calculated according to **equation 1**.

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

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

2.2.2. Total solids determination

To determine the total solids in the waste, the procedure is the same as in section 2.2.1. Total solids percentage (grams of solid per 100 grams of humid waste) was calculated according to **equation 2.**

$$\textbf{TS (\%)} = \frac{\text{WCDS - WC}}{\text{WCHS - WC}} \text{ x } 100 \qquad \textbf{(eq.2)}$$

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

2.2.3. Ash determination

A known quantity of waste, about 1 g, with a known humidity was added in a porcelain container (previously exposed to 575°C during 30 min in the muffle). The porcelain container with the waste was placed in the hot air oven at 105 °C for about 24 h and in the muffle at 575 °C for about 2 h (until constant weight). After cooling in desiccator containing silica gel and then it was weighed. This determination was performed in triplicate. Ash percentage (grams of ash per 100 grams of dry waste) was calculated according to **equation 3**.

$$\textbf{Ash (\%)} = \frac{\text{WCA - WC}}{\text{(WCHS - WC) x (1 - H)}} \text{ x } 100 \qquad \textbf{(eq.3)}$$

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

2.2.4. Nitrogen, carbon and metals determination

Total nitrogen and organic carbon were determined by a Thermo Finningan Flash Element Analyzer 1112 series, San Jose, CA (USA) and metals, such as Ca, K, Mg, Na, Zn, Cu, Fe, Mn, Cr, Ni and Pb were analysed in ashes using Flame Atomic Absorption and Atomic Emission Spectrometry (FFAS/FAES) FAAS/FAES [127] .

2.2.5. Cellulose, hemicellulose and lignin determination

The organic constituents of olive pomace 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 °C for 1 h and the second stage after dilution to 4% wt H_2SO_4 at 121 °C for 1 h.

A sample of about 0.5 g was weighed in a test tube. It was added 5 mL of 72% wt H_2SO_4. The test tubes were placed in the water bath at 30 °C during 1 h with periodic agitation (first stage). After this period, the test tube content was transferred for flasks and the waste that stayed adhered to the walls was dragged with distilled water. To increase the volume of dissolution up to 148.67 g distilled water was added. The flasks were closed, weighed and introduced in to autoclave during 1 h at 121 °C (second stage). Posteriorly the flasks were cooled and was determined the originated losses during the second stage by weighing. The entire content of each of the flasks was filtered through a Gooch container with known weight. The Gooch container used to filter the product resulting from QHA were introduced in the hot air oven at 105 °C. After 24 h it was cooled in desiccator containing silica gel and then weighed. 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. Retention times in minutes were, respectively, 8.075 min for glucose, 8.61 min for xylose, 9.327 min for arabinose and 13.18 min for acetic acid.

With the data of sugars concentrations (glucose, xylose, arabinose and acetic acid) was calculated the content in polymers (CP) which hydrolyse gave rise to monomers measured in the waste. The CP, glucan, CG_n, xylan, CX_n, arabinan, CAr_n, and acetyl groups, CG_A, (grams of polymer per 100 grams of dry waste) were calculated according to **equation 4**.

$$\textbf{CP (\%)} = F \times SCF \times \frac{[S]}{\rho} \times \frac{W + WHS \times H}{WHS \times (1 - H)} \times 100 \qquad \textbf{(eq.4)}$$

wherein F is a factor which corrects degradation of sugars (1.04 for CG_n, 1.088 for CX_n/CAr_n and 1.00 for CG_A), SCF is a stoichiometric correction factor to take account the increase in molecular weight during hydrolysis (162/180 for CG_n, 132/150 for CX_n/CAr_n and 43/60 for CG_A), 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 5** and **equation 6**, respectively.

$$\textbf{Cellulose (\%)} = CG_n \qquad \textbf{(eq.5)}$$

$$\textbf{Hemicellulose (\%)} = CX_n + CAr_n + CG_A \qquad \textbf{(eq.6)}$$

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 7.**

$$\textbf{Klason lignin (\%)} = \frac{WCDS - WC}{WCHS \times (1 - H)} \times 100 \qquad \textbf{(eq.7)}$$

wherein WCDS is the weight of Gooch container with dry sample in grams, WC is the weight of Gooch container in grams, WCHS is the weight of Gooch container with humid initial sample who underwent to the QHA in grams and H is the humidity in grams of water/grams of humid waste.

2.2.6. Lipids determination

The total fats contents were extracted with diethyl ether, in a Soxtec System HT2 1045 Extraction Unit. The temperature of the process was 90 °C.

About 5 g of humid waste (with the diameter of the particles less than 1 mm) were weighed for a thimbles which were covered with a thin layer of cotton and a thimble adapter. Thimbles were inserted in the condensers of the extraction unit (1045 Extraction Unit). Extraction cups with glass beads were weighed and were added 50 mL of diethyl ether.

The extraction cups were dried in the hot air oven at 105 °C for about 30 min. After cooling in the desiccator containing silica gel weight was determined. This determination was performed in duplicate. Lipids percentage (grams of lipids per 100 grams of dry waste) was calculated according to **equation 8**.

$$\textbf{Lipids (\%)} = \frac{WCL - WC}{WHS \times (1 - H)} \times 100 \qquad \textbf{(eq.8)}$$

wherein WCL is the weight of extraction cups with lipids in grams, WC is the weight of extraction cups in grams, WHS is the weight of humid initial sample in grams and H is the humidity in grams of water/grams of humid waste.

2.2.7. Reducing sugars determination

Free reducing sugars were measured by the DNS method [128] and in order to analyse them in olive pomaces an extraction with water (S:L ratio, 1:5 w/v) was performed.

To each tube was added 0.5 mL of DNS reagent to 0.5 mL of sample (0.5 mL of distilled water for blank) in triplicate. Tubes were placed in a bath at 100 °C during 5 min. After cooling, was added 5 mL of water to the mix. Absorbance was read at 540 nm. Calibration curve was constructed with glucose standard solutions between 0 g/L and 4 g/L.

2.2.8. Total phenols determination

Total phenols were assessed by the Folin-Ciocalteu method (Commission Regulation (EEC) No. 2676/90) and in order to analyse them in olive pomace an extraction with water (S:L ratio, 1:5 w/v) was performed.

In each tube 100 µL of sample (100 µL of distilled water for blank) was added, as well as 2 mL of Na_2CO_3 at 15%, 500 µL of Folin-Ciocalteu reagent and 7.4 mL of distilled water in triplicate. Tubes were placed in a bath at 50 °C during 5 min. After cooling to room temperature, tubes were vortexed. Absorbance was read at 700 nm. Calibration curve was constructed with caffeic acid standard solutions between 0 g/L and 2 g/L.

2.2.9. Proteins determination

Proteins were measured by the Bradford method [129] and in order to analyse them in olive pomace an extraction with water (S:L ratio, 1:5 w/v) was performed.

In each well microplate was added 10 µL of sample (10 µL of distilled water for blank) to 300 µL of Coomassie Blue reagent in triplicate. Microplate was stirred during 5 min and waited for 10 min at room temperature. Absorbance was read at 595 nm. Calibration curve was constructed with bovine serum albumin (BSA) standard solutions between 0 g/L and 1 g/L.

2.3. Microorganisms

Different species of *Aspergillus* section Nigri and *Trichoderma reesei* were used in this study and are listed in **table 3**. They were obtained from MUM (University of Minho, Braga, Portugal) and CECT (Valencia, Spain) culture collection, where they were preserved in glycerol stocks stored at -80 °C. They were revived on malt extract agar (MEA) plates (20 g/L malt extract, 1 g/L peptone, 20 g/L glucose and 20 g/L agar). To obtain inocula for SSF, the selected fungi were subcultured on MEA 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.

Table 3 | Filamentous fungi screened to verify ability to produce extracellular cellulases.

Fungi	Code
Aspergillus niger	01Uas107
Aspergillus niger	01Uas181
Aspergillus niger	01Uas183
Aspergillus niger	CECT 2088
Aspergillus niger	CECT 2700
Aspergillus niger	CECT 2915
Aspergillus niger	MUM 92.13
Aspergillus ibericus	MUM 03.49
Aspergillus ibericus	MUM 2004
Aspergillus ibericus	03Uas268
Aspergillus uvarum	MUM 08.01
Aspergillus foetidus	01Uas162
Aspergillus carbonarius	01Uas130
Trametes versicolor	MUM 04.100
Trichoderma reesei	03Uas095

2.4. Screening of filamentous fungi

2.4.1. Screening by Congo red test

The strains were initially screened using the Congo red test. The screening of cellulases producing microorganisms was performed on agar plates using a cellulosic substrate, carboxymethylcellulose (CMC) as carbon source.

These fungi were inoculated in agar plates with specific medium, Mandel Weber medium. The composition of the medium consisted of 1.4 g/L $(NH_4)_2SO_4$; 2 g/L KH_2PO_4; 0.3 g/L $CaCl_2.2H_2O$; 0.3 g/L $MgSO_4.7H_2O$; 0.005 g/L $FeSO_4.7H_2O$; 0.0016 g/L $MnSO_4.H_2O$; 0.0014 g/L $ZnSO_4.7H_2O$; 0.002 g/L $CaCl_2.6H_2O$; 5 g/L CMC; 0.1 g/L peptone; 0.1 g/L tween-80; 20 g/L agar. The medium was autoclaved at 121 °C for 15 min. After, around 20 mL of medium was poured into sterile Petri plate and allowed to solidify. The fungal cultures were inoculated at the centre of plate containing sterile medium and incubated at 25 °C during 5 days. At the end of 5 days the growth of the microorganism was measured by the diameter of the colony. Each culture plate was stained with 10 mL aliquot of Congo red dye (2.5 g/L). After 15 min, the solution was discarded and the cultures were washed with 10 mL of 1 mol/L NaCl. Cellulases production was detected by appearance of clear zone of medium around growth colony, pale halo and orange edges around the fungal colony indicative of areas of hydrolysis. This halo was measured for subsequent calculation of the enzymatic index (EI) using the **equation 9**.

$$EI = \frac{H}{C} \qquad \textbf{(eq.9)}$$

wherein H is the diameter of hydrolysis zone and C is the diameter of the colony.

The strains that showed higher EI were considered to be potential producers of cellulases. Two replicates per strain were performed for this screening step.

2.4.2. Screening by SSF

The three fungi that showed more diversity in enzyme production and stronger activities were subjected to a second screening. These fungi were proved in SSF (section 2.7.1.) using COP and EOP as solid subtract. Two replicates per condition were performed for this screening step. The condition that exhibited higher enzymes activities (cellulases and xylanases) was selected for the following steps.

