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ORIGINAL ARTICLE

Screening of winery and olive mill wastes for lignocellulolytic enzyme production from *Aspergillus* species by solid-state fermentation

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Abstract Wastes from olive oil and wine industries (as exhausted grape marc, vineshoot trimmings, two-phase olive mill waste, vinasses, and olive mill wastewater) were evaluated for lignocellulolytic enzyme production (as endocellulases, endoxylanases, and feruloyl esterases) by solid-state fermentation (SSF) with *Aspergillus niger*, *Aspergillus ibericus*, and *Aspergillus uvarum*. To study the effect of different solid medium composition and time in enzyme production, a Plackett–Burman experimental design was used. Variables that had a higher positive effect in lignocellulolytic enzyme production were urea, time, and exhausted grape marc. The maximum values of enzymatic activity per unit of substrate dry mass were found with *A. niger* for feruloyl esterase. Enzymatic extracts from SSF with *A. niger* achieved maximum feruloyl esterase activity (89.53 U/g) and endoxylanase activity (3.06 U/g) and with *A. uvarum* for endocellulase activity (6.77 U/g). The enzyme cocktails obtained in the SSF extracts may have applications in biorefinery industries.

Keywords Olive mill wastes · Winery wastes · Plackett–Burman · Solid-state fermentation · Lignocellulolytic enzymes

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1 Introduction

Since the last two decades, lignocellulolytic enzymes (LCEs) are gaining enormous attention for their potential in biotechnology processes [1]. Principal LCEs are cellulases, hemicellulases, ligninases, and pectinases. LCEs are used in saccharification processes, for the subsequent production of ethanol by fermentation. Among the proposal to obtain simple sugars, they have applications on bioremediation processes and in the paper, textile, chemical, and food industries [1].

In biorefinery processes, LCEs are involved in different fractionation steps due to their ability to degrade lignin, cellulose, and hemicellulose from lignocellulosic materials. However, the use of enzymes increases the costs of these processes. LCE produced by submerged fermentation is expensive, about US$0.4–0.6/gal ethanol [2]. Cellulase cost can be assumed to be US$0.25/gal of produced ethanol [3]. Therefore, the exploitation of agro-industrial wastes as a low-cost substrate for the production of enzymes can contribute to make those processes more profitable. In addition, a cost-effective enzyme system should contain balanced activities of cellulases and hemicellulases to reduce the cost of bioethanol production [4].

Winery and olive mill wastes are commonly generated in Mediterranean countries. Several wastes from these industries are of lignocellulosic nature such as vineshoot trimmings (VTS), exhausted grape marc (EGM), and two-phase olive mill waste (TPOMW). The use of agro-industrial residues to produce high-value products and energy, in biorefinery concept, is an alternative to manage these wastes. Thus, they could be suitable substrates for LCE production by filamentous fungi in solid-state fermentation (SSF). Most commercial LCEs are currently produced by submerged fermentation [5]. However, SSFs are considered optimal for enzyme production

because it may reach higher production yields than submerged fermentations. Besides, it is better because of its operational simplicity, low-energy requirements, simple cultures, and simple design bioreactors [6]. Compared with submerged fermentation, SSF enzymatic complexes showed a greater efficiency in lignocellulose saccharification [7]. Due to these facts, SSF can be an alternative technology for LCE production, using filamentous fungi that produce an optimal enzyme complex for the degradation of the host lignocellulose [2]. Lignocellulosic materials act as both the carbon source and as an inducer for LCE enzyme production [8]. In addition, SSF with fungi can be used as biological pretreatment to enhance enzymatic hydrolysis [9].

SSF is well adapted to the metabolism of fungi, so are the microorganisms most commonly in SSF processes [10]. Fungi produce a complete set of cellulases, cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4), and β-glucosidases (EC 3.2.1.21), that are necessary to efficiently hydrolyse cellulose [11]. To this, a synergistic action of these enzymes is required to complete the hydrolysis of cellulose [12]. Cellulases carried out the hydrolysis of cellulose by breaking β-1, 4-glucosidic bonds. Endocellulases can hydrolyze these links internally in the cellulase chains, whereas cellobiohydrolases act at the end of the chains. The products of these enzymes are disaccharides or oligosaccharides which are hydrolyzed by β-glucosidases. Fungal strains that produce cellulases are mainly comprised of *Trichoderma*, *Aspergillus*, *Penicillium*, and *Fusarium* genera [4]. *Aspergillus niger* are frequently used to produce cellulases [12, 13]. However, production of cellulases by *Aspergillus uvarum* and *Aspergillus ibericus* has not been studied. *A. uvarum* is morphologically close to *Aspergillus japonicus* [14]. This species has been used to produce cellulases by SSF of different agro-industrial wastes [14–16].

