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RESEARCH ARTICLE

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Integrated biovalorization of wine and olive mill by-products to produce enzymes of industrial interest and soil amendments

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Abstract

An integral and affordable strategy for the simultaneous production of lignin-modifying and carbohydrate active enzymes and organic amendment, with the aid of a saprobe fungus was developed by using olive oil and wine extraction by-products. The polyporal fungus *Trametes versicolor* was cultivated in soy or barley media supplemented with dry olive mill residue (DOR) as well as with grape pomace and stalks (GPS) in solid state fermentation (SSF). This strategy led to a 4-fold increase in the activity of laccase, the principal enzyme produced by SFF, in DOR-soy media as compared to controls. *T. versicolor* managed to secrete lignin-modifying enzymes in GPS, although no stimulative effect was observed. GPS-barley media turned out to be the appropriate medium to elicit most of the carbohydrate active enzymes. The reuse of exhausted solid by-products as amendments after fermentation was also investigated. The water soluble compound polymerization profile of fermented residues was found to correlate with the effect of phytotoxic depletion. The incubation of DOR and GPS with *T. versicolor* not only reduced its phytotoxicity but also stimulated the plant growth. This study provides a basis for understanding the stimulation and repression of two groups of enzymes of industrial interest in the presence of different carbon and nitrogen sources from by-products, possible enzyme recovery and the final reuse as soil amendments.

Additional key words: dry olive mill residue; grape pomace and stalks; lignin-modifying enzyme; carbohydrate active enzymes; *Trametes versicolor*; phytotoxicity.

Abbreviations used: CMC-ase (endoglucanase); DOR (dry olive mill residue); EEZ (Estación Experimental del Zaidín); GH (glycoside hydrolases); GPS (grape pomace and stalks); HPSEC (high-performance size-exclusion chromatography); LiP (lignin peroxidase); Lac (laccase); LME (lignin-modifying enzyme); MnP (manganese peroxidase); Po-ase (endopolymethylgalacturonase); RDW (root dry weight); SDW (shoot dry weight); SSF (solid state fermentation); Xyl-ase (xyloglucanase).

Authors' contributions: Conceived and designed the experiments: IGR, CL and EA. Performed the experiments: RR and RU. Analyzed the data: RR, IGR, CL, and EA. Contributed reagents/materials/analysis tools: RU, CL, IGR and EA. Wrote the paper: RR, IGR, CL and EA.

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Introduction

The development of agro-industrial activity over the last 100 years has increased the amount of lignocellulosic products, representing a serious environmental problem in producing countries (Sánchez, 2009). In order to consider a circular economy aiming at "zero waste" society, the wastes through mechanical, chemical or biological processes are used as raw material for new products and applications (Mateo & Maicas, 2015).

According to FAO data, the most commonly produced commodities in Spain are beer of barley, wine as well as olive and soybean oil (http://faostat.fao.org/site/339/default.aspx; http://www.oiv.int/es/bases-de-datos-y-estadisticas/estadisticas). As a result of these agro-industrial activities, millions of tonnes of these wastes are produced every year, which need to be managed appropriately in order to avoid adverse environmental effects. "Alpeorujo" or dry olive residue (DOR) is a phytotoxic by-product resulting from

the process of olive oil production which involves a second hexane extraction to reduce oil content (Alburquerque *et al.*, 2004), leading to a low-humidity phenol-rich material. DOR also contains celluloses and hemicelluloses rich in xylans and xyloglucans (Jiménez *et al.*, 2000) as well as soluble carbohydrates like mannitol, sucrose and fructose (Alburquerque *et al.*, 2004) which make it a suitable substrate for fungal growth. The valorisation options for dealing with DOR includes the production of biofuel, biogas, organic amendment, animal feed, enzymes, polymer and phenol production and other (Dermeche *et al.*, 2013). Grape pomace and stalks (GPS) are the main by-products generated by the wine industry. Though not toxic residue themselves, their high organic matter content and the seasonally high production levels can potentially lead to environmental problems (Spigno *et al.*, 2013). GPS have high tannin content, thus making it a potentially rich source of antioxidants, and quite high hemi-cellulose content mainly composed of xyloglucans. Some studies have proposed that grape stalks be used as bio-sorbent material for the removal of toxic compounds, as composting or to obtain high added-value compounds such as phenolic compounds with antioxidant activity, lignin and cellulose (Spigno *et al.*, 2008).

Microbiological processes for the treatment of wastes have seen worldwide application, and they are considered to be environmentally friendly, reliable and, in most cases, cost effective. Some fungi are among the known organisms that can oxidize lignin compounds (Kirk & Farrell, 1987), although their purpose is probably to gain access to hemicellulose and cellulose in order to use them as carbon sources (Ten Have & Teunissen, 2001). White-rot basidiomycetes are considered to be the most extensive degraders of lignin which they are able to completely break down to carbon dioxide and water due to their oxidative enzymatic mechanisms (Papadopoulos, 2012). These fungi are able to secrete a set of oxidative enzymes such as lignin peroxidase (LiP) (EC 1.11.1.14) and manganese peroxidases (MnP) (EC 1.11.1.13) as well as laccases (Lac) (EC 1.10.3.2) among other, which present broad substrate specificity, allowing them to gain access to the 3D phenylpropanoid network. In addition, carbohydrate active enzymes such as hydrolytic enzymes play an important role in the conversion of lignocelluloses among other functions. Thus, glycoside hydrolases (GH) such as endoglucanases, xyloglucanases and endopolymethylgalacturonase (EC 3.2.1) participate in the hydrolysis of cellulose and hemicellulose breaking the non-covalent interactions present in the amorphous cellulose polymer, the most abundant in plants (Coughlan, 1985).

Lignin-modifying enzyme (LME) and GH enzymes seem to be a promising tool for solving numerous environmental problems. However, their industrial application is limited by the high cost, limited operational stability and low output of these enzymes when synthetic media are used. Thus, the utilization of natural substrates, such as agro industrial by-products, could be an alternative means of obtaining higher yields for purification purposes. DOR has been successfully used for lipases, laccases, Mn-dependent peroxidases and pectinases (Cordova *et al.*, 1998; Sampedro *et al.*, 2012). Winery by-products are also used to produce pectinolytic and cellulolytic enzymes (Romero *et al.*, 2007).