2.5. Ultrasound pretreatment

The ultrasound pretreatment (US) of EOP was carried out with a high intensity ultrasonic processor Cole-Parmer 750 model (Illinois, USA) operating at 750 W and 20 kHz. To optimize the

optimal operational conditions this pretreatment for xylanases and cellulases production by SSF a full factorial design 3^2 were planned (section 2.9.2).

The solid waste (30 g solid dry) was added to vessel and mixed with distilled water. Different liquid:solid ratio and time of treatment were studied in the experimental design. The vessel was placed in a protective box and the tip (diameter 1/2") allocated into the vessel. After pretreatment, the solid was recovered by vacuum filtration and dried at 50 °C for 24 h in a hot air oven. The treated waste was used as solid substrate in SSF to produce enzymes (section 2.7.2). This process is shown in **figure 4**.

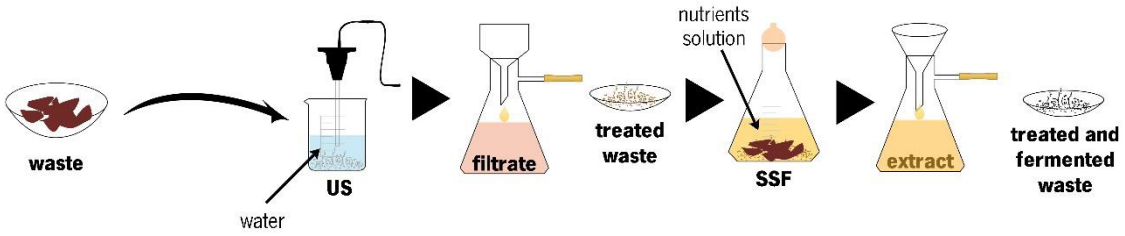

Figure 4 | Diagram which represents US treatment of olive pomace.

2.6. Evaluation of other pretreatments

Other pretreatments such as acid hydrolysis with diluted acid (AH) or ultrasound combined with acid hydrolysis (US+AH) were studied (**figure 5**) to compare with US.

The AH of EOP was carried with dilute H_2SO_4 (3%) during 30 min in flasks inside autoclave at 121 °C with a L:S ratio of 8 g/g [130]. The hemicellulosic fraction solubilized in the liquid was separated by vacuum filtration and the solid was neutralized (pH 6) by washing with distilled water and dried at 50 °C for 24 h in a hot air oven. The treated waste was used as solid substrate in SSF to produce enzymes (section 2.7.3.).

US+AH was carried out in two steps. In the first step the pomace (60 g solid dry) was subjected to US in the optimal operational conditions previously optimized in section 2.5. and this pretreatment was performed in same conditions mentioned above. In the second step the treated moist waste was submitted to the AH. This pretreatment was performed in the same conditions mentioned above. The treated waste was used as solid substrate in SSF to produce enzymes (section 2.7.3.).

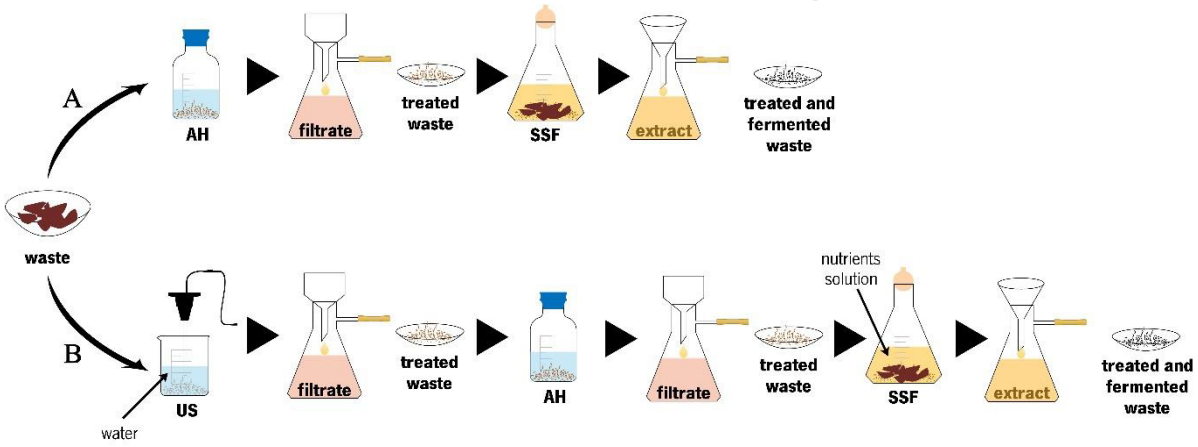

Figure 5 | Diagram which represents AH (A) and US+AH (B) pretreatment of olive pomace.

2.7. Solid-state fermentation

2.7.1. Screening of filamentous fungi by SSF

COP and EOP were used as substrates in SFF experiments in order to compare its adequability for enzymes production and it was also used the three fungi selected in screening stage with stronger activities.

SSF was carried out in 500 mL Erlenmeyer with 10 g of dried solid substrate. Moisture level was adjusted to 75% (wet basis) with nutrients solution (5 g/L peptone, 5 g/L yeast extract, 0.2 g/L KH_2PO_4). Erlenmeyers with solid medium were sterilized at 121 °C for 15 min.

For the inoculation, fungal spores selected in Congo red test were grown in MEA medium slants tubes and were suspended in a sterile solution (1 g/L peptone, 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 the spore suspension and incubated at 30 °C for 6 days.

The extraction of enzymes was performed at final of each experiment with a solution composed of 1% NaCl and 0.5% Triton-X100 at room temperature in an L:S ratio of 1:5 and with agitation for 1 h. Following, extracts were centrifuged (4000 g, 15 min), filtered through Whatman N° 1 filter paper and verified the extract volume.

2.7.2. Ultrasound pretreatment

The experiments were planned to observe the optimal operational conditions of US to improve xylanases and cellulases production by SSF in according to the experimental design (section 2.9.2.). For this, it was used the optimal conditions obtained from the screening of filamentous fungi by SSF (section 2.4.2.) and US pretreatment was carried out (section 2.5).

The SSF, inoculation, incubation and extraction of enzymes were carried out in same conditions of section 2.7.1.

2.7.3. Other pretreatments

One experiment was planned to observe the differences between AH and US (section 2.6.). For this, it was used the optimal conditions obtained from the screening of filamentous fungi by SSF (section 2.4.2.) and was carried out the AH.

The other experiment was planned to observe the differences between US+AH and US. For this, it was used the optimal operational conditions previously optimized (section 2.5.) to carry out the US followed by AH.

The SSF, inoculation, incubation and extraction of enzymes were carried out in same conditions of section 2.7.1.

2.7.4. Study of kinetic to enzymes production

The experiments were planned to observe over time the production of enzymes and the behaviour of the sugars, proteins and phenols. For this was used the optimal operational conditions previously optimized to carry out the US. Eleven Erlenmeyers with the same conditions were used and in each time one Erlenmeyer was taken for analysis.

The SSF, inoculation and extraction of enzymes were carried out in same conditions of section 2.7.1. The incubation was at 30 °C during seventeen days. Until the fifth day one Erlenmeyer was taken per day. After the fifth day until the eleventh day one Erlenmeyer was taken of two in to two days. The last Erlenmeyer was taken in the seventeenth day.

2.7.5. Effects of nutrients

To evaluation the effect of nutrients several tests were performed and are described below.

2.7.5.1. Low cost nutrients

In the first test, the experiment was planned to observe the effect of the low cost nutrients in the xylanases and cellulases production by SSF. For this was used the optimal operational condition previously optimized to carry out the US.

In the SSF the moisture level was adjusted to 75% (wet basis) with a low cost nutrients solution (0.04 g_{urea} / $g_{subtract}$ and 7.5 mL vinasses) [131]. The other conditions of the SSF, inoculation, incubation and extraction of enzymes were carried out in same conditions of section 2.7.1.

2.7.5.2. Importance of nutrients

In this test, the experiment was planned to observe the importance of nutrients in the xylanases and cellulases production by SSF. For this, it was used the pomace (10 g solid dry) selected in section 2.4.2. that was mixed with distilled water in an L:S ratio of 3 and the sonication time was 5 min. The US was performed in same conditions mentioned in section 2.5.

In the SSF the moisture level was adjusted to 75% (wet basis) with the filtrate resulting of the filtration after pretreatment. The other conditions of the SSF, inoculation, incubation and extraction of enzymes were carried out in same conditions of section 2.7.1.

2.7.5.3. Importance of liquid fraction of ultrasound pretreatment

In this test (**figure 6**), the experiment was planned to observe the importance of the liquid of pretreatment in the xylanases and cellulases production by SSF. Firstly the pomace (10 g solid dry) selected in section 2.4.2. was used and it was mixed with nutrients solution (5 g/L peptone, 5 g/L yeast extract, 0.2 g/L KH_2PO_4) with an L:S ratio of 3 and a sonication time of 5 min. In a second assay the pomace (30 g solid dry) selected in section 2.4.2. was used and it was mixed with nutrients solution in an L:S ratio and a sonication time of the optimal operational condition of the US previously optimized (section 2.5.). The US was performed in same conditions mentioned in section 2.5 for both assays.

In the SSF the moisture level was adjusted to 75% (wet basis) with the filtrate resulting of the filtration after pretreatment. The other conditions of the SSF, inoculation, incubation and extraction of enzymes were carried out in same conditions of section 2.7.1.

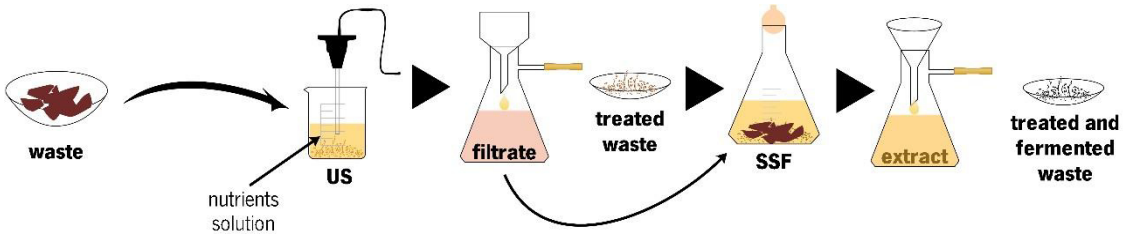

Figure 6 | Diagram which represents US of olive pomace and the moisture adjust of solid to carry out the SSF (75%) it was performed with liquid fraction of ultrasounds pretreatment.

2.8. Analysis of enzyme activities

The activity of cellulases (endo-1,4-ß-glucanase) was determined with the enzymatic kit Azo-CM-Cellulose S-ACMCL 094/12 (Megazyme International, Ireland).