Among cellulase production, fungi can produce hemicellulases which are a diverse group of enzymes due to the heterogeneity of hemicelluloses. These enzymes improve the hydrolysis of cellulose by exposing their fibers, thus making them more accessible to hydrolysis by cellulases [17]. Xylanases are glycosidases (*O*-glycoside hydrolases, EC 3.2.1.x) which catalyze the hydrolysis of 1,4-β-D-xylosidic linkages in xylan [18]. Endoxylanases can hydrolyse β-1,4 links in xylan chains to oligomers which are hydrolyzed to xylose by β-xylosidases. Other enzymes such as β-mannoses, arabinofuranosidases, and α-L-arabinases can act depending on hemicellulose composition. Endoxylanases are much more common than β-xylosidases [19]. Some genera that produce xylanases include the *Aspergillus*, *Trichoderma*, *Streptomyces*, *Phanerochaetes*, and *Chytridiomycetes* [18]. On an industrial scale, xylanases are produced by *Aspergillus* spp. [20]. Owing to *Aspergillus* secretions at high concentrations, a large variety of hemicellulases work synergistically [17].

Feruloyl esterases (FAEs, EC 3.1.1.73), also known as ferulic acid esterases, are another type of hemicelluloses; they are a subclass of carboxylic acid esterases (EC 3.1.1.1). FAEs are capable of releasing the phenolic acids such as ferulic and *p*-coumaric acid and their dimers. It has been demonstrated that FAEs act in synergy with xylanases by cleaving diferulic bridges between xylan chains, opening the structures, and releasing lignin [21]. In industries, *A. niger* FAEs are used for ferulic acid extraction from agro-industrial wastes [22]. Besides, FAEs are used in pulp and paper industry for lignin removal from cellulose [23]. In the processes of saccharification of lignocellulosic materials, combined treatment with cellulases, xylanases, and FAEs allows the use of lower quantities of enzymes and reduces the cost of the process [11]. Therefore, it may be interesting to obtain enzymatic extracts with these activities. In addition, production of LCE by *A. ibericus* and *A. uvarum* has been reported.

In the present work, we evaluate different wastes from olive oil and wine industries, TPOMW, EGM, VT, vinasses, and olive mill wastewater (OMWW), for endocellulase, endoxylanase, and FAE production by solid-state fermentation with three strains of *Aspergillus*.

2 Experimental

2.1 Raw material and chemical characterization

The waste samples were collected from industries in the area in season 2011/2012 and stored at −20 °C. The solid residues were TPOMW, EGM, and VT. Their particle sizes were less than 1 mm. The liquid wastes were V and OMWW. The solid residues were characterized by quantitative acid hydrolysis in a two-stage acid treatment (the first stage with 72 wt% sulfuric acid at 30 °C for 1 h and the second stage after dilution of the medium to 4 wt% sulfuric acid at 121 °C for 1 h) to determine cellulose, hemicelluloses, and Klason lignin. Total nitrogen in liquid residues was determined by the test kit from Hach Lange LCK 338. For chemical oxygen demand (COD) determination, the test kit from Hach Lange LCK914 was used according the manufacturer's method. Total organic carbon in liquid residues was quantified by the test kit from Hach Lange LCK 387. Nitrogen and carbon in solid residues were analyzed using a Thermo Finningan Flash Elemental Analyzer 1112 series, San Jose, CA (USA). To determine free reducing sugars, total phenols, and proteins in solid residues, extraction with water 1:5 (*w*/*v*) was performed. Reducing sugars were determined by dinitrosalicylic acid method. Lipids (total fat contents) were extracted with diethyl ether in a Soxtec System HT2 1045 extraction unit. Protein was determined by Bradford method. Total phenols were determined by the Folin–Ciocalteau method using caffeic acid as a standard. Total solids were analyzed by oven drying to a constant weight at 102 °C.

