Appl Biochem Biotechnol (2011) 163:40–51
DOI 10.1007/s12010-010-9014-0

Enzyme Production by Solid Substrate Fermentation of *Pleurotus ostreatus* and *Trametes versicolor* on Tomato Pomace

Donata Iandolo · Alessandra Piscitelli · Giovanni Sannia · Vincenza Faraco

Received: 15 January 2010 / Accepted: 11 June 2010 /
Published online: 27 June 2010

Abstract A process of solid state fermentation (SSF) on tomato pomace was developed with the white-rot fungi *Pleurotus ostreatus* and *Trametes versicolor*, using sorghum stalks as support. Operative parameters (humidity, water activity, and size of substrate particles) guaranteeing a good colonization of tomato pomace by both fungi were defined and conditions for production at high titers of the industrially relevant enzymes laccase, xylanase and protease were identified. Significant laccase activity levels (up to 36 U g^{-1} dry matter) were achieved without any optimization of culture conditions, neither by nutrient addition nor by O_2 enrichment. Furthermore, protease activity levels up to 34,000 U g^{-1} dry matter were achieved, being higher than those reported for the fungi typically considered as the best protease producers such as *Aspergillus* strains. Moreover, as one of the most significant results of this study, analysis of *P. ostreatus* tomato SSF samples by zymogram revealed two bands with laccase activity which had not been detected so far.

Keywords Fungi · Solid state fermentation · Laccase · Xylanase · Protease

Introduction

Among biotechnology products, enzymes encompass one of the biggest market share with considerable selling prices entailing high costs in the following industrial processes. Worldwide enzyme production is mainly based on microbial submerged fermentation (SmF) processes, with high production costs due to the exploitation of genetically modified microorganisms, expensive culture conditions, and downstream processing for product recovery. Microbial solid state fermentation (SSF) holds tremendous potential for enzyme

D. Iandolo · A. Piscitelli · G. Sannia · V. Faraco (✉)
Department of Organic Chemistry and Biochemistry, University of Naples "Federico II",
Complesso Universitario Monte S. Angelo, via Cintia, 4, 80126 Naples, Italy
e-mail: vfaraco@unina.it

D. Iandolo · V. Faraco
School of Biotechnological Sciences, University of Naples "Federico II", Naples, Italy

production, being a better system than SmF from both economical and environmental points of view [1, 2]. SSF involves the growth of microorganisms in the absence or near absence of free water [3, 4]. Basidiomycetous fungi represent the most appropriate microorganisms for producing enzymes through SSF because of the similarity between their natural environment and the conditions in which SSF processes are carried out [1, 2]. Agro-industrial residues are generally considered the best substrates for SSF processes and many studies have been focused on application of SSF to adding extra value to these wastes through enzyme production and metabolite synthesis [5–7]. Different organic wastes such as bagasse, ballico seed, banana waste, canola roots, corn, cotton, grape, sawdust, wheat bran, wheat straw, and wood have shown to be good substrates for enzyme production by fungal SSF due to the presence of significant concentrations of soluble carbohydrates and inducers of enzyme synthesis [8, 9]. Until now, several industrially relevant enzymes have been obtained through fungal SSF on agricultural and food farming residues, mainly belonging to the class of ligninolytic enzymes, such as laccases, manganese peroxidases (MnP) and lignin peroxidases (LiP) [10], and of carbohydrate hydrolysing enzymes, such as amylases, xylanases, cellulases, and pectinases [11]. Obtaining good yields of other enzymatic activities such as proteases, chitinases, and tannases has also been achieved by fungal SSF [12].

Tomato processing residues represent one of the most abundant wastes of agro-industrial sector. In 2005, 10 million tons of tomato was processed in Europe [13] and 6 million tons of these were transformed in Italy, where the Campania Region (South Italy) contributed with 500,000 tons of processed tomato [14]. A ratio of transformed product to waste of around 50 [15] gives a total amount of solid tomato residues (peels and seeds, called pomace) of about 10,000 tons in this region. Animal feeding represents the main way currently adopted for disposal of tomato pomace, whilst very few studies have been reported so far on conversion of this waste, such as its fungal transformation improve its digestibility [16].