The procedure for determination of cellulases' activity was to add 0.5 mL of suitably diluted enzyme solution in 0.1 M sodium acetate buffer (pH 4.6) and pre-equilibrated at 40 °C to 0.5 mL of pre-equilibrated substrate solution (CM-Cellulose 4 M). For the reaction blank was added precipitant solution to substrate solution. Then, the mixture was stirred and incubated at 40 °C for exactly 10 min. The reaction was terminated and not hydrolysed substrate was precipitated by the addition of 2.5 mL of Precipitant Solution with vigorous stirring for 10 sec on a vortex mixer. The reaction tubes was allowed to equilibrate to room temperature for 10 min. Was stirred the tube contents again and centrifuged the tubes at 1,000 g for 10 min. Was measured the absorbance of the supernatant solution at 590 nm and was determined enzyme activity by reference to a standard curve. For prepare the Precipitant Solution was dissolved 40 g of $C_2H_3NaO_2.3H_2O$ and 4 g of $Zn(O_2CCH_3)_2$ in 150 mL of demineralised water. Was adjusted the pH to 5.0 with 5 M HCl and the volume to 200 mL with demineralised water. Was added 200 mL of this solution to 800 mL of ethanol (95%), was mixed well and stored at room temperature in a well-sealed bottle. To prepare the buffer solution was added 6 g of $C_2H_4O_2$ to 800 mL of distilled water. The pH was adjusted to 4.6 by the addition of 5 M NaOH solution. The final volume was adjusted to 1 L.

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 4.5. The values of cellulases' activity were expressed in U per gram of dry subtract.

The activity of xylanases (endo-1,4-ß-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 U per gram of dry subtract

2.9. Statistical analyse

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

2.9.1. Analysis of variance

Statistically significant differences of the several assays tested in section 2.4 were evaluated by a one-way ANOVA. A significant difference was considered if $P < 0.05$ applying the Tukey multiple-comparisons. Statistical analyses were performed using GraphPad (San Diego, USA) software.

2.9.2. Full Factorial Design

For evaluation of sonication effect of EOP in xylanases and cellulases production by SSF a full factorial design 3^2 was carried out. To fix the range of operation conditions for studying in experimental design through preliminary experiments (select of fungus and type of pomace, section 2.4) were performed. The two studied variables were the time of treatment and the L:S ratio (v/w), the dependent variables studied were xylanases and cellulases activity. The independent variables considered and their variations ranges are shown in **table 4**. The correspondence between coded and uncoded variables was established by linear equations deduced from their respective variation limits.

This design allows estimating the significance of parameters and their interaction using Student's *t* – tests. The experimental design was performed in 11 experiments with three replicates in the centre point. For statistical calculation, the variables were coded according to **equation 10**.

$$x_i = (\chi_1 - \chi_0)/\ \delta\chi \qquad \textbf{(eq.10)}$$

wherein x_i is the dimensionless coded value of the independent variable, x_o is the value of independent variable at the centre point, and δX is the step change.

Table 4 | Levels of independent variables and dimensionless coded variables definition (x_i) i of the experimental design – optimization of US.

Independent variables	**Units**	**Levels**			x_i
		-1	0	1	
Time ($\boldsymbol{\chi_1}$)	min	5	10	15	(T – 10/5)
L:S ratio ($\boldsymbol{\chi_2}$)	g/g	3	7	11	(L:S – 7/4)
Dependent variables	**Units**				
Cellulases activity	U/g solid substrate				
Xylanases activity	U/g solid substrate				

The interrelationship between dependent and independent variables was established in the **equation 11**.

$$Y=b_0 + b_1 \cdot x_1 + b_{11} \cdot {x_1}^2 + b_2 \cdot x_2 + b_{22} \cdot {x_2}^2 + b_{12} \cdot x_1 \cdot x_2 + b_{112} \cdot {x_1}^2 \cdot x_2 + b_{122} \cdot x_1 \cdot {x_2}^2 + b_{1122} \cdot {x_1}^2 \cdot {x_2}^2 \qquad \textbf{(eq.11)}$$

wherein Y is the dependent variable, b are the regression coefficients (calculated from experimental data by multiple regression using the least-squares method) and x are independent variables (coded).

All experiments were carried out in duplicate and in randomized run order.

The experimental data were evaluated by response surface methodology using Statistica 5.0 software. Dependent variable were optimized using an application of commercial software (Solver, Microsoft Excel 2007, Redmon, WA, USA).

Chapter 3

Results and Discussion

3.1. Physical-chemical characterization of olive pomace

The solid wastes from olive mills were characterized to assess the potential as solid substrate in SSF. **Table 5** shows the physical and chemical composition of COP and EOP. It was analysed the content of moisture, total solids, ash, organic constituents (such as lignin, hemicellulose and cellulose) and lipids. In addition, it was measured the concentration of proteins, reducing sugars and phenols, nitrogen, carbon, and micronutrients according to the methods described in the section 2.2.

The COP was directly collected after olive oil extraction obtaining a wet solid waste, having a very high humidity, around 74%. EOP was recovered after extraction of residual olive oil and dried, having a low humidity, around 10%. The moisture content and total solids were very different between wastes due to the drying processes. Roig *et al.* (2006) [2] proposed valorisation methods for the TPOMW, between them, the author considers that the drying and second extraction of oil is a physicochemical treatment.

The content of lignin, hemicellulose and cellulose were high in both residues, which was roughly accounted for 35% (mass per mass of dry solid), 39% and 34%, respectively, for COP and 42%, 24% and 11%, respectively, for EOP. The content of the main organic constituents were similar to the other residues studied in literature [2]. The higher content of cellulose and hemicellulose indicated that these wastes have potential to be used as solid substrate in SSF and can induce the production of lignocellulolytic enzymes [132].

Other important components in these residues were lipids, reducing sugars and proteins. All of these compounds were higher in COP because these compounds could have been extracted from EOP when the recovered of residual olive oil was performed.

The analysis of olive pomace showed other valuable compounds, phenolic substances, which were high and similar in both residues. TPOWM usually are rich in polyphenols, which could be used in pharmaceutical, cosmetic and food industries and its extraction may be an alternative for revalorization of olive pomaces [31], however their presence it can be harmful for SSF due to they can inhibit fungal growth [133].

The analysis of the content of C and N showed an increase of N content and decrease of C after extraction of residual oil. C/N ratio of the COP and EOP, were, respectively, 83 and 36. Carbon and nitrogen sources are important components for fungal growth and should be given significant consideration [134]. The optimum ratio for SSF is closed to 15 [135].

Table 5 | Physical-chemical composition of COP and EOP. The results represent the average of three independent measurements ± SD.

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 (%)	35 ± 1	41.62 ± 0.04
Hemicellulose (%)	39 ± 5	24.1 ± 0.2
Cellulose (%)	34 ± 1	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

As regards the olive pomaces mineral content, the values was not different in both residues, except to the Fe content that was higher in EOP and the content of Na that was higher in COP. These residues were rich in K, which is a common characteristic in OMW and by-products as reported by Albuquerque *et al.* (2003). The macro and micronutrients content in residues was similar to other residues studied in literature [2]. The concentration of heavy metals in residues of

TPOMW is almost non-existent. Madejón *et al.* (1998) showed a concentration lower than 1 mg/kg for Pb, Cd, Cr and Hg. In analysis of the COP and EOP, heavy metals such as Cr, Ni and Pb showed a concentration lower than 22 mg/kg.

3.2. Screening of filamentous fungi

The aim of this study was to screen several strains of filamentous fungi and selection of most efficient cellulases-producing microorganisms. The screening of filamentous fungi correlates qualitative screening using agar plate's assays, initial screening step, with quantitative measurements of cellulases and xylanases production during cultivation for 6 days under SSF, second screening step.

3.2.1. Screening by Congo red test

The initial screening step consisted of observation of the growth of fifteen strains of filamentous fungi on agar plates using CMC as carbon source, and measurement of the hydrolysis halo that is used for calculation of the EI. The diameter of the halo zone is useful for selection of strains that can efficiently degrade polysaccharides such cellulose [136]. The halo produced by hydrolysis of cellulose is directly related to the region of action of cellulolytic enzymes, since the dye only remains attached to regions where there are ß-1,4-D-gluconohydrolase bonds [137] and as hydrolysis indicator was used Congo red dye [138–140].

The clear zone around the colonies is represents in **figure 7**, which corresponds to the zone of CMC degradation and was observed for the strains studied in two experiments independents and compared with a control.

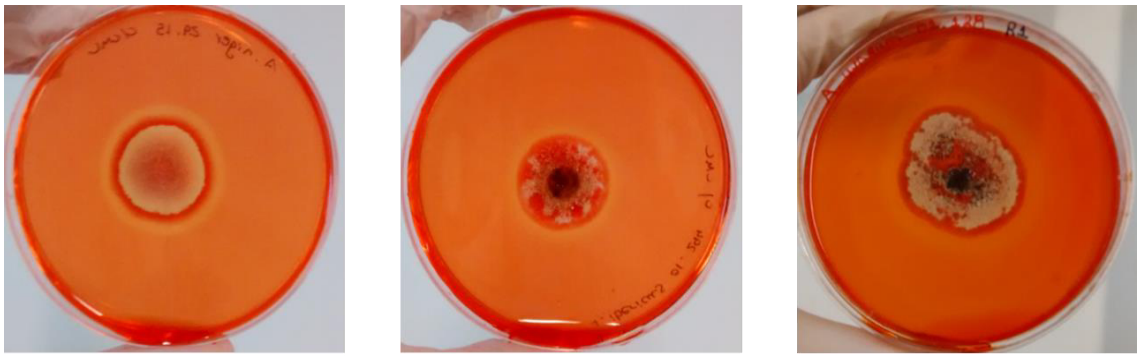

Figure 7 | Observation of the clear zone around a colony of *A. niger* CECT 2915 (**A**), *A. ibericus* MUM 03.49 (**B**) and *A. uvarum* MUM 08.01 (**C**) using Congo red dye.

The EI can be used as a rapid and simple methodology to select strains within the same genus that have potential for the production of enzymes [141]. **Figure 8** shows the results of the assays carried out on agar plate containing CMC as carbon source, in which for each strain was determined the EI. These results were analysed by one-way ANOVA followed by a multiple comparison test, Tukey's Multiple Comparison Test. The differences observed between the EI values for the D, F, G, H, K, M, N and O were found to be statistically significant (p-value = 0.0005). From these results it was possible to select the three strains that presented the highest EI.