Although it is difficult to comprehend the gene regulation of ligninolytic enzymes in filamentous fungi, the enhancement of hydrolytic enzymes by small inducer molecules liberated from cellulosic biomass is clearly understood (Tani *et al.*, 2014). Nevertheless, the effect of complex polymers on the induction or repression of lignocellulolytic enzymes and, most importantly, their high yields for industrial application require further explanation (Kapich *et al.*, 2004; Amore *et al.*, 2013).

Some saprobic fungi which LME and GH enzymes, such as *Fusarium* spp, *Phanerochaete* spp, *Pleurotus* spp and *Trametes versicolor*, have been used for biopulping or fungal pretreatment of wood for the production of mechanical or chemical pulps, forage upgrading, bioremediation of soils, wastes and recalcitrant contaminants (Wan & Li, 2012). The conspicuous polyporal *T. versicolor* produces several Lacs, class II peroxidases and dye-decolorizing peroxidases, but information concerning the simultaneous production of hydrolytic and oxidative enzymes is scarce (Elisashvili & Kachlishvili, 2009; Carabajal *et al.*, 2013). *T. versicolor* is a well-known fungus on which genomic and proteomic studies have already been carried out (Floudas *et al.*, 2012) enabling us to gain a rough understanding of the protein set capable of secretion. This has provided us with a starting point to determine which components trigger these activities, since one of the biggest challenges facing the biotechnology industry is to find a suitable expression medium to activate enzymatic production and subsequently to improve their marketing.

Traditionally, most studies related with wastes bioremediation have been focused on obtaining a single high-value product discarding the possibility of a double valorisation. Thus, the objective of this work was to study for the first time the use of two agronomic wastes, DOR and GPS, as source of both enzymes and agronomic amendment. In this study it was analysed firstly the use of phenol-rich agricultural residues as a

basal medium for growth microorganisms and enzyme production. However, as the effects of these enzymes are the elimination of the phenol fraction, which are mainly responsible for the phytotoxicity cause, we also determined the potential agronomic applications of the DOR and GPS after fungal treatment. In the case of DOR, the exhausted residues, according to different fungal treatment, usually presents less phytotoxicity that the original one (Reina *et al.*, 2014), being suitable for a second valorisation using them as soil amendment. However, little information about fungal bioremediated GPS is available, and its potential use as soil amendment.

For this reason, the aim of this study was to find a suitable method for a double valorisation of both agricultural by-products DOR and GPS, firstly trying to possibly use them as natural enhancers for enzyme production by the well-known fungus *T. versicolor* and investigating secondly the importance of exhausted residue for application as an amendment.

Material and methods

By-products

DOR was obtained from the Sierra Sur olive oil company in Granada, Spain (2009-2010 harvest). It was sieved, autoclaved in three cycles and stored at 4 °C before use.

GPS from a cultivar in La Herradura (Granada, Spain) were kindly provided by wineries in Southern Andalucía during the vintage 2012. GPS was generated from a mixture of grapevine varieties ('Moscatel', 'Garnacha', 'Garnacha Tintorera', 'Monastrel', 'Airen', 'Jaen black' and 'Jaen white'). The samples were collected immediately after the pressing operation and dried for 1 week at 40 °C. The dried stalks and pomace were then milled, autoclaved in three cycles and stored at 4 °C.

Organisms and fungal inoculum

DOR and GPS degradation studies require the preculture of the polyporal fungus *T. versicolor*. The strain (JAO-EEZ 13) was obtained from the culture collection of the Estación Experimental del Zaidín, CSIC, Granada, Spain. *T. versicolor* was cultivated at 25 °C on 2% malt extract agar (MEA) plates for 1 week to obtain fresh inoculum. To produce the inoculum for the experiment, an agar plate containing fungal mycelia was homogenized in 80 mL of a sterile water and the suspension was added to the solid medium (0.5% v/w).

Solid state fermentation (SSF)

Solid state fermentation was performed using barley and soy as organic support according to Reina *et al.* (2013). One third of the flasks were mixed with DOR (50% w/w), another third with GPS (28% w/w) and the remaining flasks were kept as barley and soy controls. The amount of by-products added was previously optimized (data not shown). The same treatments were carried out on the heat-inactivated mycelium as control in order to detect any possible phenol adsorption. The inoculated flasks were harvested after 0, 1, 2, 3, 4 and 5 weeks after the addition of DOR and GPS until the end of the experiment. DOR and GPS were manually separated from barley and soy and dried for 3 days in an oven at 60 °C for phytotoxicity experiments (Reina *et al.*, 2013). Before drying, an aliquot was separated to obtain an aqueous extract using distilled water (1:5 w/v) by shaking on a rotary shaker for 2 hours. The extracts were centrifuged, filtered and used to measure extracellular enzyme activity, pH, total phenol content and the molecular mass distribution of water-soluble aromatic fragments released from DOR and GPS by fungal treatment.

Enzyme assays

MnP was measured by monitoring the formation of Mn^{3+}-malonate complexes at 270 nm (ε_{270nm}: 11.59 mM/cm) using Na-Malonate buffer at pH 4.5 (Wariishi *et al.*, 1992). LiP was monitored using veratryl alcohol at 310 nm (ε_{310nm}: 9.3 mM/cm) using tartrate buffer at pH 3.0 and H_2O_2 (final concentration 0.1 mM) (Liers *et al.*, 2011). Laccase was determined by monitoring the oxidation of 2,20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ε_{420nm}: 36 mM/cm) using a combination assay in 50 mM Na-Malonate buffer at pH 4.5 (Wolfenden & Willson, 1982). Ligninolytic enzyme activities were expressed as U/g of DOR or GPS in SSF experiments. A unit of activity is defined as the amount of enzyme that catalyses the conversion of 1 µmol of substrate/minute. Mean values of triplicate measurements were calculated and error bars represent standard deviation.