In this study, operative parameters guaranteeing a good colonization of tomato pomace by SSF of the basidiomycetous fungi *Pleurotus ostreatus* and *Trametes versicolor* were defined and conditions for laccase, xylanase, and protease activity production were identified. SSF of *P. ostreatus* on tomato pomace led to the production of laccase isoforms not detected in any of the conditions which have been analyzed so far.

Materials and Methods

Lignocellulosic Substrates

Commercially available wheat employed for fungal pre-culture was weighted, washed, and moisturized with bi-distilled water (1:1 weight/volume) before autoclaving for 1 h at 110 °C. After the addition of 1% (*w*/*w*) $CaCO_3$ and of bi-distilled water (1:1 weight/volume), a new sterilization cycle was run.

Tomato residues were collected in a local tomato processing farm in Campania (Italy). The residues (peels and seeds) were dried at 65 °C, reduced to small pieces and sieved to have 0.8–2.0 mm dimension particles. Sorghum stalks, after milling and sieving, were added to tomato residues up to 15% of the total dry matter (2.6 g in each inoculum flask, prepared as described in the paragraph 2.3). Before sterilizing in autoclave for 1 h at 110 °C, a moisture content of 65% (*w*/*w*) was achieved by water addition and 1% (*w*/*w*) $CaCO_3$ was added in order to get a final pH of 6.0 after sterilization.

Fungal Strains

Strains of the white rot fungi *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida; ATCC MYA-2306) from ATCC, the Global Bioresource Centre and *T. versicolor* (NBRC4937) from the fungal collection Nite Biological Resource Center (Department of Biotechnology National Institute of Technology and Evaluation, Japan) were used in this study.

The fungi were maintained through periodic transfer at 4 °C on agar (1.5% *w*/*v*) plates containing PDY medium (24 g l^{-1} potato dextrose (Difco, Detroit, MI, USA) and 5 g l^{-1} yeast extract (Difco)).

Solid State Fermentation

Pre-cultures were prepared by pre-inoculating 100 g of pre-treated wheat in 1 L Erlenmeyer flasks with five agar plugs of *P. ostreatus* (11 mm diameter) or ten agar plugs of *T. versicolor* (9 mm diameter) mycelia from the edge of a 5-day-old agar culture. The different pre-cultures conditions were chosen to achieve a comparable growth for both the fungal strains.

After fungal growth in a temperature-controlled incubator at 28 °C for 6 days, the wheat, after mechanical stirring, was used to inoculate 500 g of wet tomato waste prepared as above described. Of the material, 50 g, made homogeneous by stirring, were transferred to 250 ml flasks then incubated at 28 °C, statically. The fermentations of *P. ostreatus* and *T. versicolor* were followed for 18 and 22 days, respectively, due to the different growth rates of the analyzed microorganisms. At suitable time intervals, three flasks were sampled and the content of each flask was used to prepare samples for the analytical determinations. Freeze dried and milled samples were suspended (1:10 weight/weight) in bi-distilled water for reducing sugars determination and in 50 mM Sodium Phosphate pH 6.8 and added with 1 mM phenylmethylsulfonylfluoride (PMSF) for enzymatic assays. When samples were used for protease activity assay, PMSF was not added to the protein extract. After vigorous mixing by vortex, samples were centrifuged for 30 min at 7,500 rpm at 4 °C (Beckman Coulter, Inc.), and supernatants were subjected to analytical determinations, as described below.

The results of the analytical determinations reported in the figures correspond to mean values of the three replicates with a standard deviation lower than 15%.

Analytical Procedures

Humidity and Weight Loss Evaluation

The percentage of humidity was determined gravimetrically. In a pre-weighed glass plate (W1) 1–2 g of sampled material (W2) were allowed to dry in a ventilated oven at 65 °C for about 24 h. After cooling in a dessiccator, plates were weighed to get the dry weight (W3). Percentage of humidity was evaluated as {((W2-W1)-(W3-W1))/(W2-W1)} *100. The dry mass was determined at each sampling time.