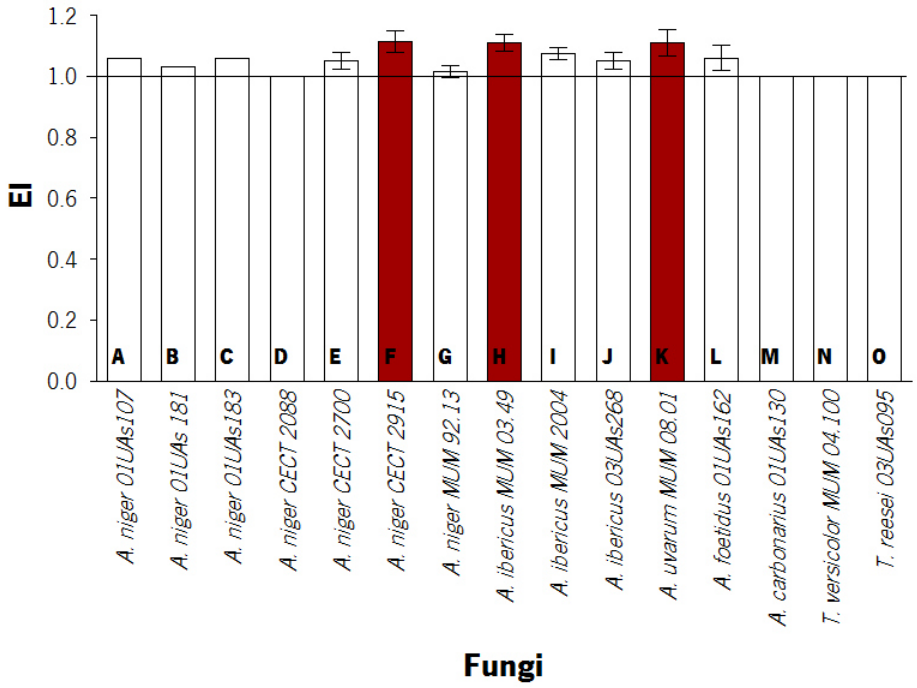

Figure 8 | EI of fifteen strains of filamentous fungi. The results represent the average of two independent experiments and error bars represent SD.

Florencio *et al.* (2012)[104] observed which the between-isolate variability of EI was low for the strains of the genus Trichoderma where an obvious hydrolysis halo was visible.

In other work, Congo red plates with CMC presented low hydrolysis zone intensities when compared to a method using Gram's iodine reagent for selection of bacteria-producing cellulases [142]. However, concession of a longer time for reaction of the dye with the medium could increase the visibility of hydrolysis zone, while the diameter of the halo can aid selection of strains possessing high polysaccharide degradation activity [136].

Based on these preliminary data, *A. niger* CECT 2915, *A. ibericus* MUM 03.49 and *A. uvarum* MUM 08.01 were selected for further studies.

3.2.2. Screening by SSF

In second screening step filamentous fungi selected in initial screening step were evaluated by cultivation under SSF during a period of 6 days using as solid substrate, the solid waste, COP and EOP. **Figure 9** shows the activities of cellulases and xylanases achieved. When COP was used as solid subtract, the activity for cellulases and xylanases was not verified. When EOP was used, *A. niger* achieved maximum activity for cellulases (38 U/g of solid subtract) and for xylanases (28 U/g of solid subtract).

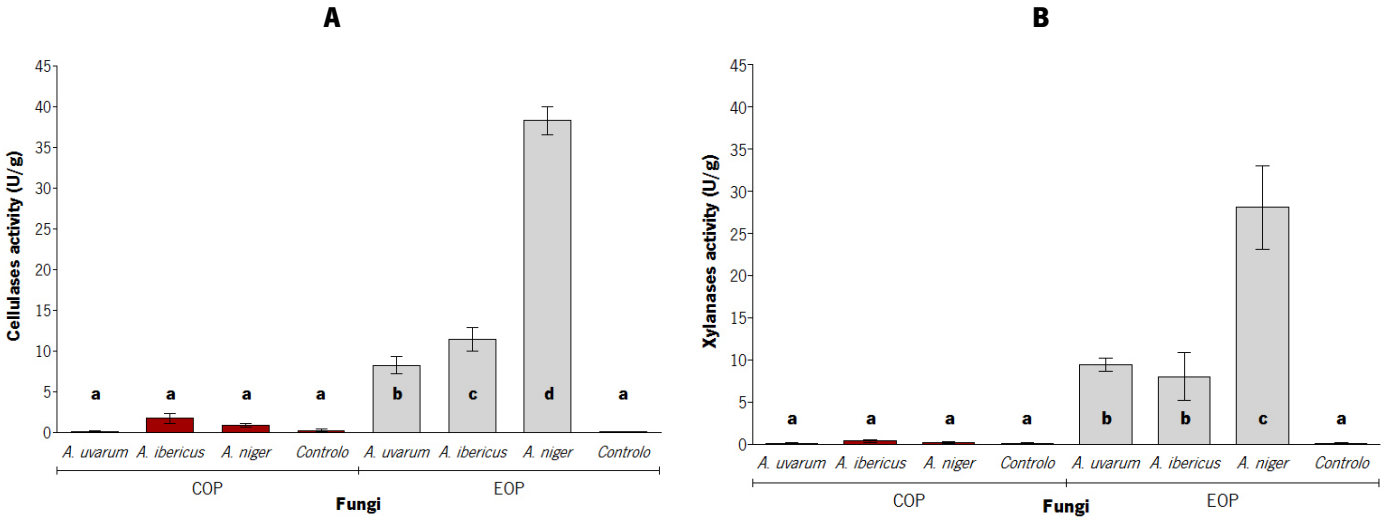

Figure 9 | Cellulases production (**A**) and xylanases' production (**B**) during SSF using COP (**red**) or EOP (**grey**) as solid subtract and the strains were screened in Congo red test. The results represent the average of two independent experiments and error bars represents SD.

These results were analysed by one-way ANOVA followed by a multiple comparison test, Tukey's Multiple Comparison Test. The differences observed between the cellulases activities for the *A. uvarum* on EOP, *A. ibericus* on EOP and *A. niger* on EOP were statistically significant (p-value < 0.0001). The differences observed between the xylanases activities for the *A. uvarum* on EOP, *A. ibericus* on EOP and *A. niger* on EOP were statistically significant (p-value < 0.0001), but differences statistically significant between *A. uvarum* on EOP and *A. ibericus* on EOP were not obtained.

The production of enzymes in these wastes was considerably different. A SSF with EOP as a solid subtract enhanced the production of cellulases and xylanases by any of the three studied strains.

The fact undetectable enzymatic activity in COP is probably caused by the presence of high initial concentration of fats and phenols with recognized antimicrobial properties in TPOMW, which delays the beginning of the fermentation and, on the other hand, to the high concentration of lignin, which needs specific enzymes and high temperatures to be degraded. Several authors, such as Baeta-Hall *et al.* (2005)[143], Alburquerque *et al.* (2006)[34], Cayuela *et al.* (2006)[144], Cayuela *et al.* (2010)[145] and Salgado *et al.* (2014)[131] also referred that in composting processes of TPOMW long composting periods are needed for this kind of material and concentrations of fats and phenols can inhibit growth of fungi. Kumar *et al.* (2009)[44] refers that enzymes produced by a variety of microorganisms are also capable of breaking down lignocellulosic materials to sugars but require longer retention times. In addition, the content of N in COP (0.6%) was lower than in EOP (1.27%). This difference causes an increase of C/N ratio of COP (83) versus C/N ratio of EOP (36) which it is closer to the optimum value for SSF.

Otherwise, the EOP was subjected to a second extraction. This process may have functioned as a pretreatment of the waste and the accessibility of fungi improved, reflecting on the increase of enzymes activities. Agreeing with the characterization physical-chemical (section 3.1.) this waste present half of the lipids content and the percentage of N is higher compared to COP. There are evidences in literature which showed as that solvents can improve the access of fungi to lignocellulosic material. The organosolv process involves simultaneous prehydrolysis and delignification of lignocellulosic biomass supported by organic solvents and, usually, dilute aqueous acid solutions [44]. An organic or aqueous organic solvent mixture with inorganic acid catalysts is used to break the internal lignin and hemicellulose bonds [146,147]. Swelling of lignocelluloses with water and polar solvents creates a very large internal surface area [148].

The sugars have an important role in SFF. In COP sugars concentration is very high so it may inhibit the lignocellulosic enzymes. In literature is reported that cellobiose, gentibiose at higher concentration inhibited about 80% of the ß-glucosidase activity and similarly, laminaribiose and glucose also led to a 55–60% inhibition in the enzymatic activity [149]. In EOP the sugar concentration may induce cellulases and xylanases production. Several investigations so far have indicated that cellulases are inducible enzymes. It is reported that cellobiose may act as an effective inducer of cellulases synthesis in *Nectria catalinensis* [150]. Xylanases biosynthesis was also induced by xylose or cellobiose added to the culture medium during growth [151].

EOP was selected to evaluate the effect of US on cellulases and xylanases production by *A. niger* in SSF.

In **figure 10** is represented the course of reducing sugar, proteins and phenols during a SSF by 6 days. It was observed a reduction of the concentration of reducing sugars in all of the fermentations comparatively to the control. This data can indicate the use of the free sugars in the medium for the growth of fungi.

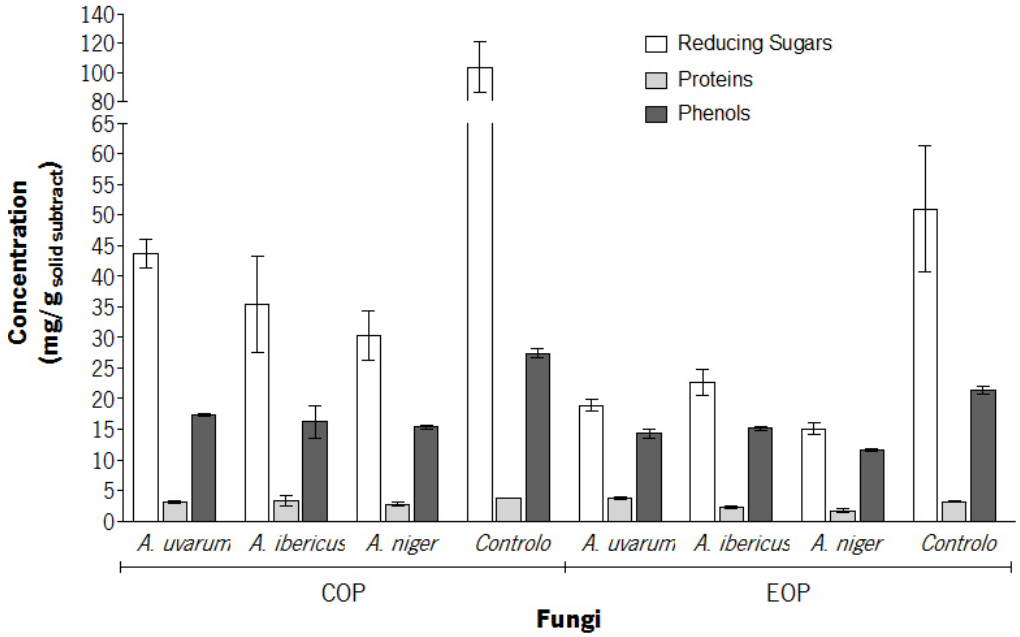

Figure 10 | Reducing sugars, proteins and phenols concentration in extract after SSF. The results represent the average of three independent measurements and error bars represents SD.