The GH activities endoglucanase (CMC-ase) (EC 3.2.1.4), xyloglucanase (Xyl-ase) (EC 3.2.1.151) and endopolymethylgalacturonase (PO-ase) (EC 3.2.1.15) were determined with the aid of the viscosimetry method (Rejón-Palomares *et al.*, 1996). The substrates used were carboxymethylcellulose, xyloglucan extracted from *Tropaeolum majus* seeds and citrus pectin, respectively (McDougall & Fry, 1989). The reaction mixture contained 1 mL of substrate (0.3% carboxym-

ethylcellulose, 0.3% xyloglucan and 1% pectin for each enzyme in a 50 mM citrate–phosphate buffer (pH 5)) and 0.1 mL of sample. The reduction in viscosity was determined at intervals of 0-30 min by the changes in drainage time using a calibrated 0.1 mL syringe. Reactions were performed at 37 °C. Enzyme activity was expressed as relative activity (RA) calculated as the inverse of the time needed to reduce viscosity by 50% according to the following formula $RA=1/T_{50}\cdot 1000$, being $T_{50}=50T_{30}/\%X$ and $\%X=(T_0-T_{30}/T_0)\cdot 100$ (Rejón-Palomares *et al.*, 1996).

Total phenol content of the aqueous extracts was determined in triplicate spectrophotometrically by using the method described by Ribéreau-Gayon *et al.* (1968) using tannic acid for the standard curve.

Total sugar content was assessed in triplicate using an anthrone-modified method described by Dubois *et al.* (1956). The diluted sample (1 mL) was added to 5 mL of anthrone reagent, mixed and immediately heated at 30 °C for 10 min and rapidly cooled in iced water. Absorbance was measured at 620 nm, and total sugar content was calculated based on a glucose standard curve.

Mineral composition of DOR and GPS

Mineral quantitative determination of DOR and GPS was carried out using inductively coupled plasma-optical emission spectrometry (Varian ICP 720-ES model), and nitrogen and carbon were determined by the Dumas method (Kirsten & Grunbaum, 1955) using a Leco TruSpec CN analyzer.

Chromatographic analyses

DOR and GPS residue were milled to powder to evaluate the phenolic composition. Samples (0.5 g) were extracted with methanol/water according to the method described by Sampedro *et al.* (2004). Phenols in DOR and GPS were analyzed by ultra performance liquid chromatography-mass spectrometry (Acquity UPLC System, Waters) by the Scientific Instrumentation Centre of the University of Granada (CIC, Granada, Spain) using a Waters ACQUITY UPLC™ HSS T3 column (2.1 × 100 mm, 1.8 µm). Acetonitrile and acetic acid (0.5%) were used in a gradient of 5 to 95% (until min 15.0) and from 95 to 5% (from min 15.0 to 15.1). The detector used was a high definition spectrophotometer (SYNAPT G2 HDMS Q-TOF, Waters). Measurements were performed by using negative electrospray ionization. Column temperature, flow and injection volume were 40 °C, 0.4 mL/min and 10 µL, respectively. Phenol concentrations were expressed as the mean value of the four biological replicates and errors were expressed as standard deviation.

The analysis of aromatic water-soluble DOR and GPS fragments were done by HPSEC. Separation by size exclusion was performed using a HP 1090 LC (Hewlett–Packard, Waldbronn, Germany) equipped with a diode array detector (HP 1100) according to the Liers *et al.*'s (2011) protocol using a HEMA-Bio linear column (8 × 300 mm, 10 µm) from Polymer Standard Service (Mainz, Germany).

Determination of phytotoxic effect of agro-industrial by-products in *Solanum lycopersicum* plants

The phytotoxic experiment was performed as previously described by Reina *et al.* (2013). Fermented DOR and GPS after different incubation periods and the non-inoculated negative control (DOR and GPS 5% v/v) were applied to the 300 mL pots of soil. No residue was added to positive plant controls.

A completely randomized design was used for the experiments. Tomato plants were grown with two different residues to the following treatments: controls without residue, controls with the unfermented residue and residues treated with *T. versicolor* during 0, 1, 2, 3, 4 and 5 weeks with 4 replicates per treatment. After the different times, tomato plants were harvested. The shoot and root dry weight were measured after being oven-dried for 48 hours.

Statistics

The statistics were analyzed using the IBM SPSS Statistics 21 program. The Shapiro-Wilk and Levene tests were used to check normality and variance homogeneity, respectively. Depending on the Levene test results, different posthoc tests were carried out The Tukey test was used for homogeneous variances and the Dunnet test for non-homogeneous variances.

Results

Chemical characterization of DOR and GPS

Table 1 provides information on the mineral composition of DOR and GPS. The analyses performed by ICP show variations in the composition of minerals such as Al, B, Ca, Cd, Cr, Cu, Fe, K, Ni, Zn, Li, Ti and V, which were present in DOR in considerably higher concentrations. On the other hand, concentra-

Table 1. Mineral composition of dry olive mil residue (DOR) and grape pomace and stalks (GPS). Data represents means of three replicates and ± standard deviation.

