Research Article

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Mediterranean agro-industrial wastes as valuable substrates for lignocellulolytic enzymes and protein production by solid-state fermentation

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Abstract

BACKGROUND: Mediterranean agro-food industries (such as wineries, breweries and olive mills) dispose of great amounts of waste. This generates environmental problems, and the waste has a low nutritional value for use as animal feed. In this sense, solid-state fermentation (SSF) can increase the nutritional value of these wastes and simultaneously produce lignocellulolytic enzymes.

RESULTS: All fermented wastes were enriched in protein by the three fungi studied. *Aspergillus ibericus* was the fungus with the biggest increase of protein, which ranged from 1.4 times to 6.2 times with respect to unfermented wastes. Likewise, *A. ibericus* achieved the maximum cellulase and xylanase activities. The relationships among substrates composition, fungi used and SSF performance were evaluated by principal components analysis. The high content of cellulose and hemicellulose favoured lignocellulolytic enzymes production, and the phenolics content was negatively correlated with enzymes production and with the increase of protein by SSF. Furthermore, the scanning electron microscopy analysis showed the growth of fungi over solid wastes, the formation of conidiophores and the changes in their structures.

CONCLUSION: The nutritional value of Mediterranean wastes was improved and other value-added products such as lignocellulolytic enzymes were produced in the same process, which could facilitate the efficient reuse of these wastes.

Keywords: Mediterranean wastes; solid-state fermentation; lignocellulolytic enzymes; scanning electron microscopy

INTRODUCTION

Presently, there is a growing interest in the exploitation of wastes generated by the food industry. This is key to a circular economy, which is an alternative to a traditional linear economy, and it searches to use the resources for as long as possible, to extract the maximum value from them, and then to recover and reuse the wastes. Agro-food industries from the Mediterranean region are dominated by production of olive oil and wine. According to the Food and Agriculture Organization Corporate Statistical Database (FAOSTAT), in 2014 the production of virgin olive oil and wine was 93% and 51% of world production respectively. In terms of quantity in tonnes, beer from barley is the third processed crop in the Mediterranean region (FAOSTAT, 2014). The three products sum to about 27×10^6 t in the Mediterranean region, which can generate in their production about 9×10^6 t of solid wastes each year. Thus, to search for applications for these wastes that are more profitable and environmentally friendly must be a permanent priority.

In this sense, the utilization of agro-food wastes has been proposed for animal feed. Wastes utilization as an alternative source for animal feed needs huge attention because their recycling and reduction are good ways to minimize environmental pollution and improve the present living conditions.[1] Currently, 33% of croplands are used for livestock feed production, which is a key factor in deforestation. By using agro-industrial wastes as animal feed, food supply can increase and reduce the environmental impact of these wastes. In addition, the price of cereals has increased in recent years, and so alternative sources of feedstuffs should be considered in order to reduce production costs.[2] However, the nutritional quality of these wastes does not meet the standards that are required for animal feed, mainly due to low protein content. In this sense, olive mills and wineries wastes such as olive pomace, grape marc and vine-shoot trimmings (VST) are noted for their low nitrogen (N) content of between 0.6 and 1.7% of dry solids.[3] On the other hand, brewers' spent grain (BSG) has a higher N content.[4] The mixture of wastes and the application of biotechnology processes such as solid-state fermentation (SSF) can enrich them for use directly in animal feeds.[5] SSF is considered an alternative technology to obtain new products and use wastes as cheap raw material.[6]

In recent years there has been further focus on improving the protein content of lignocellulosic wastes by SSF.[1] Bioprocessing of

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wastes by SSF is a low-cost technique to obtain nutritive animal feed.[7] Several workers have demonstrated the protein enhancement of agro-food wastes by fungi of around 5–26%.[8–10] On the other hand, lignocellulosic wastes are considered as being of low digestibility,[11] but SSF by fungi can improve the digestibility by enzymes production, since these can break the bonds of hemicellulose and cellulose with the lignin. In addition, the SSF of lignocellulosic materials can increase the degradability of dietary protein in the rumen.[12] Thus, several nutritional aspects of agro-food wastes can be improved in the same biotechnology process, increasing the value of wastes.

Fungi comply with several of these aspects as they have high protein content, are safe for human and animals and have the ability to grow in agro-industrial wastes.[10] *Aspergillus niger* is a filamentous fungus considered as generally regarded as safe by the US Food and Drug Administration. *Aspergillus ibericus* and *Aspergillus uvarum* are species isolates from grapes unable produce mycotoxins.[13] Their ability to colonize agro-industrial wastes has been demonstrated.[3] However, the potentiality of *A. ibericus* and *A. uvarum* to increase protein content of agro-industrial wastes was barely studied.

This study was aimed to increase of nutritional value of winery, brewery and olive mill wastes and produce lignocellulolytic enzymes simultaneously. Three fungi were tested in four different wastes. The effect of fungi growth on lignocellulose composition and phenolic compounds was evaluated. In addition, the relationship between substrate composition, fungi used and the results of SSF was studied. Finally, the structure of unfermented and fermented solid wastes was analysed by scanning electron microscopy (SEM) to show the changes produced by fungi.

EXPERIMENTAL

Agro-industrial wastes

During this work, four wastes were used: Brewers' spent grain (BSG) obtained from the brewery industry after mixing hot water and malt, exhausted olive pomace (EOP) collected from the olive oil industry after recovery of residual olive oil from crude olive pomace, exhausted grape mark (EGM) and vine-shoots trimming (VST) from the winery industry after distilling of the residual alcohol and after the pruning respectively. These residues were all obtained from industries in the north of Portugal during season 2015–2016. The wastes were dried at 65 °C for 24 h and stored at room temperature.