The phenol concentration has also reduced in all conditions comparatively with the control. Besides the phenolic compounds being toxic to microorganisms, the fungi have the capacity to degrade the phenol compounds present in wastes. It also shows that the phenols concentration has increased in both residues in comparison to the characterization of initial waste which showed 8.4 ± 0.3 mg/g of dry waste in COP and 8.9 ± 0.2 mg/g of dry waste EOP. In COP control there was 27.4 ± 0.9 mg/g of dry waste and in EOP 21 ± 1 mg/g of dry waste. The solid subtract was sterilized at 121 °C for 15 min before fermentation, and this step can function as a pretreatment, breaking the lignin bounds. A wide range of phenolic compounds is generated due to the lignin break down varying widely between different raw materials [152].

3.3. Ultrasound pretreatment

The recalcitrant structure of the biomass hinders the efficient hydrolysis [153]. A high content of lignin and the degree of binding of this component to the other organic constituents in lignocellulosic materials [154] may difficult the ability of microorganisms and their enzymes to degrade the residues. Due to the complex structure of the lignocellulosic biomass, a pretreatment becomes a prerequisite so that the enzymatic hydrolysis of the polysaccharides to fermentable sugars is performed effectively. The ultrasounds pretreatment is a physical treatment with a low environmental impact that can favour the accessibility of fungi to hemicellulose and cellulose fraction.

EOP was selected to study the effect of US. The L:S ratio and sonication time were optimized using a full factorial design 3^2 to maximize the production of cellulases or xylanases.

Table 6 describes the corresponding experimental matrix and the results obtained. The range of variation of L:S ration was set from 3 to 11 and the sonication time varied from 5 to 15 min. The two responses studied were cellulases and xylanases activities per mass of dry solid subtract (U/g). According to the results of eleven SSF, the cellulases activity varied from 13.38 to 37.88 U/g and xylanases activity varied from 7.53 to 70.86 U/g.

The predict response by the model (Y) and experimental data observed in experiments (y) are represented in **figure 11** and it shows that no significant differences between the predicted model and observed data were found.

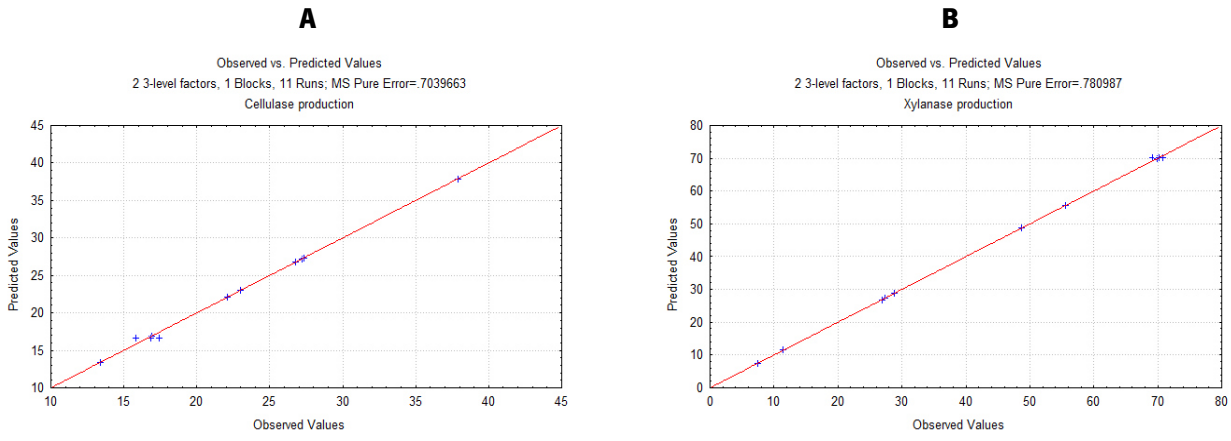

Figure 11 | Relation between observed and predicted values in cellulases (**A**) and xylanases (**B**) production response.

Table 6 | Response variables obtained according to the studied full factorial 3^2 design to optimize US.

Runs	Independent variables		Dependent variables			
			Cellulases production (γ_1)		*Xylanases production (γ_2)*	
	Real levels		observed	predicted	observed	predicted
	χ_1 (Time, min)	χ_2 (L:S ratio)	γ_1 (U/g)	Y_1 (U/g)	γ_2 (U/g)	Y_2 (U/g)
1	5	3	37.88 ± 4.47	37.88	27.35 ± 5.84	27.35
2	5	7	27.18 ± 4.75	27.18	28.84 ± 2.34	28.84
3	5	11	13.38 ± 3.33	13.38	7.53 ± 1.92	7.53
4	10	3	22.10 ± 6.78	22.10	11.46 ± 1.06	11.46
5	10	7	15.80 ± 3.88	16.70	69.11 ± 3.89	70.05
6	10	11	16.91 ± 2.39	16.91	26.88 ± 3.27	26.88
7	15	3	27.34 ± 5.71	27.34	55.52 ± 4.99	55.52
8	15	7	26.74 ± 2.03	26.74	69.86 ± 3.17	69.86
9	15	11	23.02 ± 7.91	23.02	48.64 ± 3.67	48.64
10	10	7	17.46 ± 1.33	16.70	70.86 ± 8.83	70.05
11	10	7	16.85 ± 0.66	16.70	70.17 ± 3.87	70.05

χ_1: time (uncoded); χ_2: L:S ratio

γ_1: cellulases activity; γ_2: xylanases activity

The statistical analysis of the results yielded an empirical coded model for cellulases and xylanases activity as a function of the sonication time and L:S ratio. **Table 7** lists the regression coefficients, their statistical significance (based on a t test, with significance levels of α= 0.05) and the statistical parameters, such as, F value, determination coefficient (R^2) and significance level.

R^2 is the coefficient of determination and it is used to measure goodness of fit. The values of R^2 were found equal to 0.9972 to cellulases activity and 0.9997 to xylanases activity. The values of R^2 were close to 1 showing a good fitting of the data to the model and indicating that 99% of the variability in the response could be explained by the model.

Table 7 | Regression coefficients and correlation and statistical significance parameters of experimental design for cellulases and xylanases production.

	Regression coefficients	Standard error	t	P
Cellulases production				
b_0	16.70***	0.4844	34.4761	0.0008
b_1	-0.22	0.5933	-0.3742	0.7442
b_{11}	10.26***	0.7659	13.3947	0.0055
b_2	-2.60**	0.5933	-4.3765	0.0484
b_{22}	2.80*	0.7659	3.6568	0.0673
b_{12}	5.04***	0.4195	12.0223	0.0068
b_{112}	0.00	0.7266	-0.0055	0.9961
b_{122}	-4.61**	0.7266	-6.3383	0.0240
b_{1122}	-4.3*	1.0558	-4.1272	0.0540
Xylanases production				
b_0	70.05***	0.5102	137.2827	0.0001
b_1	20.51***	0.6249	32.8247	0.0009
b_{11}	-20.69***	0.8067	-25.6515	0.0015
b_2	7.71***	0.6249	12.3365	0.0065
b_{22}	-50.87***	0.8067	-63.0603	0.0003
b_{12}	3.23**	0.4419	7.3195	0.0182
b_{112}	-3.19*	0.7653	-4.1723	0.0529
b_{122}	-14.38***	0.7653	-18.7940	0.0028
b_{1122}	36.28***	1.1120	32.6281	0.0009

Correlation and statistical significance parameters

	R	R^2	R^2 Adjusted	F_{exp}	P
y_1	0.9986	0.9972	0.9863	91.3837	0.0108
y_2	0.9998	0.9997	0.9987	967.7274	0.0010

*significant coefficient at 90%; **significant coefficient at 95%; ***significant coefficient at 99%

P: probability; R: multiple correlation coefficient; R^2: determination coefficient. $\alpha = 0.05$

γ_1: cellulases activity ; γ_2: xylanases activity .

The interaction of the independent variables and the optimum levels that have the most significant effect on dependent variable can be determined through the three-dimensional response surface curve plot. **Figure 12** shows the effect of sonication time and L:S ratio on cellulases and xylanases production.

As it can be observed, the US had a positive effect in xylanases production, however the cellulases activity decreased after US. Yang *et al.* (2012) [155] observed that exists positive effect on xylanases production by *A. japonicus* using rice hull as solid subtract in SSF with ultrasonic treatment. This positive effect of ultrasounds pretreatment of lignocellulosic materials could improve the accessibility of fungi to hemicellulose fraction and to induce the xylanases production. The formation of microbubbles during sonication improves diffusivity or mass transfer processes [156]. Salgado *et al.* (2013) [157] also observed that *A. niger* was more effective in producing hemicellulose enzymes such as endoxylanases, whereas, maximum endocellulases production was achieved by *A. uvarum*.

The optimal conditions that led to maximum xylanases activity (75.32 U/g) were calculated with Solver tool showing 12.41 min and 7.27 of liquid and solid ratio as optimal parameters of US.

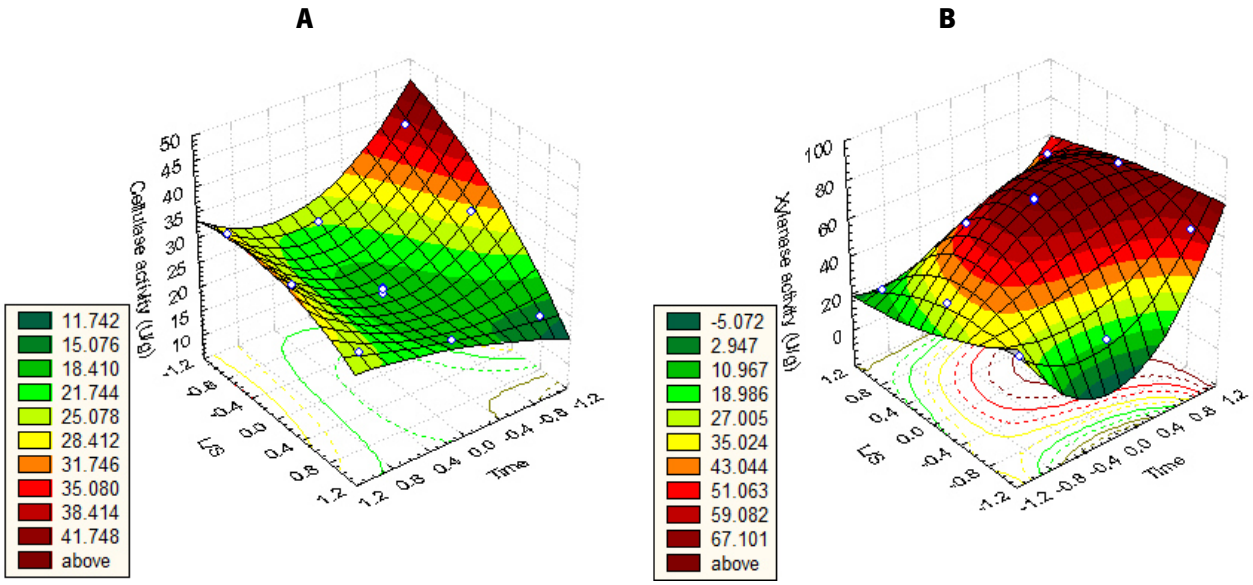

Figure 12 | Response surface for cellulases (**A**) and xylanases (**B**) production as a function of the time (coded) and L:S ratio (coded).