2.2 Microorganisms

Black *Aspergillus* species were used (*A. niger* 01UAs101, *A. ibericus* 01UAs294, and *A. uvarum* 01UAs128). They were obtained from MUM culture collection (Braga, Portugal). They were revived on malt extract agar (MEA) plates (2 % malt extract, 2 % glucose, 0.1 % peptone, and 2 % agar) from preserved glycerol stocks stored at −80 °C, subcultured on MEA slants, and incubated at 25 °C for 7 days to obtain inocula for SSF. During the experimental period, strains were preserved at 4 °C and cultured monthly on fresh MEA slants.

2.3 Solid-state fermentation

Fermentations were carried out in a 500-mL Erlenmeyer flask with 30 g of dry solid substrate. Compositions of media were defined in Table 1. Moisture level was adjusted to 75 %. Erlenmeyers with substrate were sterilized at 121 °C, for 15 min. Independent fermentations were performed with *A. niger*, *A. ibericus*, and *A. uvarum*. For the inoculation, spores of fungus grown in MEA medium slant tubes were suspended in a sterilized solution composed of 0.1 % of peptone and 0.01 % of Tween 80. The inoculum spore concentrations were adjusted to 10^6 spores/mL using a Neubauer counting chamber. Each flask was inoculated with 2 mL of the spore suspension and incubated at 25 °C for 7 or 14 days.

The extraction of enzymes was performed at final time (7 or 14 days) of each experiment with a solution composed of 1 % NaCl and 0.5 % Triton-X100 at 4 °C, with agitation for 2 h. Following, extracts were centrifuged (12, 225×*g*, 10 min) and filtered through a Whatman no. 1 filter paper.

2.4 Plackett–Burman experimental design

To evaluate the effect of supplementation of TPOMW on the enzyme productions by SSF, seven independent variables were screened in eight combinations (Table 1) organized according to the Plackett–Burman design. This is a fraction of a two-level factorial design and allows investigation of $n-1$ variables with at least n experiments. The levels +1 and −1 represent the lower and the higher values of the process parameters. In addition, three center points were added to the eight experiments to ensure enough degrees of freedom for error evaluation. Experiments were carried out in randomize order to avoid protection against the effects of lurking variables. The factors were addition of EGM, VT, OMWW, V, basal medium, urea, and fermentation time. Xylanase, cellulose, and feruloyl esterase activities were taken as the responses. The factors that have confidence level above 95 % were considered the most significant factors affecting enzyme production. The main effect of the substrate composition, regression coefficients, *P* values, and correlation coefficients were determined using Statistica version 5.0 (Statsoft, USA).

2.5 Enzyme activity assays of SSF extracts

Cellulase (endo-1,4-ß-glucanase) activity was determined with the enzymatic kit Azo-CM-Cellulose S-ACMC 04/07 (Megazyme International, Ireland). 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.

Xylanase (endo-1,4-ß-xylanase) activity was determined with the enzymatic kit Azo wheat arabinoxylan AWX 10/2002 (Megazyme International, Ireland). One unit of enzyme activity was defined as the amount of enzyme

Table 1 Experiments of Plackett–Burman design

Runs	EGM/TPOMW (g/g) Uncodded (codded values)	VTS/TPOMW (g/g)	OMWW (mL/g)	Vinasses (mL/g)	Nutrients[a] (mL/g)	Urea (g/g)	Time (h)
1	1 (+1)	1 (+1)	0.5 (+1)	0.5 (+1)	0.5 (+1)	0.01 (+1)	14 (+1)
2	1 (+1)	1 (+1)	0 (−1)	0.5 (+1)	0 (−1)	0 (−1)	7 (−1)
3	1 (+1)	0 (−1)	0.5 (+1)	0 (−1)	0.5 (+1)	0 (−1)	7 (−1)
4	1 (+1)	0 (−1)	0 (−1)	0 (−1)	0 (−1)	0.01 (+1)	14 (+1)
5	0 (−1)	1 (+1)	0.5 (+1)	0 (−1)	0 (−1)	0.01 (+1)	7 (−1)
6	0 (−1)	1 (+1)	0 (−1)	0 (−1)	0.5 (+1)	0 (−1)	14 (+1)
7	0 (−1)	0 (−1)	0.5 (+1)	0.5 (+1)	0 (−1)	0 (−1)	14 (+1)
8	0 (−1)	0 (−1)	0 (−1)	0.5 (+1)	0.5 (+1)	0.01 (+1)	7 (−1)