Fungi

Three species of *Aspergillus* from MUM (Braga, Portugal) were used: *A. niger* 01UAs181, *A. uvarum* MUM08.01 and *A. ibericus* MUM03.49. The fungi were cultivated on malt extract agar (20 g L^{-1} malt extract, 1 g L^{-1} peptone, 20 g L^{-1} glucose and 20 g L^{-1} agar) slants and incubated at 25 °C for 6 days. Strains were preserved at 4 °C and cultured monthly on fresh malt extract agar slants.

SSF of agro-food wastes

BSG, EOP, EGP and VST were used as substrate in SSF experiments to evaluate the three fungi *A. uvarum*, *A. niger* and *A. ibericus*. The SSF process was carried out in 500 mL Erlenmeyer flasks with 10 g of dry substrate. Moisture level was adjusted to 75% (wet basis); this moisture was optimized in previous work.[14] The ratio carbon (C)/N was fixed to 15 (value of BSG) for comparison of the substrates. Different concentrations of urea were added to EOP, EGP and VST to achieve a C/N ratio of 15. Erlenmeyer flasks with solid medium were sterilized at 121 °C for 15 min. For inoculation, the fungi were suspended in a sterile solution (1 g L^{-1} peptone and 0.1 g L^{-1} Tween 80). The inoculum spore concentration was adjusted to 10^6 spores mL^{-1} using a Neubauer counting chamber. Each Erlenmeyer flask was inoculated with 2 mL of spore suspension and incubated at 30 °C for 6 days. A control experiment was performed for each sterilized waste without fungi inoculation.

Extraction of enzymes after SSF

At the end of each experiment, a representative sample (5 g dry substrate) was used to extract enzymes with a solution of 10 g L^{-1} sodium chloride and 5 g L^{-1} Triton X-100, ratio liquid : solid of 5, at room temperature with mechanical agitation for 1 h. This solution was optimized in previous studies (data not shown). Triton X-100 is a non-ionic surfactant, and it can improve the solubilization of the enzyme from the solid substrate to the solution. Sodium chloride was used to increase the stability of the enzyme, based on literature.[15] Subsequently, the extract was filtered through a nylon net and the liquid fraction was centrifuged at 6000 × *g* for 10 min. The enzyme extract was recovered and stored at −20 °C until its analysis. A representative sample of fermented solid (5 g of dry substrate) was removed before enzyme extraction; the solid was dried at 50 °C for 48 h and characterized.

Analysis of enzyme activities

The activity of cellulases (*endo*-1,4-β-glucanase) was determined with the Azo-CM-Cellulose S-ACMCL (Megazyme International, Ireland) enzymatic kit. Enzymatic extracts were diluted with 0.1 mol L^{-1} sodium acetate buffer (pH 4.6) to a final volume of 0.5 mL and mixed with 0.5 mL substrate solution (CM-Cellulose 4 mol L^{-1}). Before analysis, the solutions were pre-equilibrated at 40 °C. A blank was performed with water and substrate solution. Then, the mixture was stirred and incubated for 40 °C and 10 min. To end the reaction, substrate was precipitated by the addition of 2.5 mL of precipitant solution (40 g L^{-1} $C_2H_3NaO_2 \cdot 3H_2O$, 4 g L^{-1} $Zn(O_2CCH_3)_2$ in ethanol solution at 76%, pH 5) with vigorous stirring for 10 s on a vortex mixer. The reaction tubes were stabilized 10 min at room temperature. The tubes were stirred again and centrifuged at 1000 × *g* for 10 min. The supernatant solution absorbance was measured at 590 nm and the enzyme activity was determined by reference to a standard curve performed with *A. niger endo*-cellulase. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of glucose reducing sugar equivalents from carboxymethyl cellulose in 1 min at 40 °C and pH 4.6. The values of cellulases activity are expressed in units per gram of dry substrate (U g^{-1}).

The analysis of *endo*-1,4-β-xylanase was carried out using azo-wheat arabinoxylan S-AWAXP (Megazyme International, Ireland) as substrate. The procedure was performed in the same way as cellulases activity analysis (as already described), except the buffer solution was 0.1 mol L^{-1} sodium acetate (pH 4.5), the precipitation solution was ethanol (95% v/v) and centrifugation was performed at 1500 × *g*. The supernatant solution absorbance was measured at 590 nm and the enzyme activity was determined by reference to a standard curve performed with *A. niger endo*-xylanase. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of xylose reducing sugar equivalents from wheat arabinoxylan in 1 min at 40 °C and pH 4.5. The values of xylanases activity were expressed in units per gram of dry substrate (U g^{-1}).