Component	DOR	GPS
Al (ppm)	787.89 ± 12.3	54.20 ± 2.64
B (ppm)	30.12 ± 2.08	Undetermined
Ca (%)	0.62	0.36
Cd (ppm)	<0.5	<0.025
Cr (ppm)	4.31 ± 0.84	2.89 ± 0.31
Cu (ppm)	14.41 ± 2.51	5.95 ± 0.54
Fe (ppm)	566.37 ± 22.5	93.73 ± 2.63
K (%)	2.12	0.68
Mg (%)	0.16	800.85
Mn (ppm)	16.45 ± 1.22	12.85 ± 0.45
Na (%)	0.03	62.38
Ni (ppm)	2.38 ± 0.14	1.23 ± 0.068
Pb (ppm)	0.53 ± 0.05	0.59 ± 0.03
P (%)	0.20	0.21
S (%)	0.13	Undetermined
Zn (ppm)	31.50 ± 1.42	10.28 ± 0.54
Hg (ppm)	<0.025	<0.025
Li (ppm)	0.62 ± 0.015	0.05 ± 0.011
Mo (ppm)	<0.025	<0.025
As (ppm)	0.89 ± 0.025	1.27 ± 0.094
Co (ppm)	<0.025	<0.025
Se (ppm)	<0.025	<0.025
Ti (ppm)	17.77 ± 0.76	3.37 ± 0.27
V (ppm)	0.69 ± 0.10	0.08 ± 0.01
N (%)	1.57	0.93
C (%)	51.47	43.69
Organic C (%)	49.2	41.31
Total phenols (mg/g)	16.42 ± 0.84	6 ± 0.75
Total sugar content (mg/g)	174.50 ± 3.21	260.04 ± 10.26

tions of Mg and Na were much greater in GPS than in DOR. Total phenolic content in both these by-products differs considerably, being nearly 3-fold higher in DOR (16.42 mg/g) as compared to GPS (6 mg/g). The main phenols present in DOR (Table 2) were p-tyrosol, hydroxytyrosol and protocatechuic acid (105, 128, 140 µ/g DOR, respectively) whereas, in GPS, only three simple phenols were detected and quantified: hydroxytyrosol, protocatechuic acid and gallic acid (17, 18 and 133 µg/g GPS, respectively). Gallic acid was not detected in DOR samples (Table 2).

Total sugar content was 174 mg/g in DOR and 260 mg/g in GPS at the beginning of fermentation (Table 1).

Enzyme secretion

T. versicolor showed a noteworthy capacity to secrete Lac, especially in soy-based cultures supplemented with DOR, peaking at 42.6 U/g in SSF cultures after 5 weeks (Fig. 1). When grown in a DOR-barley medium, maximum Lac activity was found to be

Table 2. Abundance of simple phenols in dry olive mil residue (DOR) and grape pomace and stalks (GPS) analysed by LC-MS. Data represents means of three replicates and ± standard deviation.

Phenol content (µg/g of DOR or GPS)	DOR	GPS
Gallic acid	ND	133 ± 10
Protocatechuic acid	140 ± 10	18 ± 3
Hydroxybenzoic acid	100 ± 9	ND
p-tyrosol	105 ± 10	ND
Caffeic acid	47 ± 8	ND
Syringic acid	81 ± 8	ND
Ferulic acid	31 ± 3	ND
Methoxycinnamic acid	42 ± 3	ND
3,4-Dimethoxycinnamic acid	90 ± 5	ND
Hydroxytyrosol	128 ± 10	17 ± 1

ND: not detected.

2.4 U/g in the second week of incubation but a non-stimulating effect of this activity was observed for GPS-soy and barley. The highest MnP activity detected was 1.3 U/g in DOR-soy cultures at week 3 of fermentation, with no significant differences being found with respect to cultures to which GPS had been added. The maximum LiP activity was found after grown *T. versicolor* during 2 and 3 weeks in DOR-barley cultures (1.9 U/g) which showed a similar pattern to that of Lac. The activity levels found in GPS-barley cultures were unremarkable. The 80% of phenol conversion occurred within two weeks of incubation in both cases. The pH remained stable for 5 weeks in DOR-barley cultures and recorded a slight increase in barley-control cultures. However, pH in soy based cultures rose more sharply from 4.8 to 7.2.

There was no significant difference in the CMC-ase activity of *T. versicolor* during SSF culture between soy and DOR-soy cultures (Table 3). Despite this activity was found to increase after 2 weeks of incubation with both medium, CMC-ase activity in GPS-supplemented soy cultures was lower. The CMC-ase secretion profile was different in the three barley-based media used. Although maximum activity levels in barley and soy did not differ substantially, the CMC activity in DOR and GPS-supplemented barley cultures was higher than in the soy medium. The higher stimulation was found at week 4 of fermentation where this activity was 2-fold higher in GPS than in barley control cultures.

Among the three hydrolytic activities measured, Xyl-ase showed the highest values, especially in DOR-soy and soy cultures where maximal activity levels were reached during weeks 2 and 3 of fungal growth, peaking at 150 and 134, respectively (Table 3). Xyl-ase activity measured in GPS-soy cultures was considerably lower than that in soy control and DOR-soy cul-