All data are expressed per gram of dry solid substrate

EGM exhausted grape marc, *TPOMW* two-phase olive mill waste, *OMWW* olive mill wastewater, *VTS* vineshoot trimmings

[a] Basal medium: 3 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L $CaCl_2 \cdot 2H_2O$, 1 mL/L of trace metal solution, 0.1 g/L peptone, 0.1 g/L yeast extract

required to release 1 μmol of xylose reducing sugar equivalents from wheat arabinoxylan in 1 min at 40 °C and pH 4.5.

Feruloyl esterase activity was determined according to Mastihuba et al. [24]. Enzyme extracts from SSF (3 mL) were mixed with 9 mL of 1.33 mM ethyl ferulate in 50 mM sodium phosphate buffer (pH 6) with 1 % (*w*/*v*) ethanol. The reaction was incubated at 40 °C for 30 min. A solution of 0.35 M H_2SO_4 (4 mL) was added to stop the reaction. The samples were filtered and the free ferulic acid was measured by high-performance liquid chromatographic (HPLC) with UV absorbance detection. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of ferulic acid per minute, under the assay conditions.

Laccase activity was determined by spectophotometric method [25]. This method is based on monitoring the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) by measuring the increase of absorbance at 420 nm. One unit of laccase activity corresponded to oxidation of 1 μmol of ABTS per minute at 25 °C.

Lignin peroxidase activity was quantified by the method proposed by Cai and Tien [26]. One unit of lignin peroxidase activity represented the oxidation of 1 μmol of veratryl alcohol per minute at 30 °C.

Mn peroxidase activity was determined by oxidation of Mn^{2+} to Mn^{3+}, measuring the increase of absorbance at 270 nm [27]. One unit of Mn peroxidase activity was defined as the amount of enzyme that produced 1 μmol of Mn^{+3} per minute at 25 °C.

2.6 HPLC analytical methods

Glucose, xylose, arabinose, and acetic acid were measured using HPLC system. Chromatographic separation was carried out using a Metacarb 87 H column (300×7.8 mm, 8–10 μm, Varian, USA) under the following conditions: mobile phase, 0.005 M H_2SO_4; flow rate, 0.7 mL/min; and column temperature, 60 °C. The system was comprised of a Jasco chromatograph 880-PU intelligent pump (Jasco, Tokyo, Japan) equipped with a Jasco 830-IR intelligent refraction index detector (Jasco, Tokyo, Japan) and a Jasco AS-2057 Plus intelligent autosampler (Jasco, Tokyo, Japan).

Ferulic acid was determined by HPLC model 1200 (Agilent, Palo Alto, CA, USA), using a UV detector (at 276 nm) and a quaternary pump. Separation was achieved at 35 °C using Zorbax SB-Aq reverse-phase column (4.6 mm ID×150 mm, 5 μm, Agilent, Palo Alto, CA, USA) with a guard column and a linear gradient run in 35 min from 100 to 52 % of A at a flow rate of 1 mL/min consisting of two solvents: solvent A (2.5 % formic acid in water, *v*/*v*) and solvent B (100 % methanol).

3 Results and discussion

3.1 Agro-industrial waste characterization

The nature of agro-industrial wastes affects the growth of fungi in SSF. Depending on their composition, microorganisms can produce specific products. Carbon and nitrogen sources are the most important components and should be given significant consideration [28]. The ratio between the mass of carbon and nitrogen is often optimized for the production of certain products as enzymes [29, 30]. Table 2 shows the composition of winery wastes (VT, EGM, V) and olive mill wastes (TPOMW, OMWW). TPOMW presented low nitrogen content and high concentration of phenolic compounds that can inhibit fungal growth [31]. Reducing sugar concentration was low in EGM because most of them are consumed in alcoholic fermentation during winemaking. The high organic content (high COD) of OMWW is also noteworthy that is why biological treatments are needed to reduce its pollutant load [32]. Lignocellulosic residues are excellent substrates for fungal growth in SSF [33]. Lignocellulose may be a substrate for the production of value-added products, such as biofuels, biochemicals, and enzymes [2]. Cellulose, hemicelluloses, and lignin present in these wastes can induce the production of lignocellulolytic enzymes [34]. VTS showed higher cellulose content which can induce the production of cellulases by fungi. TPOMW had a low cellulose and hemicellulose content; therefore, it is necessary to mix with other waste to improve the production of LCE. Vinasses can be a good source of N and minerals, and their use as a nutritional supplement for submerged fermentations has already been tested [35].