Table 1. Composition of agro-food wastes

Parameter	EGM	EOP[b]	VST	BSG
Humidity (g kg^{-1})	109 ± 1	96.6 ± 1.2	63.3 ± 5.3	735 ± 4
Total solids (g kg^{-1})	891 ± 1	903.4 ± 1.2	936.7 ± 1	265 ± 4
Ash (g kg^{-1})	90.95 ± 5.58	33.6 ± 1.68	35.87 ± 4.32	12.75 ± 0.05
Lignin (g kg^{-1})	666.26 ± 5.09	550 ± 15	340.85 ± 5.54	153.68 ± 17.58
Hemicellulose (g kg^{-1})	101.78 ± 3.81	289.16 ± 0.54	237.79 ± 3.81	339.96 ± 5.05
Cellulose (g kg^{-1})	144.49 ± 2.11	128.77 ± 2.42	423.96 ± 5.25	362.16 ± 10.24
Crude protein (g kg^{-1})	134.74 ± 3.97	57.51 ± 3.58	37.9 ± 0.2	217 ± 21
Soluble protein[a] (g kg^{-1})	0.54 ± 0.08	0.54 ± 0.08	0.54 ± 0.08	1.07 ± 0.33
Reducing sugars[a] (g kg^{-1})	4.1 ± 0.07	33.07 ± 1.59	4.1 ± 0.07	132.53 ± 0.41
Phenols[a] (g kg^{-1})	1.72 ± 0.01	7.07 ± 0.07	1.72 ± 0.01	2.39 ± 0.04
N (g kg^{-1})	21.56 ± 0.64	9.2 ± 0.57	6.06 ± 0.03	34.67 ± 3.36
C (g kg^{-1})	482.4 ± 16.1	460.7 ± 12.9	462.6 ± 2.2	471.4 ± 0.01
C/N	22.38	50.1	74.85	12.23
Calcium (g kg^{-1})	3.04 ± 0.19	1.8 ± 0.2	2.02 ± 0.12	2.45 ± 0.06
Potassium (g kg^{-1})	7.14 ± 0.22	14.2 ± 0.7	4.76 ± 0.15	0.72 ± 0.04
Magnesium (g kg^{-1})	0.14 ± 0.01	0. 47 ± 0.01	0.09 ± 0.01	1.81 ± 0.06
Zinc (mg kg^{-1})	9 ± 2.55	10.5 ± 0.7	6 ± 1.7	154 ± 4.24
Copper (mg kg^{-1})	50.1 ± 8.9	11 ± 1	33.4 ± 5.9	12.5 ± 0.7
Iron (mg kg^{-1})	1255.5 ± 69.16	147 ± 33	817 ± 46	138.5 ± 4.95
Manganese (mg kg^{-1})	43.44 ± 4.16	10.2 ± 0.4	28.96 ± 2.77	40.5 ± 0.71
Chromium (mg kg^{-1})	47.4 ± 3.82	<22	31.6 ± 1.7	<22
Nickel (mg kg^{-1})	45.9 ± 3.8	<22	30.6 ± 2.55	<22
Lead (mg kg^{-1})	<22	<22	<22	<22
Sodium (mg kg^{-1})	6110.1 ± 485.78	92 ± 5	4073.4 ± 323.85	56 ± 1.4

All values are expressed per kilogram of dry material; BSG, brewers' spent grain; EGM, exhausted grape marc; EOP, exhausted olive pomace; VST, vine-shoot trimmings.
[a] Value in aqueous extract.
[b] C and minerals data were published in Leite *et al.*[19]

Chemical analysis of unfermented and fermented wastes

The unfermented and fermented wastes were characterized to observe the effect of fungal growth on their composition. To estimate free sugars, free protein and phenolic compounds in wastes, an extraction with distilled water with liquid : solid ratio of 5 was performed. Free reducing sugars were analysed by the 3,5-dinitrosalicylic acid method.[16] Proteins were measured by the Bradford method. Total phenols were determined by the Folin–Ciocalteu method (Commission Regulation (EEC) No. 2676/90). N content was analysed by the Kjedahl method.[17] The crude protein content was calculated on the basis of N determined by the Kjeldhal method with an appropriate factor of 6.25.[18] Determinations of cellulose, hemicelluloses, lignin, C and minerals were carried out following the methods described by Leite *et al.*[19] Ashes were determined by high temperature treatment at 575 °C for 2 h in a muffle furnace. C and minerals were analysed following the process described by Salgado and co-workers[19] Table 1 shows the initial composition of solid wastes.

SEM

The unfermented and fermented wastes were characterized using a desktop scanning electron microscope coupled with energy-dispersive X-ray spectroscopy (EDS) analysis (Phenom ProX with EDS detector (Phenom-World BV, Netherlands)). All results were acquired using the ProSuite software. The samples were added to aluminium pin stubs with electrically conductive C adhesive tape (PELCO Tabs™), with the excess removed using compressed air. Samples were imaged without coating. The aluminium pin stub was then placed inside a Phenom charge reduction sample holder.

RESULTS AND DISCUSSION

The main wastes from breweries, wineries and olive mills (BSG, EOP, EGM and VST) were inoculated with three different filamentous fungi: *A. uvarum* MUM 08.01, *A. ibericus* MUM 03.49 and *A. niger* 01UAs183. The goal was the selection of fungus that produces higher cellulose and xylanase activities and increases the protein content of agro-industrial wastes. In previous work,[19] these strains showed potential for producing lignocellulolytic enzymes on agar plates. In addition, *A. uvarum* MUM 08.01 showed good results in the increase of protein of agro-food wastes mixtures.[20]

Increasing the protein content of agro-food wastes

Fungal biomass protein, also known as single-cell protein, is a suitable product to be used as animal feed. It is estimated that the protein content in *A. niger* is 270 g kg^{-1} with balanced essential amino acids.[21,22] Thus, *Aspergillus* strains were tested on agro-food wastes to increase their nutritional value.

The protein content was determined in solid substrates before fermentation, in control (solid substrate after addition of urea and sterilization) and in fermented solids. As can be observed in Fig. 1, the protein content of the four fermented wastes was increased with respect to initial waste and control.

BSG was the waste with a higher initial protein content; however, the protein content in other wastes was very low, particularly