Analysis of Soluble Reducing Sugar Content

Reducing sugars were measured by the 3,5-dinitrosalicylic acid (DNS) method using (100–500 μg) D-glucose as a standard, according to Miller [17]. Sugar content was assayed on 0.5 ml of the appropriately diluted sample. A DNS solution containing 10 g l^{-1} 3,5-dinitrosalicylic acid (Sigma Aldrich), 10 g l^{-1} sodium hydroxide, 0.5 g l^{-1} anhydrous sodium sulfite and 2 g l^{-1} phenol was prepared. A 1:1 (volume/volume) mixture of diluted

sample and DNS solution was incubated at 95 °C for 5 min, after mixing by vortex. Mixtures were let to cool to room temperature and, after addition of 3 ml of bi-distilled water; absorbance was measured at 580 nm.

Enzyme Assays

(a) Laccase activity

Laccase activity was assayed using 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate [18]. The assay mixture contained 2 mM ABTS and 0.1 M sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε=36,000 M^{-1} cm^{-1}).

(b) Manganese peroxidase

MnP activity was determined using manganese sulfate as substrate [19]. The reaction mixture contained 0.5 mM manganese sulfate and 0.1 mM Hydrogen peroxide in 50 mM sodium malonate buffer, pH 4.5. Oxidation of Mn^{2+} to Mn^{3+} was followed by absorbance increase at 270 nm (ε=11,590 M^{-1} cm^{-1}) due to the formation of malonate–Mn^{3+} complex.

(c) Lignin peroxidase

LiP activity was determined using veratryl alcohol as substrate [20]. The reaction mixture contained 2 mM veratryl alcohol and 0.5 mM hydrogen peroxide in 50 mM sodium tartrate buffer, pH 2.5. Oxidation of veratryl alcohol was followed by measuring the absorbance increase at 310 nm (ε=9,300 M^{-1} cm^{-1}).

All the enzyme activities were expressed in International Units (IU), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate in 1 min.

(d) Filter paper activity

Filter paper activity was assayed by a modified version of the protocol reported by Ghose [21]. A strip of 50 mg (1×6 cm) of Whatman #1 filter paper was used as a substrate, by incubating it for 1 hour at 50 °C, in a mixture of 1 ml of 50 mM sodium citrate pH 4.8 and 0.5 ml of the appropriate sample dilution (in 50 mM sodium citrate at pH 4.8). Of this reaction mixture, 0.5 ml was used to determine the released reducing sugars by DNS method. A mixture of the analyzed sample and DNS solution (1:1, volume/volume) was incubated at 95 °C for 5 min. Then, the assay mixture was let cool and, after addition of 4 ml of bi-distilled water, absorbance was measured at 580 nm. Enzyme activity was inferred using D-glucose as a standard.

(e) Xylanase activity

Xylanase activity assay was performed according to Bailey et al. [22]. The reaction mixture consisting of 1.8 ml of a 1.0% (*w*/*v*) suspension of birch-wood xylan in 50 mM Na citrate at pH 5.3 and 0.2 ml of enzyme dilution (in 50 mM sodium citrate at pH 5.3) was incubated at 50 °C for 5 min. Released reducing sugars were determined by DNS method, by adding 3 ml of DNS solution and then incubating the mixture at 95 °C for 5 min. Absorbance was measured at 540 nm. One unit of enzyme is defined as the amount of enzyme catalyzing the release of 1 μmol of xylose equivalent per minute.

(f) Protease activity

The substrate used for protease activity determination is azoalbumin (Sigma-Aldrich). The substrate stock solution (15 mg ml^{-1}) was prepared in 3-(N-Morpholino)-propane-

sulfonic acid (MOPS) buffer 50 mM pH 7. Of the appropriately diluted sample, 250 μl were incubated in 24-well plates with 400 μl of substrate solution for 30 min at 37 °C. Reaction was stopped by adding trichloroacetic acid 20% (650 μl). The mixture was centrifuged (600×*g*, 15 min), and 325 μl of sodium hydroxide 10 M were added to 650 μl of digested substrate. Absorbance was registered at 440 nm, against a blank constituted by substrate and MOPS, in the same proportion used for the reaction. One unit of enzyme is defined as the amount of enzyme catalyzing the release of the amount of azodye producing 0.001 unit change in the absorbance read at λ=440 nm.

Evaluation of Total Protein Content

Protein concentration was determined using the BioRad Protein Assay (BioRad) based on the method developed by Bradford [23], with bovine serum albumin as a standard.