For this reason, it was considered that it could be interesting to mix TPOMW with other residues in order to reduce the concentration of phenolic compounds and to counteract the lack of nitrogen.

3.2 Screening of substrate composition for SSF using a Placket–Burman design

In this study, selected fungi were inoculated on winery and olive oil mill waste for production of enzymes by SSF. In initial tests, a poor growth of fungi on TPOWM was observed (data not shown). In other work, *A. niger* also exhibited slower growth on TPOWM than on sugar beet waste and rice hulls [36]. Additionally, Aloui et al. [37] observed that *A. niger* was inhibited by TPOMW, but the mixtures of TPOMW with sugarcane bagasse clearly increased the fungal growth. This strong inhibition was probably caused by the high initial concentration of phenolic compounds. In composting processes of TPOWM, long composting periods are needed for this kind of material due to the presence of fats and phenols [38]. The use of other wastes could stimulate the growth of fungi

Table 2 Main characteristics of solid wastes from wineries and olive oil industries

	Solid wastes		
Parameters	TPOMW	Vineshoot trimmings	Exhausted grape marc
C (g/kg)	516.61±15.43	453.56±2.21	482.37±16.07
N (g/kg)	8.59±1.92	5.62±0.71	16.97±6.26
C/N	60.16	80.76	28.42
Cellulose (g/100 g)	6.76±0.24	29.56±0.03	14.37±0.01
Hemicelluloses (g/100 g)	4.18±0.21	9.73±0.01	5.84±0.01
Klason lignin (g/100 g)	58.16±0.41	37.34±0.02	57.67±0.01
Reducing sugars (mg/g)	24.30±1.42	55.35±0.05	3.00±0.01
Protein (mg/g)	0.30±0.03	1.27±0.03	1.30±0.00
Total phenols[a] (mg/g)	2.57±0.04	1.25±0.04	0.19±0.01
Lipids (mg/g)	102.46±0.04	29.6±0.00	21.3±0.00
Moisture (%)	75.31±0.14	6.08±0.09	11.03±0.12
	Liquid wastes		
Parameters	Vinasses		OMWW
Nitrogen (mg/L)	218.67±35.27		5.2±0.2
TOC (g/L)	3.53±0.02		21.4±1.2
COD (g/L)	48.07±1.43		122.9±0.42
Reducing sugars (g/L)	0.68±0.01		12.7±1.3
Protein (g/L)	0.31±0.08		0.04±0.01
Lipids (g/L)	–		4.1±0.42
Total phenols (mg/L)	0.54±0.03		5.91±0.09
Total solids (%)	2.24±0.09		15.43±0.51

TPOMW two-phase olive mil waste, *OMWW* olive mill wastewater, *TOC* total organic carbon, *COD* chemical oxygen demand

[a] In aqueous extract

and the production of enzymes on SSF processes with TPOMW because it could improve the transfer of oxygen and reduce toxicity [37].

To evaluate the effect of TPOMW mixture with other agro-industrial by-products in SSF, a Plackett–Burman experimental design was performed. This design assumes that there are no interactions between the different media components (x_i) in the range of variables under consideration. The following first-order polynomial model was used to describe the results:

$$Y = \beta_0 + \sum \beta_i \cdot x_i \quad (1)$$

where Y is the response value, β_0 is the model intercept, β_i is the linear coefficient, and x_i is the level of the independent variable.

An experimental design for each microorganism was performed; designs were applied with 11 different fermentation conditions as shown in Table 1. This design allowed evaluating the main effect of EGM, OMWW, V, nutrients, urea addition, and time upon enzyme production by SSF.