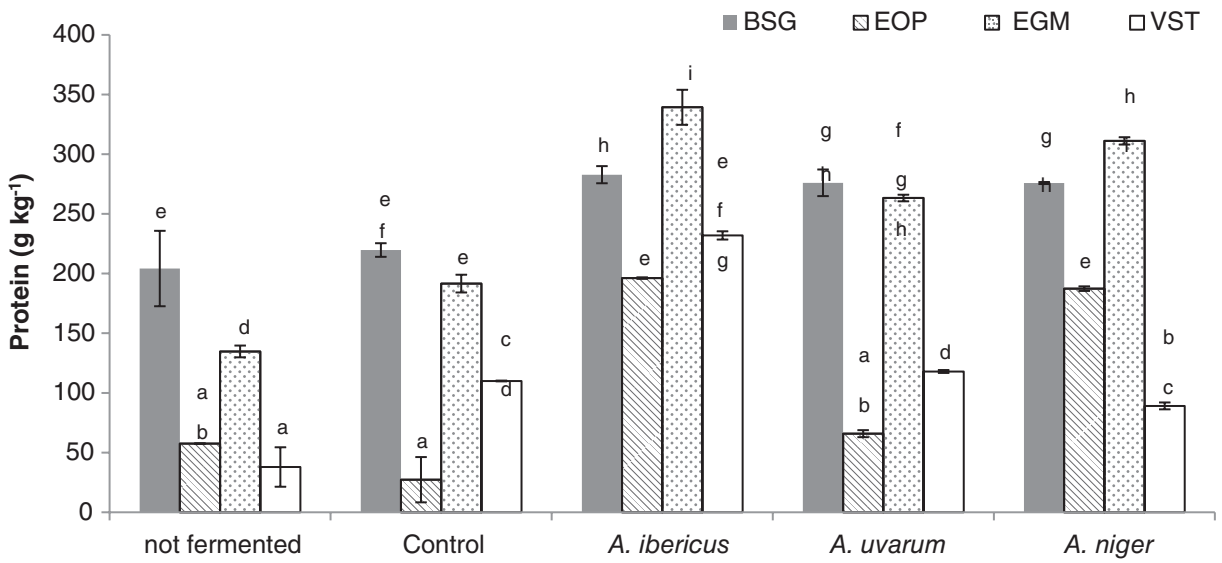

Figure 1. Protein content of the initial substrate, control (solid after sterilization) and residues after SSF with *A. ibericus*, *A. uvarum* and *A. niger*. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different. BSG, brewers' spent grain; EOP, exhausted olive pomace; EGM, exhausted grape marc; VST, vine-shoot trimmings.

in EOP (57.5 g kg^{-1}) and VST (37.9 g kg^{-1}). To observe the effect of heat treatment and the addition of urea, control assays were carried out with each waste. Owing to the protein being measured by Kjehldahl N method, the addition of urea (source of N) could increase the value of N. In addition, the heat treatment of sterilization can hydrolyse or degrade compounds of solid wastes and modify their protein content. However, the addition of urea and the sterilization process (control) barely modified the protein content; only in winery wastes were significant differences observed, where the protein content increased.

Although BSG had a significant protein content, the fermentation with three fungi increased the protein further. There were no significant differences between the three fungi; the increase of protein by SSF of *A. ibericus* was 38.5%. This value was slightly higher than that Canedo *et al.*[23] achieved in BSG fermented by *Rhizopus oligosporus*, in which the protein content was increased by 31.5% after SSF.

In the same way, the protein was increased in EOP after SSF. The increase was higher than fermented BSG; the fungi that achieved a maximum increase were *A. ibericus* and *A. niger*, with the protein content increasing by 3.4 and 3.2 times respectively. The SSF allowed the protein to increase to similar values of initial BSG, which is considered a nutritional waste to be used as animal feed. Brozzoli *et al.*[24] studied the increase of protein in stoned olive pomace by *Pleurotus* species. In this case, the olive pomace was mixed with other conventional feedstuffs, such as wheat bran, barley grans, and so on. In the best conditions, the protein was increased 1.3 times.

SSF of EGM also showed a protein increase, and *A. ibericus* and *A. niger* were also the fungi that further increased the protein content; *A. ibericus* achieved 340 g kg^{-1} of protein, although no significant differences were observed with *A. niger*. The bioconversion of EGM to nutrient-rich feed was studied by Jin *et al.*,[10] who enhanced the protein content up to 260 g kg^{-1} after SSF.

Among all wastes, the higher increase of protein was observed using VST as substrate; SSF by *A. ibericus* increased the protein content 6.1 times with respect to unfermented VST. Thus, SSF was a suitable process to increase the nutritional value of wastes with low protein content. All fungi increased the protein content; however, *A. ibericus* MUM 03.49 showed the maximum increase in each waste studied. The fungal biomass protein is considered a good source of dietary protein for the feeding of ruminants and improves animal performance.[12] In the same way, the fungal biomass protein was successfully tested in fish diets.[25]

Production of lignocellulolytic enzymes

Using the same processes, the production of lignocellulolytic enzymes was evaluated. Figure 2 shows the activities of cellulases and xylanases achieved after SSF. *A. ibericus* was the fungus that contributed to a higher cellulose activity using BSG as solid substrate. The high content of cellulose and hemicelluloses in BSG induced lignocellulolytic production. The use of BSG as solid substrate has barely been studied, though Sim and Oh[26] evaluated BSG as a solid substrate for cellulose production using the typical cellulases producer *Trichoderma reesei* and they achieved a 43 U g^{-1} BSG.

A. niger was the fungus which produced cellulases with higher activity in EGM and VST. In the case of EOP, there were no statistically significant differences ($P < 0.05$) in cellulases activity between *A. ibericus* and *A. niger*. However, no cellulase activity was detected in fermented BSG by *A. niger*.

On the other hand, the maximum xylanase activity was achieved by *A. ibericus* and *A. uvarum* using BSG as solid substrate, but no significant differences ($P < 0.05$) were observed among them. The production of xylanases and cellulases by *A. uvarum* was less compared with other strains; however, this strain increased the protein content of agro-food wastes. Thus, it is possible that fungi focus their metabolism on biomass growth.

The use of EOP as solid substrate for cellulose and xylanase production was evaluated by Leite *et al.*[19] They observed a maximum enzyme production using *A. niger* CECT 2915, achieving about 25 U g^{-1} and 35 U g^{-1} of xylanase and cellulose respectively. Salgado *et al.*[20] evaluated the mixture of EGM and crude olive pomace as solid substrate and they achieved 32 U g^{-1} of cellulose activity and 10.5 U g^{-1} of xylanase activity using *A. uvarum* as fungus.