To validate the model an experiment was performed in the optimal conditions (**figure 13**). The confirmatory experiments carried out using the predicted conditions showed similar activities between experimental (71.21 ± 8.37 U/g) and predicted value.

To verify if the increased sonication time would affect the cellulases and xylanases production, two assays were carried out with the higher sonication times **(figure 13)**. The L:S ratio was maintained the optimal and the times were 20 min and 30 min. The results obtained showed that higher sonication times do not benefit the production of xylanases, but cellulases activity does not seem to change.

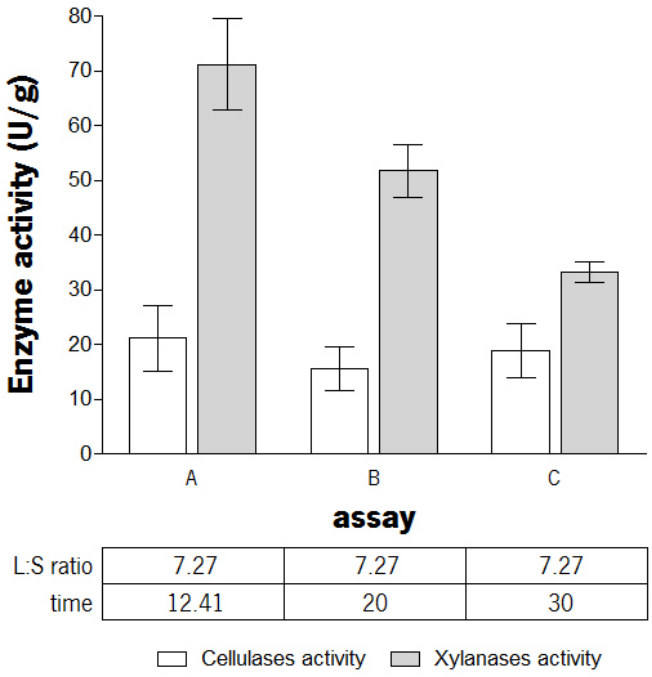

Figure 13 | Cellulases and xylanases' production during SSF using pomace treated by US. In the several assays were studied the sonication time differently and the L:S ratio was of the optimal operational condition previously optimized in experimental design. The results represent the average of two independent experiments and error bars represents SD.

There are minor researches on US from lignocellulose, but some researches have study this pretreatment. Yachmenev *et al.* (2009) [158] showed that saccharification of cellulose is enhanced efficiently by US and that the maximum effects of cavitation occur at 50°C, which is the optimum temperature for many enzymes. The effect of ultrasounds on lignocellulosic biomass have been mainly employed for extracting hemicellulose, cellulose and lignin. The extractability of the wheat straw hemicelluloses was investigated using extraction method with application of ultrasonic

irradiation in 0.5 M KOH aqueous solution. The results showed that ultrasonically assisted extraction in a period of 20–35 min produced a slightly higher yield of hemicelluloses and lignin [159]. Braguglia *et al.* (2011) [160] studied the ultrasound technology in sewage sludge treatment before anaerobic digestion with the aim to stimulate the conversion of organic matter into biogas. The shear forces generated by high-pressure waves lead the release of intracellular substances into the aqueous phase, making them available for the subsequent anaerobic digestion.

Figure 14 shows the concentration of reducing sugars that remained available in the liquid where the pretreatment occurred (known as filtrate) and in waste without pretreatment.

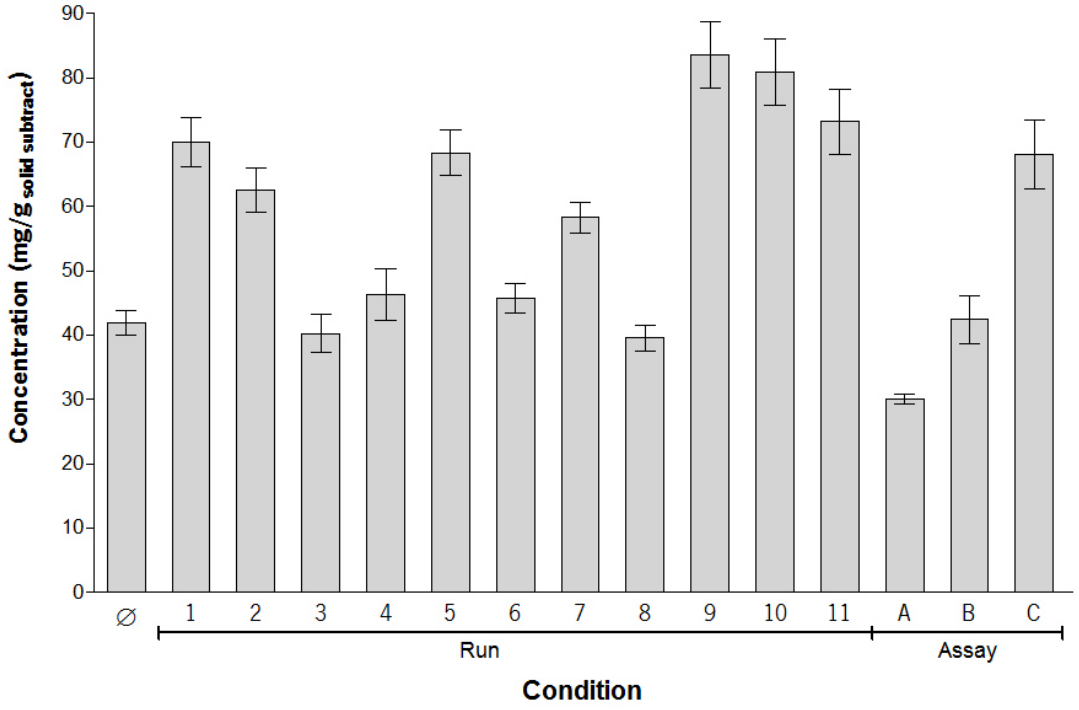

Figure 14 | Reducing sugars concentration in EOP without treatment (control) and filtrate after pretreatment. The results represent the average of three independent measurements and error bars represents SD.

After the pretreatment a great quantity of sugars is released to the reactional mean (between 30.0 ± 0.8 e 73 ± 5 mg/g solid subtract). During the pretreatment of EOP with ultrasounds the mechanical impacts, produced by the collapse of cavitation bubbles in the lignocellulosic biomass, led to the release of the polysaccharides, particularly for extracting low molecular weight substances to the mean. Sun *et al.* (2004) [161] reported a positive effect of ultrasound on the extractability of polysaccharides from corn bran, buckwheat hulls, and Salvia officinalis L. This

author also showed that ultra-sonication attacked the disruption of the cell walls and cleavage of the α-ether linkages between lignin and hemicelluloses. Consequently, the accessibility, solubility, and diffusion of the dissolved hemicelluloses and lignin from the cell walls increased.

The free sugars that were extracted during the pretreatment were lost, because the reactional mixture after the pretreatment was filtrated, and liquid fraction was not used in SSF. This data may explain the low cellulases activity. The fact that there is a low concentration of free sugars in the mean, it takes to a first action of the xylanases which degrades the hemicellulose, because the cellulases are inducible enzymes [151]. According to Sun *et al.* (2002) [162] the presence of lignin and hemicellulose makes the access of cellulases enzymes to cellulose difficult, thus reducing the efficiency of the hydrolysis.

3.4. Evaluation of other pretreatment

Acid hydrolysis with diluted acid pretreatment has received considerable research attention over the years [52,54]. The main objective of this treatment is to solubilize the hemicellulosic fraction of the biomass and to make the cellulose more accessible to enzymes [152].

The course of cellulases and xylanases was studied when the EOP was subjected to AH comparing it with the US. It was also studied a combination of both pretreatments to understand the course of the lignocellulosic enzymes.

The profile of cellulases and xylanases production is represented in **figure 15**. The data show that AH or US+AH did not enhance the enzyme activity. The US in the optimal conditions, L:S ratio of the 7.27 and sonication time of the 12.41 min is, still, the most effective pretreatment in the production of the enzymes in study.

It is reported in literature that dilute-acid at moderate temperatures effectively removes and recovers most of the hemicellulose as dissolved sugars, and glucose yields from cellulose increase with hemicellulose removal to almost 100% for complete hemicellulose hydrolysis [163]. Other authors suggest that although little lignin is dissolved, the lignin is disrupted, increasing cellulose susceptibility to enzymes [62] and that high temperature in dilute acid treatment is favourable for cellulose hydrolysis [55]. According to Zhao *et al.* (2008) [70] the poor delignification ability of sulfuric acid is still a limit of mild acid pretreatment, because lignin is believed to be a major hindrance in enzymatic hydrolysis. Hemicellulose can be readily hydrolysed by dilute acids under moderate conditions, but much more extreme conditions are needed for cellulose hydrolysis [44].

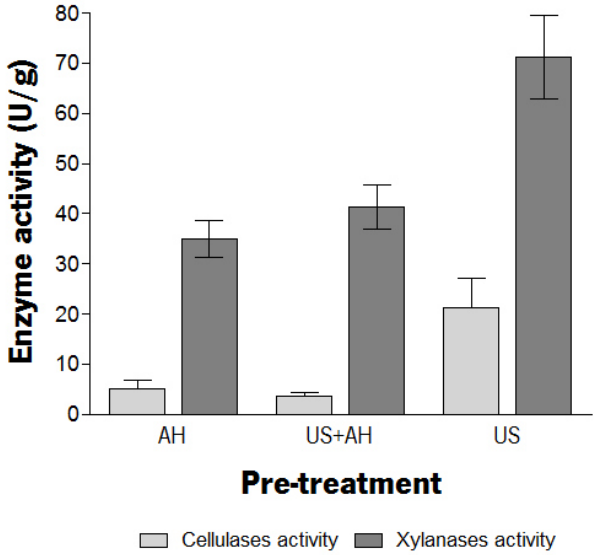

Figure 15 | Cellulases and xylanases' production during SSF using pomace treated by acid hydrolysis with diluted acid (AH), ultrasound combined acid hydrolysis with diluted acid (US+AH) and ultrasound pretreatment (US). The results represent the average of two independent experiments and error bars represents SD.