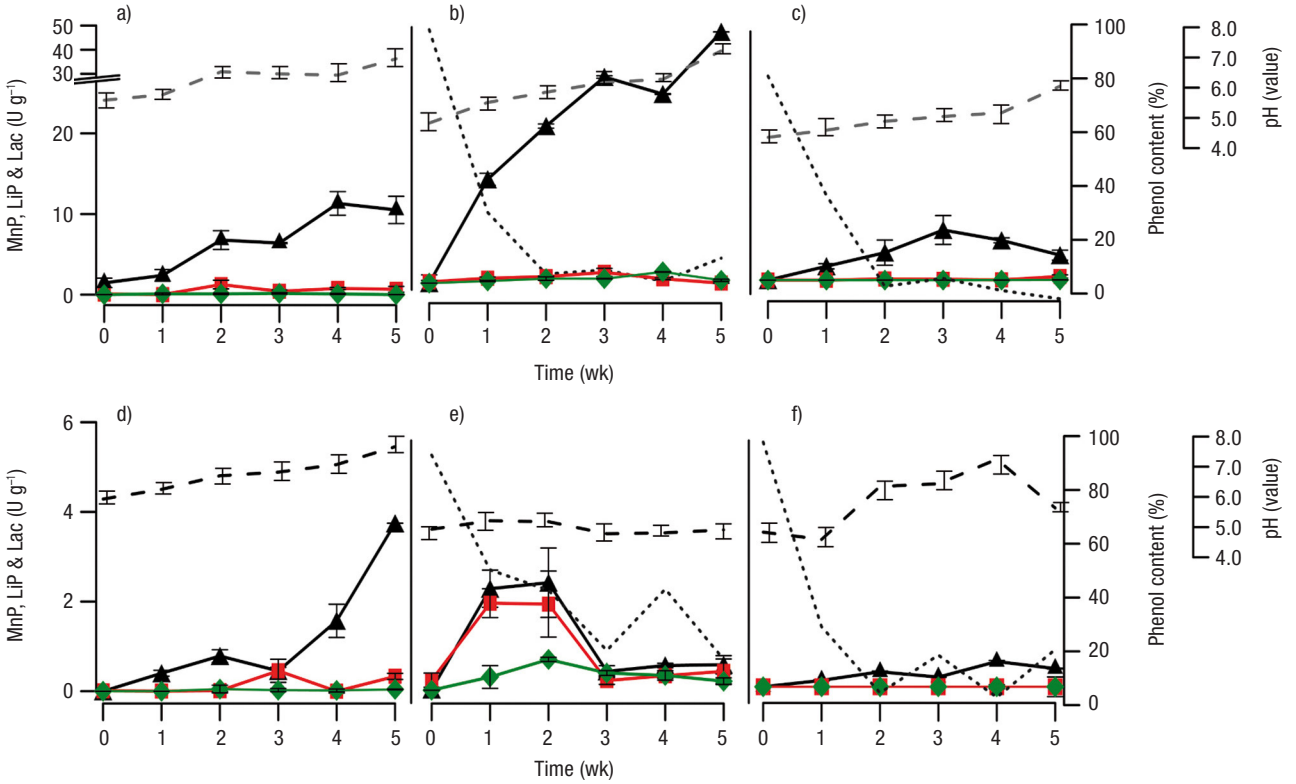

Figure 1. Time course of manganese peroxidase (MnP), laccase (Lac) and lignin peroxidase (LiP) activity of the polyporal fungus *T. versicolor* during solid state fermentation in soy (a), dry olive mill residue (DOR)-soy (b), grape pomace and stalks (GPS)-soy (c), barley (d), DOR-barley (e) and GPS-barley (f). Enzyme activities (Lac, *triangles*; LiP, *diamonds*; MnP, *squares*), total percentage of phenol content (*dotted line*), and pH value (*dashed line*).

Table 3. Carboxymethylcellulase (CMC-ase), xiloglucanase (Xyl-ase) and endopolymethylgalacturonase (Po-ase) relative activity in 30 minutes of *T. versicolor* reaction during solid state fermentation (SSF) in soy, dry olive mil residue and soy (DOR-soy), grape pomace and stalks and soy (GPS-soy, barley), dry olive mil residue and barley (DOR-barley) and grape pomace and stalks and barley (GPS-barley).

Week	Soy	DOR-soy	GPS-soy	Barley	DOR-barley	GPS-barley
			CMC-ase			
0	7 ± 1 a	1 ± 3 a	3 ± 1 a	37 ± 9 ab	0 a	0 a
1	6 ± 2 a	9 ± 2 b	14 ± 0.6 b	25 ± 4 a	50 ± 7 d	32 ± 0.4 c
2	38 ± 10 b	31 ± 10 c	5 ± 2 a	50 ± 10 b	74.9 ± 0.4 e	16 ± 5 b
3	38 ± 10 b	45 ± 5 d	4 ± 1 a	40 ± 10 b	25 ± 5 c	52 ± 6 c
4	38 ± 1 b	34 ± 5 c	5 ± 2 a	52 ± 5 bc	18 ± 7 c	96 ± 6 d
5	29 ± 10 b	27 ± 4 c	40 ± 10 c	53 ± 6 bc	6 ± 3 b	91 ± 5 d
			Xyl-ase			
0	42 ± 8 b	2 ± 1 a	51 ± 0.9 c	52 ± 3 c	2.3 ± 0.7 a	0 a
1	34 ± 6 a	122 ± 6 b	5.1 ± 0.7 a	40 ± 10 b	96 ± 4 e	4 ± 2 ab
2	150 ± 10 d	120 ± 30 b	44 ± 8 c	20 ± 6 a	64 ± 7 d	53 ± 1 c
3	79 ± 8 c	134 ± 8 bc	58 ± 6 c	14 ± 3 a	37 ± 2 c	80.3 ± 0.9 d
4	90 ± 30 c	115 ± 4 b	21 ± 7 b	31 ± 6 b	14 ± 7 b	91 ± 10 d
5	48 ± 1 b	100 ± 20 b	14.5 ± 0.9 b	13 ± 3 a	10 ± 4 b	50 ± 3 c
			Po-ase			
0	7 ± 1 a	1 ± 1 a	0 a	7 ± 3 b	0 a	0 a
1	6 ± 2 a	9 ± 6 b	2.9 ± 0,7 a	7 ± 1 b	14 ± 7 b	3 ± 1 b
2	40 ± 10 c	30 ± 3 c	4.2 ± 0.4 b	6 ± 1 b	3.2 ± 0.8 a	5 ± 1 b
3	40 ± 10 c	44 ± 8 d	17 ± 8 c	2 ± 0,1 a	5 ± 2 a	10 ± 2 c
4	37 ± 4 c	34 ± 4 c	5 ± 3 b	6 ± 1 b	3 ± 1 a	28.2 ± 0.9 d
5	28.6 ± 0.5 b	27 ± 1 c	7 ± 3 b	7 ± 5 b	5 ± 1 a	31.4 ± 0.9 e

tures. As in the case of CMC-ase activity in barley-based cultures, no reduction in Xyl-ase activity occurred in GPS-barley media. This activity peaked at 52, 96 and 91 at week 0, 1 and 4 in barley, DOR-barley and GPS-barley media, respectively.

PO-ase activity was notably lower than the CMC-ase and Xyl-ase in GPS-soy media and also this activity was lower than the DOR-soy and soy cultures, as the case of CMC-ase and Xyl-ase activities. Peak activity in all the soy-base media was reached in week 3 of incubation but DOR or GPS not stimulated this activity. Again, this did not occur in barley-based media, where activity in *T. versicolor* SSF culture measured during week 4 and 5 in GPS-barley cultures was 5-fold higher than in DOR-barley and barley cultures. PO-ase activity in barley media was lower than the other hydrolases measured when comparing equal treatments.