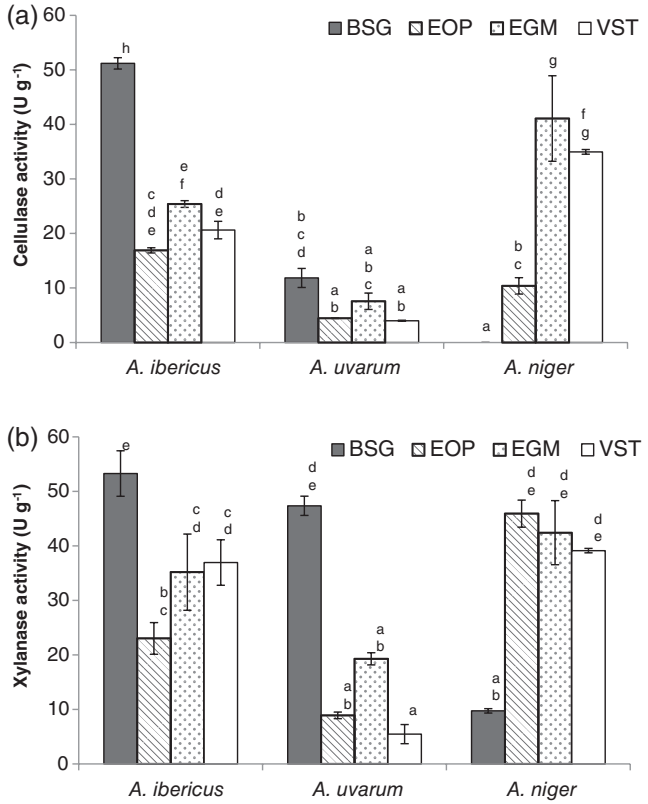

Figure 2. Cellulases (a) and xylanases (b) production during SSF. These results represent the average of two independent experiments, and error bars represent standard deviation. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different. BSG, brewers' spent grain; EOP, exhausted olive pomace; EGM, exhausted grape marc ; VST, vine-shoot trimmings.

All wastes showed that they are suitable substrates for cellulose and xylanase production. These agro-food wastes are of a lignocellulosic nature; thus, they can induce the production of lignocellulolytic enzymes.[27]

Effect of fungi on lignocellulosic composition of agro-food wastes

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

Figure 3 shows the lignocellulosic composition of the initial waste, the control and each fermented waste by different fungi. All lignocellulosic components were reduced when they were submitted to SSF. A small reduction was observed in control, which could be due to the effect of the sterilization processes. The SSF caused a decrease in the initial dry matter; this decrease was similar in all SSFs, from 6.9% to 14% of initial dry solid waste.

The maximum reduction of hemicellulose was calculated taking into account the loss of dry matter. *A. uvarum* showed the maximum reduction in BSG, EOP and VST, this being 54.2%, 46.7% and 53% of reduction respectively. The results of hemicellulose reduction were not correlated with xylanase activities. The maximum xylanase activity was achieved by *A. ibericus*; however, this fungus did not achieve the maximum reduction of hemicellulose fraction.

Shinners-Carnelley *et al.*[28] evaluated the reduction of hemicellulose of canola stubble, observing a reduction of 25% by

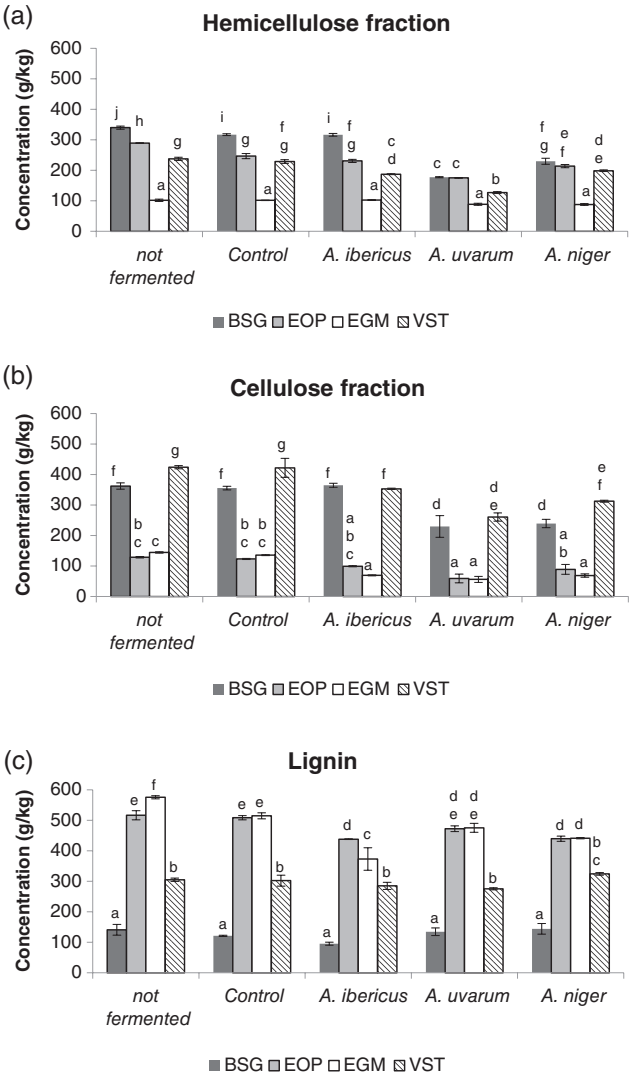

Figure 3. Lignocellulosic composition of different residues with the different fungi: (a) hemicellulose fraction, (b) cellulose fraction, (c) lignin. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different. BSG, brewers' spent grain; EOP, exhausted olive pomace; EGM, exhausted grape marc; VST, vine-shoot trimmings.

Cyathus olla in SSF. In another study, *A. niger* reduced the hemicellulose content 36% and *Rhizopus* sp. reduced 25% after 10 days of SSF of cactus pear.[29] Tuyen *et al.*[30] studied 11 fungi and observed hemicellulose reductions ranging from 7.5 to 53%. The maximum xylanase production with the least hemicellulose degradation was evaluated, with BSG and EGM fermented by *A. ibericus* showing the best ratio of xylanase activity : reduction of hemicellulose (3.1). In this way, the fermented waste keeps the hemicellulose fraction for use as an animal feed or in the biorefinery industries.