Besides this pretreatment having the advantage of solubilizing hemicellulose, mainly xylan, and converting solubilized hemicellulose into fermentable sugars, nevertheless, depending on the process temperature, some sugar degradation compounds are detected, and affect the microorganism metabolism in the fermentation step [152]. A variety of toxic compounds such as furfural and aromatic lignin degradation compounds, would be generated. In most cases, these by-products, known as inhibitors, exert negative effects on the growth, metabolism, and product formation of microbial cells [152,164]. Alvira *et al.* (2010) [152] showed that organic acids can pretreat wheat straw with high efficiency and furthermore, less amount of furfural was formed in the maleic and fumaric acid pretreatments than with sulfuric acid.

Table 8 shows the variation of the compounds (reducing sugars, proteins and phenols) in filtrate and waste after pretreatment and in the extract and waste after fermentation for the three pretreatments in study. A general analysis of the results shows that most of the compounds remains in the filtrate, after the pretreatment and in the extract, after fermentation.

Facing the sugars course, it should be noted the data obtained in the filtrate that corroborate the literature. In the end of AH, the concentration of sugars was 311 ± 19 mg/g solid subtract. In literature it is described that the acid pretreatment is effective for converting cellulose and hemicelluloses into monomeric sugars [165]. In filtrate of the US+AH the sugars concentration was

slightly lowest. This waste was subjected to two pretreatment, US followed by AH, and two filtrations. There was sugar loss in two steps, impoverishing the waste and affecting this way the fermentation.

Table 8 | Reducing sugars, proteins and phenols concentration in initial waste, filtrate and treated waste after pretreatment, extract and treated and fermented waste after SSF. The results represent the average of three independent measurements ± SD.

	Initial waste*	after pretreatment		after SSF	
		Filtrate	Treated waste*	Extract	Fermented and treated waste*
Reducing Sugars (mg/g $_{\text{solid subtract}}$)					
AH		311 ± 19	2.53 ± 0.03	3.0 ± 0.5	1.4 ± 0.1
US+AH	42 ± 2	287 ± 0	2.42 ± 0.01	2.8 ± 0.3	1.2 ± 0.3
US		38.4 ± 0.5	7.9 ± 0.3	4.7 ± 0.2	1.0 ± 0.2
Proteins (mg/g $_{\text{solid subtract}}$)					
AH		1.0 ± 0.1	n.d.	1.3 ± 0.8	n.d.
US+AH	2.6 ± 0.3	3.2 ± 0.1	n.d.	1.0 ± 0.4	n.d.
US		1.5 ± 0.2	0.59 ± 0.03	3.7 ± 0.5	0.018 ± 0.004
Phenols (mg/g $_{\text{solid subtract}}$)					
AH		27 ± 1	1.33 ± 0.02	2.8 ± 0.5	1.38 ± 0.07
US+AH	8.9 ± 0.2	17.2 ± 0.6	1.23 ± 0.01	2.9 ± 0.1	1.5 ± 0.2
US		11.8 ± 0.3	4.37 ± 0.08	4.8 ± 0.2	1.58 ± 0.06

*extraction with water 1:5 (w/v)

For proteins it is possible to observe an increase after the fermentation. The fermentation with the waste treated with ultrasounds presented the highest proteins concentration (3.7 ± 0.5 mg/g solid subtract) and also achieved more enzymatic production.

Analysing phenol compounds, the highest concentration was verified in the filtrate after the pretreatment. As it was already referred, the pretreatment increases the phenols concentration due to the breaks in the lignin. The filtrate may be a source of extracting the phenol compounds. Phenolic extracts from OMW can be used as alternatives to synthetic antioxidants in order to increase the stability of foods by preventing lipid peroxidation, and protect living systems from oxidative damage by scavenging oxygen radicals [22].

The effect of several pretreatment in the removal of lignocellulosic compounds is shown in in **table 9** and was set the percentage of elimination for each compound after each treatment.

When the residue is treated with diluted acid, there is a high percentage of elimination of hemicellulose, 78% in AH and 76% in US+AH. However, the elimination of lignin and cellulose were much reduced. In literature is referred the possibility of the almost 100% hemicellulose removal by AH but this pretreatment is not effective in dissolving lignin [42,52].

Table 9 | Lignin, hemicellulose and cellulose percentage in treated waste by AH, US+AH and US. Eliminated percentage of the lignin, hemicellulose and cellulose after pretreatments. The results represent the average of three independent measurements ± SD.

	Lignin (%)	**Hemicellulose (%)**	**Cellulose (%)**
Initial waste	41.620 ± 0.04	24.1 ± 0.2	11 ± 2
Treated waste			
AH	56.0 ± 0.2	7.9 ± 0.6	9.2 ± 0.1
US+AH	61 ± 3	9.7 ± 0.3	15.50 ± 0.01
US	38.5 ± 0.7	19 ± 1	9.4 ± 0.7
Eliminated (%)			
AH	10	78	3
US+AH	14	76	18
US	24	30	25

The eliminated percentage of the lignocellulosic material in US was reduced and practically the same for all the compounds. Its reported that the ultrasound pretreatment in distilled water was effective and gave a 7.37% reduction in Klason lignin of wheat straw compared with the untreated wheat straw [166]. Salgado *et al.* (2014) [167] observed that SSF of the olive pomace and exhausted grape marc by *A. niger* reduced cellulose, hemicellulose and lignin content of substrate by 28.08, 10.78 and 13.3%.

3.5. Study of enzymes production kinetics

Cellulases and xylanases production during SSF was evaluated in the optimal conditions of US. In this study, the course of cellulases and xylanases activities during several days were evaluated and their values were represented in **figure 16A**. The highest xylanases activity (75 ±

1 U/g solid substrate) was achieved after 7 days of fermentation, which is similar to the theoretical value predicted by the model of US. After this, the activity decreased, maybe due to proteolysis. Salgado *et al.* (2015) [167] refers that low proteolytic activity can be an advantage when developing SSF processes, may avoid inactivation of other secreted enzymes and benefit the accumulation of those in the substrate, improving production yields. Biesebeke *et al.* (2002) [168] identified two proteases that are only expressed in SSF culture.

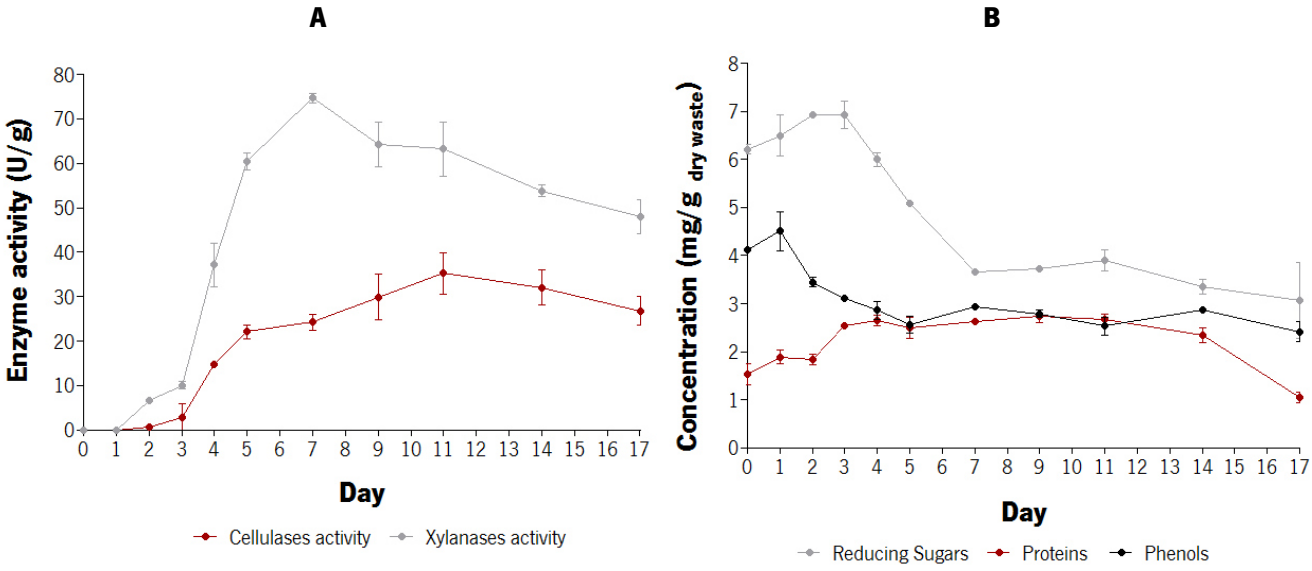

Figure 16 | Evaluation of the behaviour over time of cellulases and xylanases (**A**), just like reducing sugars, proteins and phenols contents (**B**) during SSF in the optimal operational condition of the US. The results represent the average of two independent experiments (**A**) or three independent measurements (**B**) and error bars represents SD.

The maximum cellulases activity (35 ± 5 U/g solid subtract) was achieved after 11 days.

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 [157]. According to Kumar (2008) the rapid and efficient fermentation of hydrolysates is limited because a range of inhibitory compounds in addition to monomeric sugars is generated during the hydrolysis of lignocellulosic.

Kavya *et al.* (2009) [169] observed a maximum xylanases production (12.65 U/ml) by *A. niger* in wheat bran after 6 days of incubation period. Xu *et al.* (2008) [170] achieved maximum activity at 48h. Christopher *et al.* 2005 [124] observed a maximum xylanases production by *Thermomyces*

lanuginosus after 4 days. *Park et al.* (2002) [171] also observed under the optimized conditions, the xylanases production by *A. niger* obtained after 5 days of fermentation were 5.071 IU/g of rice straw.

The cellulases carried out the enzymatic hydrolysis in the three steps: adsorption of cellulases enzymes onto the surface of the cellulose, the biodegradation of cellulose to fermentable sugars, and desorption of cellulases. Cellulases activity decreases during the hydrolysis. The irreversible adsorption of cellulases on cellulose is partially responsible for this deactivation [52,172]. The cellulases activity can be inhibited by cellobiose and to a lesser extent by glucose [173,174]. Inhibition of the cellulases by hemicellulose-derived sugars has also been shown [175].

At the start and at each time of SSF, the reducing sugars, proteins and phenols concentration were analysed to follow the change of its composition waste (**figure 16B**).

Sugars concentration is decreased rapidly after 3 days of fermentation. Protein concentration increased relative to the start of fermentation during 11 days. Phenol concentration seemed to increase slightly. According to Giannoutsou *et al.* (2012) this slight increase may be the result of the degradation of polyphenols to smaller phenolic compounds that may be an indication that the microorganism could use the specific substrate for its growth.