Water soluble aromatic fragment distribution

There was a clear shift in the polymerization profile of DOR treated with *T. versicolor* using soy as a support medium (Fig. 2a). In unfermented control samples, the highest peak contained fragments of 1.12 kDa after 5 weeks of DOR incubation, while there was a noticeable increase in water soluble aromatic compounds to 73.4 kDa. This trend was not observed when DOR was fermented using barley as a support medium (Fig. 2c), with fragment size distribution remaining stable throughout the incubation process, although a decrease in peak heights was observed.

In contrast to HPSEC DOR profiles, GPS control profiles showed a lower signal, with the highest peak containing fragments ~1 kDa. As the weeks went by, HPSEC GPS profiles were lower and more irregular when soy was used as a support medium (Fig. 2b). A decrease in fragment size occurred, peaking at ~0.65 kDa in the control sample with unfermented DOR and at ~0.28 kDa after 5 weeks of incubation. On the other hand, in fermented GPS in barley support media, the highest peak shifted from ~0.99 kDa in control samples to ~2.82 kDa after 5 days of incubation (Fig. 2d).

Plant phytotoxicity

Data obtained from *Solanum lycopersicum* phytotoxicity experiments followed a normal distribution. Variances were homogenous according to the Levene

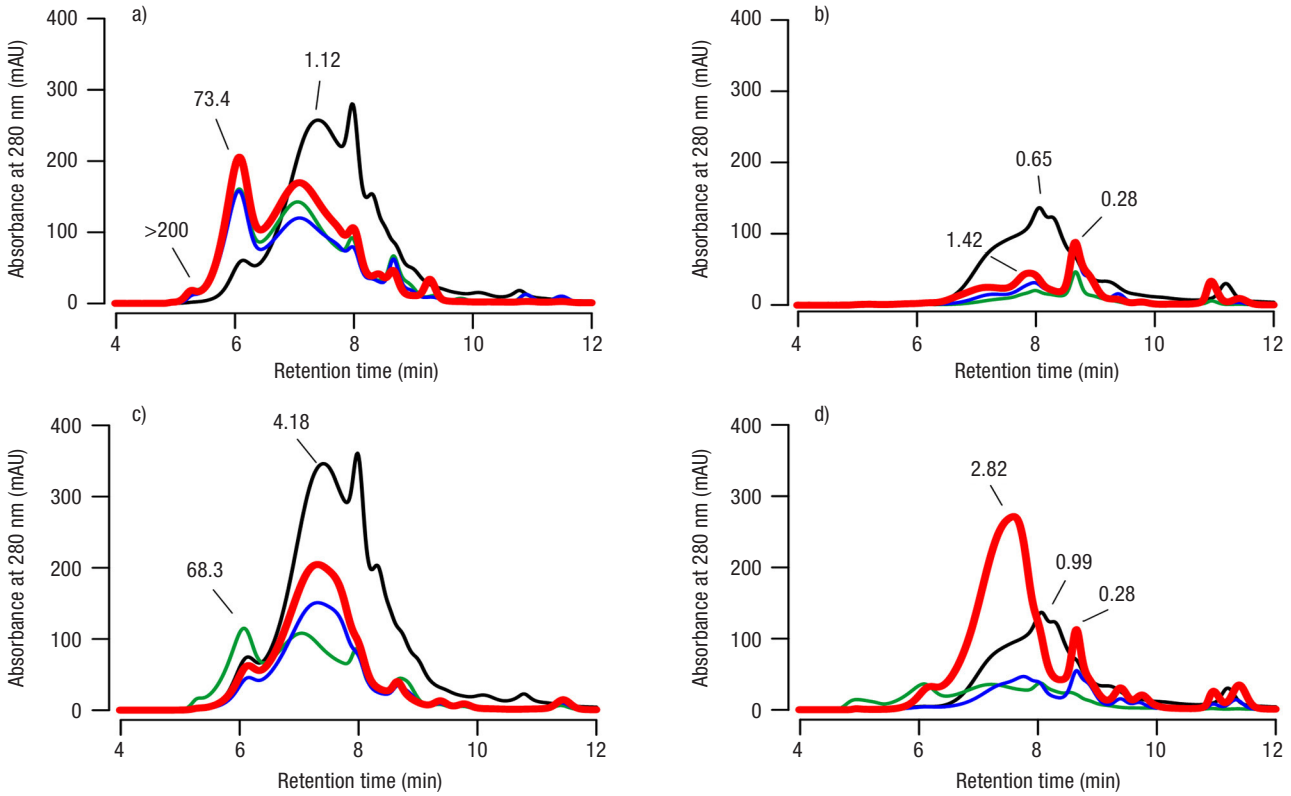

Figure 2. Changes in the molecular mass distribution of water-soluble aromatic fragments formed by *T. versicolor* during solid state fermentation in dry olive mill residue (DOR)-soy (a), grape pomace and stalks (GPS)-soy (b), DOR-barley (c) and GPS-barley (d) media. Unfermented DOR and GPS (*black line*) and residues after 1 (*green line*), 3 (*blue line*) and 5 (*red thick line*) weeks of fermentation. Numbers represent peak fragment size in kDa.

test (0.05 significance). The reduction in DOR phytotoxicity began at week 1 in DOR-soy based media with no significant differences between the shoot dry weight (SDW) and root dry weight (RDW) of the DOR-soy treatment at this time with control samples (Fig. 3a). However, the decrease in GPS phytotoxicity started at week 3 even though the reduction in phenols was similar to that in DOR (Fig. 3b). In DOR-barley media, this phytotoxic effect was dissipated at week 3 of incubation in both SDW and RDW (Fig. 3c), and there were no significant differences between dry weights of plants treated with DOR fermented for 3 and 5 weeks with *T. versicolor* in barley based media and tomato controls (Fig. 3d). The weights for tomato plants, which were treated with fermented GPS in a barley support media (Fig. 3d) gradually grew as incubation time increased. At week 4 of incubation with *T. versicolor* in a barley-based media, the phytotoxicity of GPS was totally eliminated.