The cellulose content was reduced in all fermentations. *A. ibericus* showed the least cellulose degradation in all wastes except with in EGM, which was similar in the three fungi. The best ratio of cellulase activity : reduction of cellulose was achieved by *A. ibericus* in BSG (4.9). The reduction of cellulose in SSF was observed in other fungi: *Trametes versicolor* reduced the cellulose of wheat straw 44.8% after 49 days of SSF,[30] and *A. niger* achieved a similar reduction of cellulose content (40%) in SSF of cactus pear after 10 days.[29]

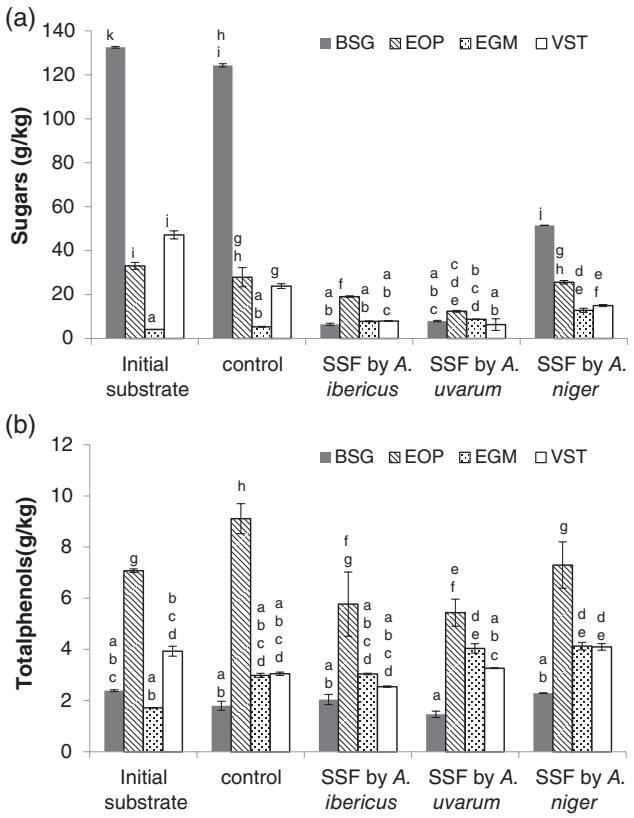

Figure 4. Content of free sugars (a) and phenolic compounds (b) in the initial substrate, control and residues after SSF with *A. ibericus*, *A. uvarum* and *A. niger*. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different. BSG, brewers' spent grain; EOP, exhausted olive pomace; EGM, exhausted grape marc; VST, vine-shoot trimmings.

The reduction of lignin content can improve the rumen fermentability of lignocellulosic wastes.[30] Figure 3(c) shows that the content of lignin decreased after SSF. The reduction of lignin content (taking into account the loss of dry matter) was higher when *A. ibericus* was inoculated for all wastes. The maximum reduction of lignin was achieved in SSF of EGM (43%), followed by SSF of BSG (40%) after 7 days. Chen *et al.*[31] observed in fermentation of cornstalk a decrease of lignin of 10% after 30 days. Carvalho do Santos *et al.*[29] observed a decrease of lignin of 28% after SSF by *A. niger* and 18% by *Rhizopus* sp. Tuyen *et al.*[30] demonstrated a high correlation between reduction of lignin and hemicellulose reduction ($r = 0.96$). In this study, we obtained a correlation of $r = 0.68$. Thus, the degradation of hemicellulose was accompanied by delignification of waste. This correlation could be due to the fungi actuating in ether and ester bonds that linked covalently the lignin and hemicellulose. Another reason could be the delignification of waste can improve the accessibility of xylanases to hemicellulose, increasing their degradation.[30]

Variation of free sugars and phenol compounds in the extract after SSF

The effect of fungus growth on sugars and phenolic compounds content was evaluated. Figure 4(a) shows the free sugars available in the control, initial substrate and solid wastes after SSF. The evaluation of sugars concentrations is very important because in high concentrations they can act as catabolite repressors of enzymes production.[32] However, in low concentrations they can improve enzyme production.[33] The low concentration of sugars can be consumed rapidly by the fungi growth at the start of fermentation, and thus they favour the fast colonization of substrate. When these sugars are consumed, the fungi should by another source of C and produce carbohydrases to extract sugars from raw material. In addition, SSF has been demonstrated to be a good strategy to minimize catabolite repression.[34] BSG was the waste with higher sugar content (130 g kg^{-1}), followed by EOP and VST with 30 g kg^{-1} and 40 g kg^{-1} of sugars respectively. EGM showed the lowest sugar concentration, with 4 g kg^{-1}, since all the sugars were consumed during winemaking.

The high concentration of free sugars of BSG did not inhibit enzyme production by *A. uvarum* and *A. ibericus*. However, *A. niger* showed a low enzyme production that could be due to the catabolite repression of free sugars. In this sense, de