Several enzyme activities were determined in extracts: cellulases, xylanases, feruloyl esterases, laccases, Mn peroxidases, and lignin peroxidases. Table 3 presents main enzyme activities detected in extract from SSF of the three selected fungal strains. In general, a wide variation can be observed in enzyme activities reported. This variation shows the influence of substrate composition and the importance of medium selection to attain a higher production of enzymes. Table 4 shows the coefficients of regression and coefficients of determination (R^2) of each design. Significant coefficients were identified by an asterisk (*). Regression coefficients allow the determination of the effect of each constituent. A high value of regression coefficient indicates that a factor has a large impact on dependent variable. A R^2 close to 1 indicates a good correlation between the predicted response value and the actual response value. A possible curvature effect was included in the model.

3.3 Lignocellulolytic activity

Comparing LCE production from three different fungi, *A. niger* showed higher activity of FAEs and xylanases, while *A. uvarum* attained the best cellulase activity. The mixture of three solid residues presented the maxima lignocellulolytic activities.

3.3.1 FAE activity

A. niger was the most suitable fungi for FAE production. A maximum activity of 89.53 U/g was attained in experiment 1 (Table 3). Strains of *Aspergillus* section *Nigri* were capable of producing FAEs [23], and Ou et al. [39] tested the production of FAEs from *A. niger* by SSF. They achieved a maximum activity of 7.68 mU/g using mixtures of maize bran and wheat

Table 3 Enzyme activities of experiments with Plackett–Burman design

Runs	*Aspergillus niger*			*Aspergillus ibericus*			*Aspergillus uvarum*	
	CA	XA	FEA	CA	XA	FEA	CA	XA
1	5.20	2.30	89.53	5.50	1.52	12.09	6.77	0.49
2	0.00	0.05	0.00	0.00	0.15	0.00	0.02	0.04
3	0.08	0.02	0.00	0.00	0.11	0.00	0.06	0.03
4	3.36	2.52	58.05	3.76	1.74	7.49	4.50	0.39
5	1.80	3.06	8.51	1.33	1.34	5.90	2.36	0.11
6	0.10	0.03	0.00	0.03	0.05	0.00	0.02	0.09
7	0.11	0.06	0.00	0.10	0.13	0.00	0.01	0.00
8	1.79	1.65	32.10	1.90	0.95	0.51	1.54	0.00
9	1.73	0.42	21.45	2.08	0.213	0.21	3.00	0.08
10	1.62	0.35	18.34	2.03	0.395	0.18	3.35	0.15
11	1.60	0.38	20.51	1.96	0.133	0.23	3.49	0.17

Units are expressed as units per gram of dry solid substrate

CA cellulase activity, *XA* xylanase activity, *FEA* feruloyl esterase activity

bran. FAE production by SSF was also tested using sugar beet pulp as a solid support, and a maximum activity of 5 nkat/g (equivalent to 0.3 μmol of ferulic acid released per minute) was attained on optimal conditions [40]. Figure 1a shows a clear positive effect of urea addition to the fermentation medium. Urea is an organic nitrogen source widely used in biotechnology processes for its low cost. Ou et al. [39] observed that urea and $(NH_4)_2SO_4$ were the best sources of nitrogen for FAE production from *A. niger* in mixtures of wheat bran with maize bran and sugarcane bagasse. Moreover, the use of EGM as substrate for SSF also proved effective for the production of FAEs.

FAE activity was also observed from *A. ibericus*, but not from *A. uvarum* (Table 3). In Table 4, it can be observed that all factors had a significant effect at 95 % on FAE production by *A. ibericus*. It has been demonstrated the potential use of this strain for lipase production [41]; however, their use in the LCE production such as FAEs was not proven.

3.3.2 Cellulase activity

The three studied strains showed endocellulase activity, and maximum activity was achieved with *A. uvarum* (6.77 U/g dry substrate) mixing all solid residue TPOMW, EGM, and VT, after 14 days of fermentation. Thus, it was observed that these solid wastes can induce endocellulase production in SSF. In another work, *A. japonicus*, morphologically close to *A. uvarum*, was used in different substrates and a maximum cellulase activity of 19.38 U/g in wheat bran substrate was observed [42]. So far, no studies on the production of LCEs by *A. uvarum* were found. Figure 1b displays the main effects of substrate composition and time of fermentation on endocellulase activity. Urea and EGM were principal factors that positively influence the endocellulase activity. The effect of OMWW and nutrient supplementation was not significant at 95 %; however, the addition of V was significantly positive at 95 %. Longer fermentation times favored endocellulase