Salgado *et al.* (2015) also studied the course of the compounds over time of the fermentation and proved the depletion of the sugars, the increase of the proteins percentage from 8.47% to 17.98% after 6 days of fermentation and degradation of phenol compounds, the fungus reduced 28.32% of phenols after 10 days of fermentation.

The existence of nutrients in fermentation may have contributed to the growth of the microorganism, and the decay of these nutrients over time may have affected to the activity of enzyme, and it was the decay of the microbial production and therefore the enzyme production [96].

Enzymes usually have an expression control mechanism that can be inhibited or stimulated by products of the medium. The end products of a particular metabolic pathway are often inhibitors of enzymes that catalyse the initial steps of the pathway [176]. Biazus *et al.* (2006) worked with corn malt and observed that in the production of enzymes the beginning is slow, then accelerates until it reaches its maximum value; thereafter, the concentration of products generated are inhibited and its activity is reduced, which was also observed. The decrease in activity with increasing incubation time may be due to the production of by products resulting from microbial metabolism, as well as nutrient depletion, inhibiting fungal growth and enzyme formation [177].

Figure 17 shows pH course through fermentation. The values of the pH vary between 3.84 and 4.57. This behaviour corroborates the described in literature. The optimal pH of the xylanases and cellulases have been studied by several authors. Pérez *et al.* (2002) [20] reported that most xylanases from fungi have pH optima between 4.5 and 5.5. Kumar *et al.* (2008) [151] refers that the effect of pH on cellulases production was analysed using *A. niger*, and was observed that pH 5.5 was optimal for maximum cellulases production. Other author also reported that maximum adsorption of cellulases from *A. phoenicus* occurred at pH of 4.8 – 5.5 [178].

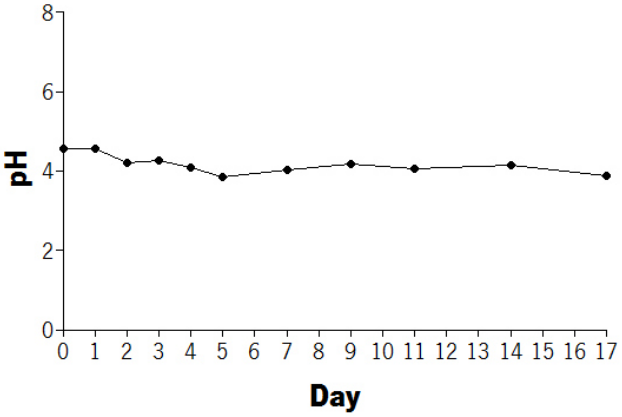

Figure 17 | Evaluation of the behaviour over time of pH during SSF in the optimal operational condition of the US.

3.6. Effects of nutrients

3.6.1. Low cost nutrients

Vinasses can be a good source of N and minerals, and their use as a nutritional supplement for submerged fermentations has already been tested [179]. Urea is an organic nitrogen source widely used in biotechnology processes for its low cost.

It was tested the production of lignocellulosic enzymes in SSF supplemented with low cost nutrients solution, 0.04 $g_{urea}/g_{subtract}$ and 7.5 mL vinasses. The conditions used in the pretreatment were the optimal and the enzyme activities obtained for cellulases and xylanases were 10 ± 1 and 19 ± 1 U/g solid subtract, respectively (**table 10**). The data suggests that the addition of the low cost nutrients are not a viable alternative to obtain good enzymatic activities of the enzymes in this study.

Table 10 | Cellulases and xylanases' production during SSF using low cost nutrients. The results represent the average of two independent experiments ± SD and the "commercial nutrients" (optimal conditions of experimental design) was used as a comparison.

	Time (min)	L:S ratio	Enzymes production	
			Cellulases (U/g)	Xylanases (U/g)
Low cost nutrients	12.41	7.27	10 ± 1	19 ± 1
Commercial nutrients	12.41	7.27	21 ± 6	71 ± 8

Salgado *et al.* (2013) [180] also studied the supplementation of TPOWM with others agro-industrial by-products. According to this author, the endoxylanases production (3.06 U/g of dry substrate) benefited from a strong effect of urea addition.

3.6.2. Importance of nutrients

In SSF, the solid material can serve as a physical support and as a source of carbon and Nutrients to sustain microbial growth or only as an inert physical support to which nutrients and the carbon source are added [87].

In order to prove how addition of nutrients is important to cellulases and xylanases production a fermentation without nutrients was carried out. The liquid of pretreatment was used in the fermentation for the humidity correction instead of the nutrients solution. **Table 11** shows the values obtained for production of enzymes, cellulases and xylanases, which were nearly zero. As can be seen, the TPOWM, by itself does not function as a source of nutrients and a support for the growth of microorganisms.

Table 11 | Cellulases and xylanases' production during SSF using the liquid of the pretreatment to adjusted the humidity in fermentation. The results represent the average of two independent experiments ± SD and the "with nutrients" (Run 1 of experimental design) was used as a comparison.

	Time (min)	L:S ratio	Enzymes production	
			Cellulases (U/g)	Xylanases (U/g)
Without nutrients	5	3	1.1 ± 0.4	0.2 ± 0.1
With nutrients	5	3	29 ± 4	27 ± 6

3.6.3. Importance of liquid fraction of ultrasound pretreatment

To confirm that the free sugars really induce the production of lignocellulolytic enzymes and that the compounds that are released to the filtrate after ultrasounds pretreatment favour the SSF. In the previous studies, this filtrate was not used and the moisture adjust, after ultrasounds treatment, was performed with nutrients solution. In the new strategy to improve cellulases and xylanases production, the ratio L:S of ultrasounds treatment was adjust with nutrients solution and after treatment the moisture adjust of solid to carried out the SSF (75%) was performed with liquid fraction of ultrasounds pretreatment. Therefore, two tests with different L:S ratio and sonication time were done and compared with the obtained enzymatic activity in the same conditions of the experimental design of the US (section 3.3.).

For the pretreatment of 5 min and an L:S ratio of 3 the enzyme activities for cellulases and xylanases were 47 ± 6 and 32 ± 2 U/g solid substrate, respectively (**table 12**). The enzyme activities obtained in previous strategy (Run 1 of the experimental design) were 29 ± 4 and 27 ± 6 solid subtract, showing an increase in both enzymes production.

The effect of the filtrate added was also tested in the optimal condition of the experimental design and obtained enzymatic activities of 46 ± 3 and 100 ± 15 U/g solid substrate for cellulases and xylanases, respectively (**table 12**). 40.8% increase of xylanases activity and 119% increase of cellulases activity were obtained compared to the initial activity.

Table 12 | Cellulases and xylanases' production during SSF using the nutrients solution as a liquid by carry out US and the liquid of the pretreatment was used to adjust the humidity in fermentation. The results represent the average of two independent experiments ± SD and the Run 1 and optimal conditions was used as a comparison.

	Time (min)	L:S ratio	Enzymes production	
			Cellulases (U/g)	Xylanases (U/g)
With filtrate	5	3	47 ± 6	32 ± 2
Without filtrate	5	3	29 ± 4	27 ± 6
With filtrate	12.41	7.27	46 ± 3	100 ± 15
Without filtrate	12.41	7.27	21 ± 6	71 ± 8

In both tests it was possible to verify that the addition of the filtrate, from the ultrasound pretreatment, in fermentation induces the production of lignocellulosic enzymes. In literature it is indicated that various soluble sugars like glucose, xylose, arabinose, galactose, which are helpful for the initiation of growth and replication of microorganisms and that are used firstly all sugars that were available for its growth [181].

Chapter 4
Conclusion

4| CONCLUSION

In this work the use of olive mill wastes in SFF for cellulases and xylanases production was evaluated analysing the effect of ultrasounds pretreatment of olive pomace, the low cost substrate used.

Two wastes from olive mills were studied, COP and EOP. The COP is rich in sugars and lipids and EOP presented intermediate sugars concentration and is poor in lipids due to its residual oil removal by extraction. Both wastes have high concentration of phenols.

A screening of several fungi was carried out to observe their ability to produce cellulases. *A. ibericus* MUM 03.49, *A. niger* CECT 2915, and *A. uvarum* MUM 08.01 were selected as the most efficient cellulases-producing microorganisms by Congo red test. In addition, the selected fungi were tested in SSF of COP and EOP seeing that the production of enzymes in both wastes was considerably different. A SSF with EOP as a solid subtract enhanced the production of cellulases and xylanases by any of the three studied strains. The maximum cellulase (38 U/g of solid subtract) and xylanase (28 U/g of solid subtract) activities were achieved using EOP as solid substrate by *A. niger*.

The ultrasounds pretreatment was optimized by full factorial design 3^2. The optimal conditions were 12.41 min and a liquid and solid ratio of 7.27. Ultrasounds treatment of EOP improved xylanase production by SSF, however it showed a negative effect on cellulase production. Thus the sonication can be an effective treatment to induce the production of xylanases by SSF and the optimal conditions led to maximum xylanases activity (75.32 U/g). Other treatments were studied, acid hydrolysis with diluted acid and sonication in combination with diluted acid, but these treatments did not to improve cellulases and xylanases production. Thus a clean treatment such as ultrasounds achieved best results that other treatment with high environmental impact as acid hydrolysis.

Kinetics of enzymes activities was monitored and the highest xylanases activity (75 ± 1 U/g solid substrate) was achieved after 7 days of fermentation and the maximum of cellulases activity (35 ± 5 U/g solid subtract) was achieved after 11 days. These results showed that the production of xylanases requires shorts periods and production of cellulases needs more time.

The data suggests that the replacement of commercial nutrients (yeast extract, peptone and KH_2PO_4) by the low cost nutrients (urea and vinasses) are not a viable alternative to obtain good enzymatic activities of the enzymes in EOP treated with ultrasounds. The addition of the nutrients is very important for the cellulases and xylanases production in SSF with EOP.

Finally, the liquid fraction obtained after the ultrasound pretreatment that was not initially used in SSF was added to solid pretreated and used in SSF. The addition of filtrate to solid pretreated showed a positive effect in xylanase and cellulase production by SSF. This liquid is rich in free sugars that can induce the enzymes production.

This study clearly improved the production of cellulases and xylanases under SSF of EOP through fungi selection, optimization of ultrasounds treatment and addition of filtrate from ultrasounds treatment. The xylanase production was increased from 28 U/g (initial SSF) to 100 U/g and the cellulase production 38 U/g (initial SSF) to 46 U/g. This friendly environment treatment was a suitable process to enhance the valorisation of olive mill wastes by improving the enzyme production.

In a future research, it would be interesting that we focus on the recovery of phenolic compounds from filtrate obtained after ultrasounds treatment, purification and characterization of enzymes produced and to search another applications for fermented solid after enzymes extraction.

Chapter 5

References

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