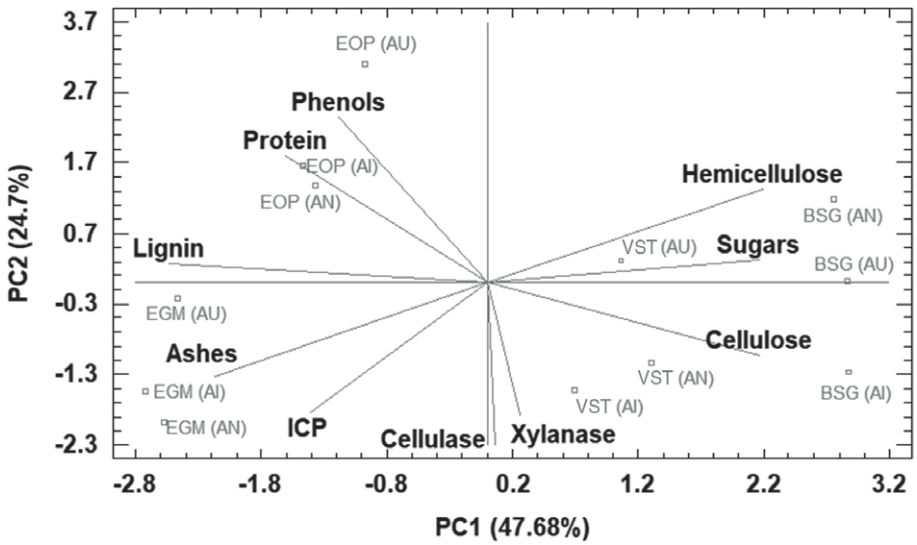

Figure 5. Principal component analysis. Biplot representation of variables and SSF experiments performed. ICP, increase content of protein; BSG, brewers' spent grain; EOP, exhausted olive pomace; EGM, exhausted grape marc; VST, vine-shoot trimmings; AU, *A. uvarum*; AN, *A. niger*; AI, *A. ibericus*.

Souza *et al.*[32] observed significant reduction of xylanase production by *Aspergillus tamarii* with free sugar concentrations over 100 g kg^{-1}. In SSF by *A. niger*, Gokhale *et al.*[33] observed the maximum repression with concentrations of glucose over 50 g kg^{-1}. Low concentrations of sugars can increase enzyme production, and Gokhale *et al.*[33] observed a positive effect of 10 g kg^{-1} glucose on cellulase production.

It is difficult to observe the kinetic profiles of reducing sugars generation and consumption during SSF. The fungus consumes the reducing sugars and then it produces enzymes that can hydrolyse the lignocellulosic wastes to more reducing sugars, which can be again consumed by the fungus. In general, a reduction of the concentration of free sugars was observed after SSF with the exception of EGM, in which the concentration of free sugars after SSF was similar to the control.

Figure 4(b) shows the results of total phenols determined in the initial substrate, control and the four residues after SSF. In these results, it is possible to observe that the concentration of total phenols after SSF was very similar to the values obtained in the control; no significant differences were observed ($P < 0.05$) with the exception of EOP, where it is possible to observe a clear decrease of total phenols concentration. It was expected that the phenolic compounds could decrease after SSF because the fungi have the ability to degrade phenolic compounds present in wastes.[35]

Relationship among substrates, fungi and SSF performance

A principal components analysis was carried out in order to assess the relationship between substrate composition, fungi used, enzymes produced and protein increase. This study allowed us to obtain a small number of lineal combinations of the 10 variables studied to explain the variability of the data. In this analysis, three components were defined that explain 87.4% of the variability of the original data. The first two principal components, PC1 and PC2, are shown in Fig. 5, accounted for 72.38% of the variability (47.68% and 24.7% respectively). PC1 was characterized positively by high concentration of cellulose, hemicellulose and sugars, and negatively with lignin and ashes content. As can be observed, the substrates with high hemicellulose and cellulose content were positively correlated with cellulase and xylanase production and negatively correlated with lignin and ashes content. For PC2, phenols and protein were characterized positively and cellulase, xylanase and increase of protein content were characterized negatively. The negative effect of phenols content on enzyme production and in the increase of protein content is clearly visible. The principal components analysis grouped the four different substrates: BSG was characterized as a waste with high content in hemicellulose, cellulose and sugars and a low content in lignin and ashes; EOP was highly correlated with a high content of phenolic compounds and low production of cellulases, xylanases and protein; EGM was clearly characterized as a waste with high content in lignin and ashes; and VST was correlated with high production of enzymes and a low content in phenolic compounds. As far as the strain of fungus used, *A. ibericus* was highly correlated with production of enzymes and increase of protein content, except with SSF using EOP as substrate.

Morphology of *A. ibericus* and structure changes on substrate during SSF

SEM was used to observe the growth of *A. ibericus* on agro-food wastes. This analysis allowed characterization of the morphology of the fungus and its distribution over the solid wastes. Figure 6 compares the four materials studied before and after SSF. As can be observed, *A. ibericus* colonized massively and dispersedly all substrates by hyphae and conidia. In general, the fungi in SSF conditions grow on the surface of the substrate, inside the substrate and as aerial hyphae forming conidiophores and conidia.[36] These regions can be observed in the micrographs. Fig. 6(B3, F2) shows clearly the formation of conidiophores in the aerial region, and huge amounts of conidia forming chains disperse and being part of the conidiophores. This type of growth was observed mainly using BSG and EGM as substrate. The surface texture of conidia showed roughness (Fig. 6(B1)); this structure had previously been noted by Serra *et al.*[37] They observed that *A. ibericus* is characterized by biseriate conidiophores and conidia with a size of 5–7 µm. In all wastes, the fungus grew on their surface; the formation of hyphae in a scattered manner can be observed in Fig. 6(B3, D3, F1, H2). The hyphae also penetrated into substrates, disseminating hydrolytic enzymes (Fig. 6(B3, D3, F1, H2)).

On the other hand, the colonization of substrate by *A. ibericus* produced a degradation of the agro-food wastes structure. This degradation observed in the micrographs is in line with the aforementioned results showing the characterization of fermented wastes and the production of lignocellulolytic enzymes. The fungus showed an aggressive attack on the structure of VST (Fig. 6(H1)), which shows different regions (Fig. 6(G1)): vessels, ray cells and fibre cells. The fungus grew preferentially in vessels and ray cells regions. This may be because vessels provide large open spaces for hyphae growth and because ray cells contain a large amount of nutrients and a low inhibitors content.[38] The degradation of structure of agro-food wastes by SSF may favour the digestibility of these wastes by animals and increase the accessibility to their nutrients.[1] In this sense, the use of fermented agro-food wastes by *Aspergillus* species has been evaluated as feed for pigs, showing a better digestibility compared with unfermented wastes.[39] To a large extent, this improvement is due to the degradation of their structure.