Table 4 Regression coefficients for enzyme activities of *A. niger*, *A. ibericus*, and *A. uvarum*

Terms	*Aspergillus niger*			*Aspergillus ibericus*			*Aspergillus uvarum*	
	CA	XA	FEA	CA	XA	FEA	CA	XA
Constant	1.555[a]	1.209[a]	23.524[a]	1.578[a]	0.748[a]	3.248[a]	1.909[a]	0.143[a]
Curvature	0.095	−0.828[a]	−3.424	0.446[a]	−0.501[a]	−3.042[a]	1.365[a]	−0.018
EGM	0.605[a]	0.012	13.372[a]	0.738[a]	0.131	1.647[a]	0.928[a]	0.094[a]
VT	0.22[a]	0.150[a]	0.986	0.138[a]	0.016	1.249[a]	0.384[a]	0.039
OMWW	0.243[a]	0.149[a]	0.986	0.155[a]	0.026	1.249[a]	0.174	−0.01
Vinasses	0.22[a]	−0.196[a]	6.884[a]	0.298[a]	−0.062	−0.099[a]	0.390[a]	0.014
Nutrients	0.234[a]	−0.213[a]	6.884[a]	0.28[a]	−0.091	−0.099[a]	0.187	0.009
Urea	1.483[a]	1.172[a]	23.523[a]	1.545[a]	0.639[a]	3.248[a]	1.882[a]	0.104[a]
Time	0.6375[a]	0.016	13.372[a]	0.77[a]	0.111	1.648[a]	0.914[a]	0.01[a]
R^2	0.9996	0.9998	0.9994	0.9998	0.9914	0.9999	0.9974	0.9807
R^2 corrected	0.9981	0.9992	0.9969	0.9988	0.9570	0.9999	0.9870	0.9035

CA cellulase activity, *XA* xylanase activity, *FEA* feruloyl esterase activity

[a] Significant coefficient at the 95 %

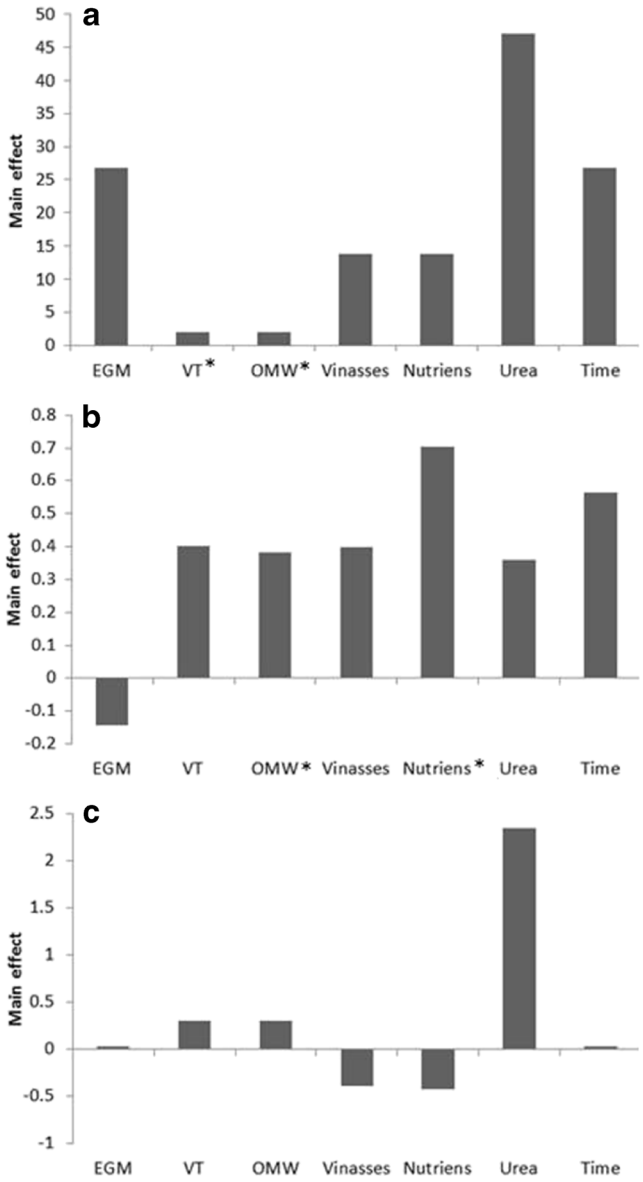

Fig. 1 The main effects of the substrate composition on **a** feruloyl esterase activity from *A. niger*, **b** cellulase activity from *A. uvarum*, and **c** xylanase activity from *A. niger*. *Asterisk* represents not significant variables