Discussion

Different white-rot fungi have been used to valorise agro-waste residues from the olive oil industry and wine by-products, such as stalks and seeds. In most cases, these studies mainly focus only to obtain only one products of added value from the residue. For example it has been studied the enzyme production from an agro-waste disregarding the analysis of those by-products that are further obtained from the originally exhausted ones. In this work it has been optimized an integral valorisation process, in which not only it has been studied the secretion of enzymes in depth, but also it has been analysed the final by-products that were obtained to measure their possible impact in the natural environment.

To this end *T. versicolor* was used. It is a well-known polyporal agaricomycete with a remarkable ability to secrete an ample set of LME (Schlosser *et al.*, 1997) as well as some valuable GH enzymes (Lahjouji *et al.*,

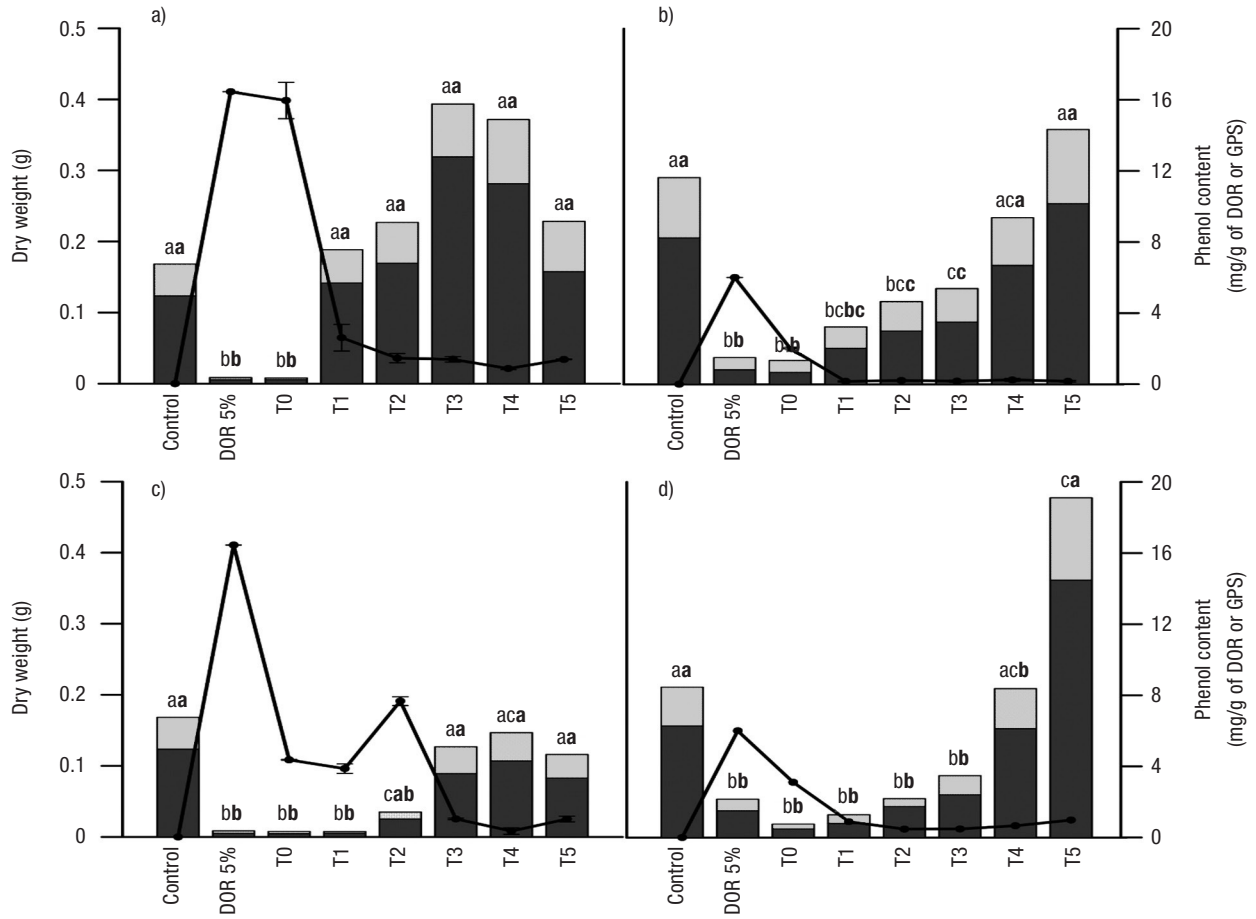

Figure 3. Shoot (*upper bar, grey*) and root (*lower bar, black*) dry weight of tomato plants (control) cultivated in the presence of 5% dry olive mill residue (DOR), grape pomace and stalks (GPS) non-fermented and fermented by *T. versicolor* during 0, 1, 2, 3, 4 and 5 weeks (T0–T5). DOR-soy (a), GPS-soy (b), DOR-barley (c) and GPS-barley (d). Lower case letters distinguish between statistically different groups for each treatment, bold letters are used for shoot dry weight and normal letters for root dry weight (p <0.05 Tukey test). The solid line represents total phenol content (mg/g DOR or GPS).

2007). Recent studies of secretome (Floudas *et al.*, 2012; Carabajal *et al.*, 2013) have provided relevant information about *T. versicolor*'s adaptation to different carbon and nitrogen sources. Laccases, peroxidases, hydrogen peroxide-producing enzymes and carbohydrate-active enzymes were identified in our studies in SSF, specially Lac. It has been described that SSF produces higher titers of biocatalysts than submerged fermentation (Viniegra-González *et al.*, 2003). SSF is more appealing from an ecological point of view given that natural substrates are better emulated as ligninolytic fungi grow in terrestrial habitats. For this reason, soy and barley have been used since it has been demonstrate the different capacity to activate different enzymatic machineries (Reina *et al.*, 2013). This study confirmed differences between the residue added to the support medium, thus demonstrating a variable response to carbon sources and their concentrations in the nutrient medium.