CONCLUSION

This study showed the potential of the barely studied strains *A. ibericus* and *A. uvarum* to increase the nutritional value of main Mediterranean agro-food wastes and to produce lignocellulolytics in the same process. The growth of fungi led to a significant increase of protein content of the four wastes studied, highlighting the increase of protein on winery wastes. In the same process, these strains produced xylanase and cellulases. *A. ibericus* was also the fungus that maximized the production of xylanases and cellulases, with BSG being the best substrate for enzymes production. The degradation of lignocellulosic compounds by fungi was demonstrated; this can facilitate the digestibility of wastes by animals. Moreover, the high hemicellulose and cellulose content favoured the production of lignocellulolytic enzymes, and the high phenols content limited the production of enzymes and the increase of protein. On the other hand, SEM analysis showed the degradation in the structure of wastes by the growth of *A. ibericus* and the liberation of lignocellulolytic enzymes.

Integral use of wastes was carried out without the generation of additional wastes. Thus, SSF may be an alternative technology to reuse Mediterranean agro-food and may offer two new products, namely fermented waste and an enzyme cocktail, with potential application in the food industry.

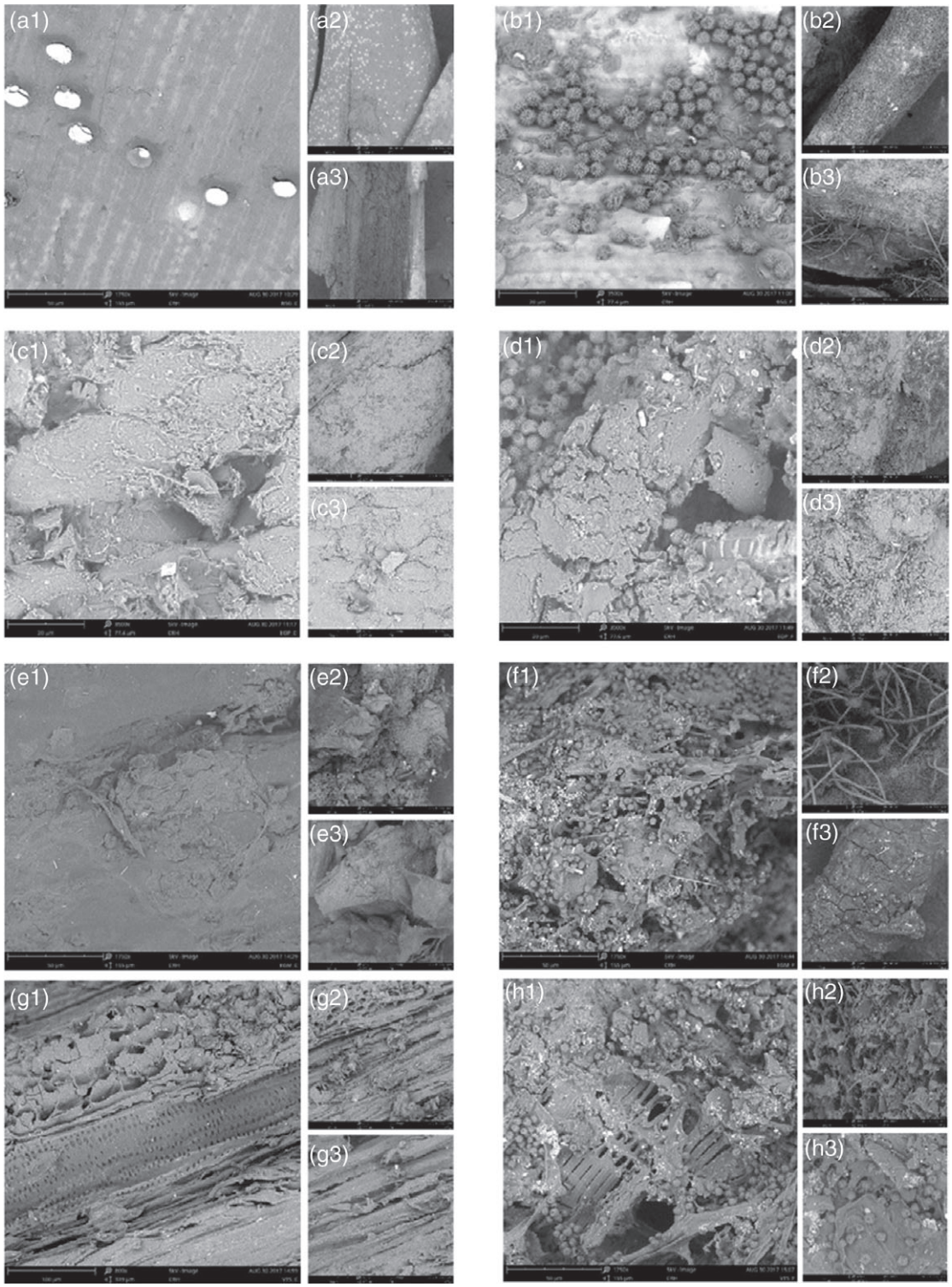

Figure 6. SEM images of unfermented and fermented wastes. (A1 – A3) Structure of unfermented BSG at different scales. (B1 – B3) View of *A. ibericus* growth on BSG at different scales. (C1 – C3) Structure of unfermented EOP at different scales. (D1 – D3) View of *A. ibericus* growth on EOP at different scales. (E1 – E3) Structure of unfermented EGM at different scales. (F1 – F3) View of *A. ibericus* growth on EGM at different scales. (G1 – G3) Structure of unfermented VST at different scales. (H1 – H3) View of *A. ibericus* growth on VST at different scales.

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