Non-denaturing PAGE

Polyacrylamide gel electrophoresis (PAGE) was performed at an alkaline pH under nondenaturing conditions. The separating and stacking gels contained 9% and 4% acrylamide, respectively. The buffer solution used for the separating gel contained 50 mM Tris hydrochloride (pH 9.5), and the buffer solution used for the stacking gel contained 18 mM Tris hydrochloride (pH 7.5). The electrode reservoir solution contained 25 mM Tris hydrochloride and 190 mM glycine (pH 8.4). The gels were stained to visualize laccase activity by using ABTS as the substrate.

Results

The basidiomycetous fungi *P. ostreatus* and *T. versicolor* were analyzed for their ability to colonize tomato pomace, using this waste as a substrate for SSF. Appropriate conditions for cultivation of fungi on tomato pomace collected in a local farm of the Campania region (South Italy) were investigated and values of particles dimension, substrate/support ratio, humidity, and pH and inoculum conditions allowing fungal waste colonization were identified, as reported in the "Lignocellulosic Subtrates" and "Solid State Fermentation" sections.

A good colonization of tomato waste was achieved with both *P. ostreatus* and *T. versicolor*, fungal growth being observed beginning from the second day of fermentation. No significant change in humidity was measured during fermentation with both fungi (data not shown).

Time courses of dry mass (g) and sugar content (mg g^{-1} of dry matter, mg g^{-1} d.m.) were analyzed during SSF (Fig. 1). A reduction of dry mass was measured for both *P. ostreatus* (Fig. 1a) and *T. versicolor* (Fig. 1b) SSFs. No significant change of sugar content was observed for *P. ostreatus* (Fig. 1a), whereas *T. versicolor* SSF (Fig. 1b) gave rise to a sugar content decrease until the fourth day, followed by stationary levels.

Production of ligninolytic (laccases, LiP, and MnP), glycosyl hydrolytic (xylanases and cellulases) and proteolytic enzymes by *P. ostreatus* and *T. versicolor* during SSF on tomato pomace was evaluated.

No peroxidase activity was detected during both *P. ostreatus* and *T. versicolor* fermentations. A maximum laccase activity production level of 15 U g^{-1} of dry matter (U g^{-1} d.m.) was achieved at the third to fourth day of *P. ostreatus* fermentation, as shown in Fig. 2a. During *T. versicolor* SSF, a maximum laccase activity value of around 35 U g^{-1} d.m. was produced at the 16th day, keeping constant at least until the 22nd day (Fig. 2b).

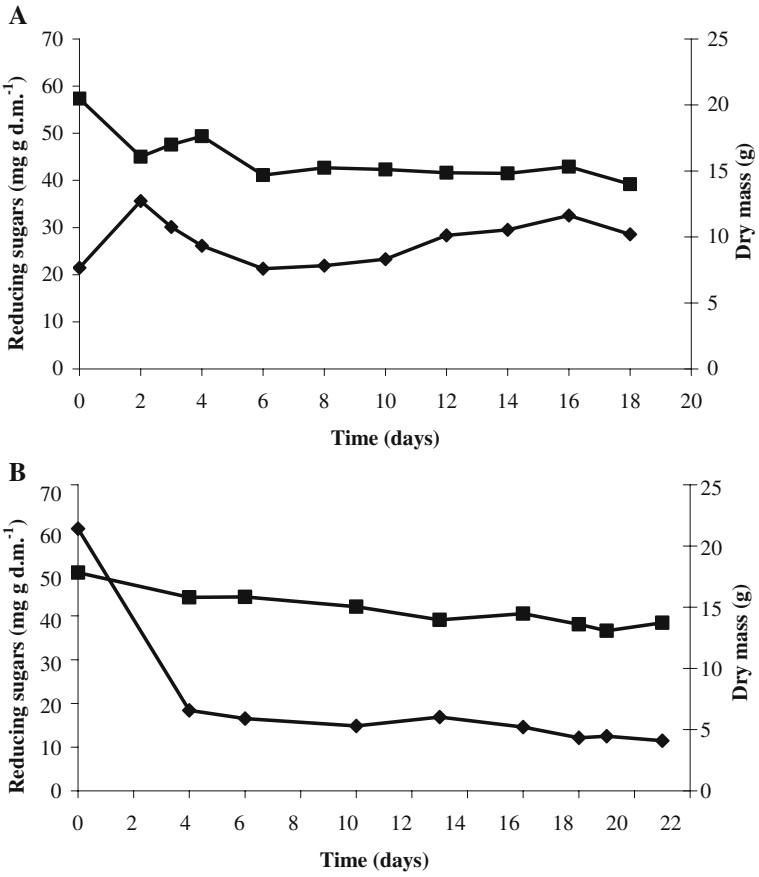

Fig. 1 Time courses of reducing sugar content and of dry mass during *P. ostreatus* (**a**) and *T. versicolor* (**b**) SSFs on tomato pomace; *filled square* dry mass, *filled diamond* reducing sugars