Our results have shown that there is a need to use different natural N and C sources as supplement for by-product conversion. That is the main reason behind the support media selected for optimizing the valorisation process. In the case of soy, it provides the appropriate support and ingredients for LME and some of the GH enzyme production, since higher activity was recorded in these media. This is aligned with the extensive use of soybean cake for fungal cultivation in SSF, which not only guarantees all nutritional requirements for fungal growth but also stimulates cellulolytic enzyme production and is an appropriate medium for LME secretion such as Lac and unspecific peroxygenases (Ullrich *et al.*, 2009; Zeng *et al.*, 2011; Delabona *et al.*, 2012). Barley has been also used for fungal cultivation to enhance certain fungi like *Phanerochaete chrysosporium* MnP and LiP (Moredo *et al.*, 2003). However, in our studies the titles of ligninolytic enzymes and GH produced by *T. versicolor* cultivated in this lignocellulosic material were modest in size in comparison with those obtained in soy.

DOR and GPS, due to their lignocellulosic nature, appear to be appropriate substrates to emulate natural conditions; however their potential as enzyme elicitors was significantly different. DOR strongly stimulated Lac production and slightly altered MnP and LiP secretion. Olive oil residues have been reported to stimulate Lac, MnP and LiP in polyporal fungi (Díaz *et al.*, 2010; Reina *et al.*, 2013, 2014). No previous evidence of GPS induction on LME has been reported, although it is capable of stimulating lipase secretion in *Aspergillus* sp. (Salgado *et al.*, 2014). Our results have shown that GPS can stimulate Lac secretion.

Although DOR is a complex mixture of ingredients that include salts, fatty acids, soluble carbohydrates, proteins and phenols (Díaz *et al.*, 2010), the latter are likely to be responsible for the stimulating effect of Lac on *T. versicolor*, as has been demonstrated with the addition of phenolic extracts from corn steep liquor (Wang *et al.*, 2014). However, the induction levels achieved by the addition of DOR have not so far been reported in relation to additional agro-industrial wastes. No previous studies have been reported using GPS as a culture medium for fungal growth and the production of enzymes involved in lignocellulose degradation. The individual aromatic phenol compounds that lead to Lac secretion enhancement are difficult to determine, with some studies suggesting that vanillic and ferulic acid are capable of stimulating its production in *T. versicolor* and *Pleurotus pulmonarius* (Pazarlioğlu *et al.*, 2005; Wang *et al.*, 2014). The majority of phenols found in our residues were protocatechuic acid, p-tyrosol and hydroxytyrosol, which could mediate this high level of stimulation.

The addition of Mn induces the secretion of MnP in polyporal fungi (Hamman *et al.*, 1999; Swamy & Ramsay, 1999). Apart from Mn regulation, MnP stimulation depends on nitrogen-limitation in most cases; when vegetative growth comes to an end and secondary metabolism begins, the fungi need to continue to mineralize lignin due to nutrient depletion (Buswell, 1991). Although this has been the generally accepted model, this pattern is not followed by all the fungi (Kaal *et al.*, 1995). MnP was not significantly stimulated by DOR or by GPS. In both cases, nitrogen content was lower than in other urban wastes, but total sugar levels were much higher in GPS, which could make the fungus lethargic, rendering the production of LME to obtain nutrients unnecessary. When DOR were added to soy and barley media, there was a slight increment of MnP activity. However, this did not occur in the GPS media used, meaning that the increment could be attributed to the fungus and the additional DOR ingredients, the form nitrogen takes or the C/N ratio rather than limited or sufficient amounts of nitrogen. In fungi such as *Bjerkandera adusta*, aqueous extract of DOR leads to an extraordinarily level of enhancement of MnP secretion (Reina *et al.*, 2014).

As white rot fungi are unable to degrade lignin as a unique energy source, they depend on co-substrates for LME production. Since these co-substrates are obtained through polysaccharide degradation (Kirk & Farrell, 1987), in the current study, higher GH activity was observed in the second week of incubation when activities were triggered, and participated not only in cellulose degradation but also in LME regulation. GPS-barley turned out to be the appropriate medium for most of the GH enzymes probably due to the ingredients presents in this waste. In fact, this by-product has been

described to produced pectinolytic and cellulolytic enzymes (Romero *et al.*, 2007). As a result we conclude in our work that olive and grape wine by-products show a greater capability to elicit LME and GH enzymes respectively.

Once the eliciting potential of the studied by-products was analysed, a further step was performed in order to reuse them as soil amendments. DOR is phytotoxic when added in agronomic doses, although once proper fungal treatment is provided, phytotoxicity is eliminated (Aranda *et al.*, 2006). The incubation of DOR with *T. versicolor* in a soy-support medium not only reduced its phytotoxicity but also appears to stimulate plant growth. Phenols are the main cause of DOR phytotoxicity (Reina *et al.*, 2013). However, their polymerization, mediated by fungal laccases and peroxidases as shown by the shift in the polymerization profile to 73.4 KDa, made these compounds inaccessible for the plant, thus leading to a reduction in phytotoxicity. Furthermore, DOR contains mineral salts that can be beneficial for plant growth. Information concerning the effect of phytotoxicity on GPS is scarce. Our data show that, despite having smaller amounts of phenols, the effects of phytotoxicity are comparable to those of DOR. This could be explained by the presence of high levels of gallic acid in GPS, which is not present in DOR samples. After 5 weeks of incubation, when evidence of phenol polymerization was greatest, there were interesting signs of plant growth caused by GPS residue when grown in a barley-based media.

Our findings show the potential use of agro-residues as enzyme elicitors, specifically DOR for the production of Lac and GPS for carbohydrate-active enzymes. With respect to biotechnological applications, it has been proposed a dual approach in which these by-products are used as an effective low-cost media to obtain high yield LME-carbohydrate-active enzyme inducers, and, once transformed, as organic fertilizers.

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