production; this may be due to the need of a prior action of the xylanases to expose the cellulose fibers, which will induce cellulase production. With *Aspergillus awamori*, xylanase activity was detected at the beginning of fermentation, then cellulase activity increased rapidly and maximum activity remained several days, but the xylanase activity declined rapidly [43]. Production of cellulases by other fungi as *Trichoderma* spp. has been widely studied. Cellulases and hemicellulases from *Trichoderma reesei* are the most commonly used in industry [44]. Florencio et al. [45] studied the production of endocellulases by different strains of *Trichoderma* (*Trichoderma harzianum* and *T. reesei* RUT C30) using sugarcane bagasse and wheat bran and observed that mutant strain (*T. reesei* RUT C30) showed higher activity (70.24 UI/g) than the other strains (18–25 UI/g).

3.3.3 Xylanase activity

Table 3 shows that *A. niger* achieved maximum endoxylanase activity (3.06 U/g of dry substrate) in run 5. It is common to use *A. niger* to produce xylanase. Kavya et al. [46] observed a maximum xylanase activity of 12.65 U/mL on wheat bran as a substrate. However, *Trichoderma longibrachiatum* is the most commonly used, which achieved high xylanase activities [47]. Endoxylanase production benefited from a strong effect of urea addition (Fig. 1c). Accordingly, a positive effect of urea in enzyme production has been observed in other works; xylanase activity was increased with urea and other nitrogen source in SSF by *A. niger* [48]. Accordingly, urea was recognized as the suitable nitrogen source along with yeast extract and $NaNO_3$ [49]. The effect of time and mixture of TPOMW with EGM were not significant at 95 %. Usually, short fermentation times are needed for xylanase production by SSF. Xu et al. [49] achieved maximum activity at 48 h. The lower endoxylanase activity detected in our study may be due to the lost activity in consequence of fermentation times used, 7 or 14 days.

Endoxylanases from *A. ibericus* and *A. uvarum* showed lower activity than *A. niger*. However, it is common to use enzyme cocktails with xylanase and cellulase activities for paper pulp industry [50]. Thereby, suitable strains for cellulase production like *A. uvarum* can have industrial interest, despite having a reduced production of xylanases.

3.3.4 Ligninase activity

Other enzymes were detected in some experiments. In the media with TPOMW, VT, OMWW, and urea (experiment 5) fermented by *A. niger*, a low activity of Mn peroxidases, laccases, and lignin peroxidases (0.64, 2.47, and 0.18 U/g of dry substrate) was observed. These enzymes can degrade lignocellulosic materials and, in addition, recalcitrant environmental pollutants, such as crude oil wastes, textile effluents, organochloride agrochemicals, and pulp effluents [51]. White rot fungi are the most common producers of laccases. *Trametes versicolor* is a good producer of laccases. In the SSF of corn stover, *T. versicolor* achieved a high laccase acivity (45.1 IU/g) under the optimized conditions; however, low xylanase and endocellulase and no Mn peroxidase and lignin peroxidase activity was detected [52].

4 Conclusions

Different enzymes were produced according to the substrate used. Mixture of olive mill wastes with winery wastes favors

the production of endocellulases, endoxylanases, and feruloyl esterases. The effect of each waste on LCE production was studied by the Plackett–Burman experimental design. In all experimental designs, good determination coefficients close to 1 were obtained. *A. niger* was more effective in producing hemicellulase enzymes such as FAEs and endoxylanases, whereas, maximum endocellulase production was achieved by *A. uvarum*. Urea showed to have a significant effect on LCE production in the three strains. Longer times of fermentation improved endocelullase and FAE production, without affecting endoxylanase production. In general, all mixtures of winery and olive mill wastes were suitable substrates for SSF. Thereby, the SSF of these wastes can be an interesting approach for its valorization.

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