Among the assayed glycosyl hydrolases, no detectable levels of cellulase were achieved with both fungi. As far as xylanase activity production is concerned, a maximum activity level of around 9 U g^{-1} d.m. was reached at the second day of *P. ostreatus* SSF (Fig. 3a), whereas a higher level of about 50 U g^{-1} d.m. was achieved at the 13th day of *T. versicolor* SSF (Fig. 3b).

Analyses of protease production by *P. ostreatus* showed achievement of a maximum activity value of around 13,000 U g^{-1} d.m. after only 4 days (Fig. 4a), while a higher protease activity level of 20,000 U g^{-1} d.m. was measured for *T. versicolor* fermentation since its beginning with a subsequent increase up to around 35,000 U g^{-1} d.m. (Fig. 4b).

Production of laccase and protease activities by *P. ostreatus* in SSF and liquid culture (in PDY medium [24] were compared (Figs. 5, 6, and 7).

Discussion

In this study, a process of fungal solid state fermentation on tomato pomace was for the first time investigated for the production of industrial enzymes, exploiting the natural ability of

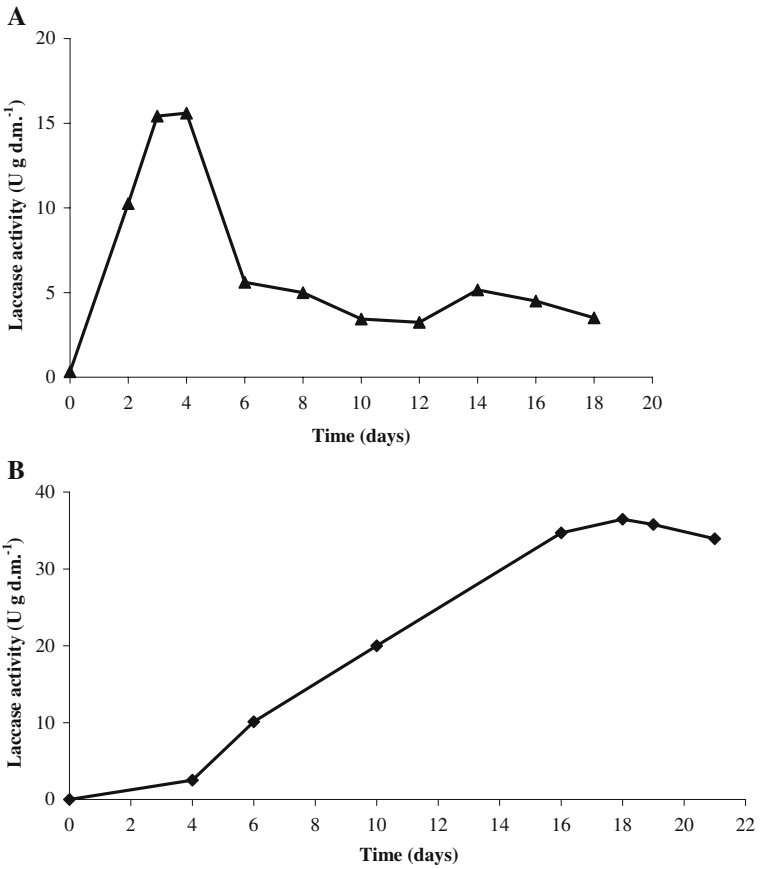

Fig. 2 Time course of laccase activity production by *P. ostreatus* (**a**) and *T. versicolor* (**b**) SSF on tomato pomace

the fungi *P. ostreatus* and *T. versicolor* to colonize lignocellulosic wastes by SSF. The reduction in dry mass measured during fungal fermentations could be ascribed to waste conversion into volatile compounds produced by fungal metabolism. *T. versicolor* was shown to be able to metabolize the sugars present in the waste, giving rise to a fast sugar consumption followed by an increase of xylanase activity level (Fig. 3b). Hence, the fungus seems to react to sugar depletion by producing enzymatic activities involved in polysaccharide hydrolysis. On the other hand, *P. ostreatus* seems not able to metabolize the monosaccharides present in the waste, and the absence of sugars to be directly metabolized could trigger the fungus to produce xylanases in a shorter time than *T. versicolor* (Fig. 3a), thus gaining the required carbon source.

Comparing *T. versicolor* and *P. ostreatus* enzyme production for all the assayed enzyme activities, higher values were achieved with *T. versicolor* but longer times were needed. Moreover, at the beginning of fermentation, higher values of both xylanase and protease activities were measured for *T. versicolor*, suggesting a higher enzyme production even during pre-culture of this fungus.

For both fungi, the correlation between production times for laccase and xylanase activities suggest their synergistic action in waste transformation.

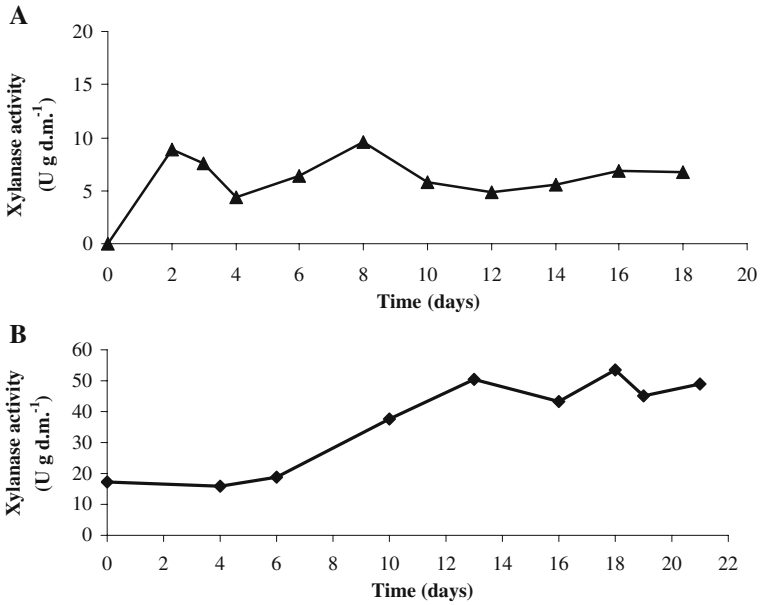

Fig. 3 Time course of xylanase activity production by *P. ostreatus* (**a**) and *T. versicolor* (**b**) SSF on tomato pomace

Lignino-cellulolytic enzyme production by *P. ostreatus* is mainly affected by the strain, substrate composition and culture conditions [25]. In comparison with other *P. ostreatus* strains previously cultivated under SSF conditions, the strain investigated in this study allowed the achievement of higher levels of laccase production after a shorter fermentation time. Kerem et al. [26] have reported a maximal laccase activity production of 0.03 U g^{-1} at the sixth day of *P. ostreatus* SSF on cotton stalks, and similar values (0.040 U g^{-1}) have been achieved by Membrillo et al. [27] at the eigth day of *P. ostreatus* SSF on sugarcane bagasse. Higher laccase activity levels (6 and 4 IU g^{-1}) have been obtained by Stajic et al. [25] and Elisashvili et al. [28] after 10 days of *P. ostreatus* SSF on grapevine sawdust (supplemented with a synthetic medium) and wheat-straw (supplemented with mineral salts and yeast extract), respectively.

Even when compared to SSF processes so far developed with other fungi, the maximum laccase activity levels achieved in this study with *T. versicolor* and *P. ostreatus* were proved significant. Comparing *Lentinus edodes*, *Pleurotus dryinus*, *P. ostreatus*, and *Pleurotus tuberregium* SSFs on wheat straw and tree leaves, Elisashvili et al. [28] have reported a maximum laccase activity value of 14 U g^{-1} by *L. edodes* SSF on both substrates supplemented with mineral salts and yeast extract. By screening different food (apple, orange, and potato) wastes as substrates for *Trametes hirsuta* SSF, Rosales et al. [29] have found a maximum laccase activity value of about 7.5 U g^{-1} within 8 days of fermentation on potato peelings. Grape seeds have shown to be a better substrate for laccase production by *T. hirsuta* SSF, giving 69 U g^{-1} on the 15th day of SSF in the presence of thiamine [30] and 40 U g^{-1} at the 13th day of SSF in the presence of a synthetic medium in a tray 1.8 l bioreactor [31]. Rosales et al. [32] have reported laccase production by *T. hirsuta* SSF on kiwi fruit wastes, with a maximum activity value of around 32.4 U g^{-1} in optimized operative conditions, such as initial concentration of ammonium, amount of support

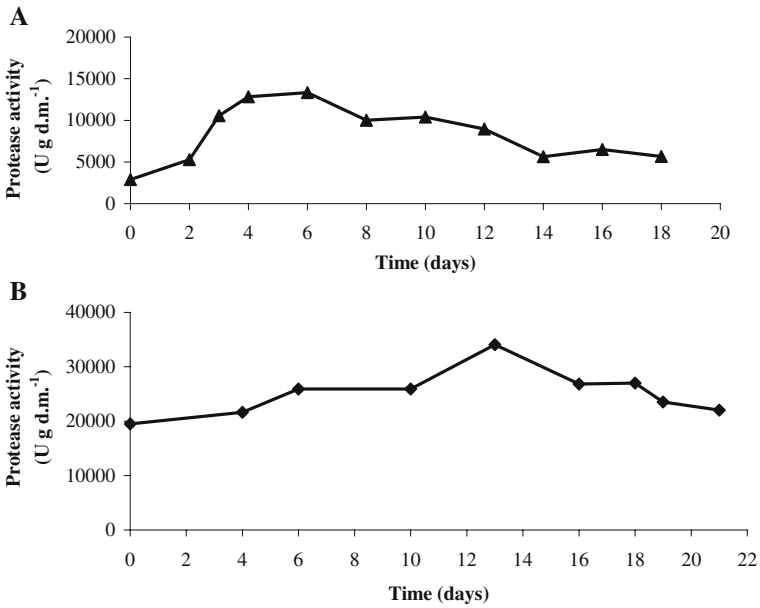

Fig. 4 Time course of protease activity production of *P. ostreatus* (**a**) and *T. versicolor* (**b**) SSF on tomato pomace

employed, need for pre-treating the support, and the part of kiwi fruit wastes used (peelings or peelings plus pulp). The achievement of a maximum laccase activity of 4.5 U g^{-1} on banana skin has been reported for *Trametes pubescens* SSF [33]. Comparing 15 fungal strains in SSF on three industrial waste materials (oat husks and waste from paper process industry fiber sludge and combined fiber and de-inking sludge, FDS), Winquist et al. [34] have achieved the maximum laccase activity level (10.65 U g^{-1}) with *Cerrena unicolor* T 71 on media containing oat husks or oat husks supplemented with 20% (*w*/*w*) of FDS. When a comparative study on *Pycnoporus sanguineus* SSF has been carried out by Vikineswary et al. [35] (on sago hampas, oil palm frond parenchyma tissue (OPFPt), and rubberwood sawdust), sago hampas and OPFPt have shown to be the best substrates, giving a maximum laccase production of 46.5 U g^{-1} at the 11th day of fermentation, through optimization of the inoculum age and density, and nitrogen supplementation.

Hence, when compared to the previously reported data, results highlight the great potential of the developed *P. ostreatus* and *T. versicolor* SSF processes for valorization of

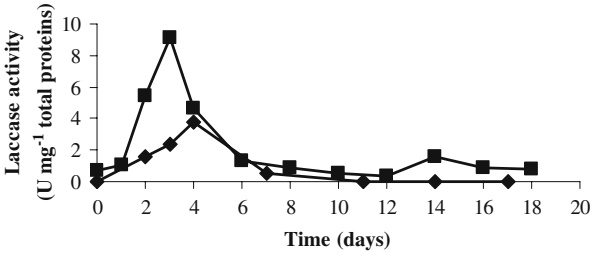

Fig. 5 Production of laccase activity referred to mg of total secreted proteins by *P. ostreatus* in SSF on tomato pomace (*filled square*) and in PDY containing liquid culture (*filled diamond*)

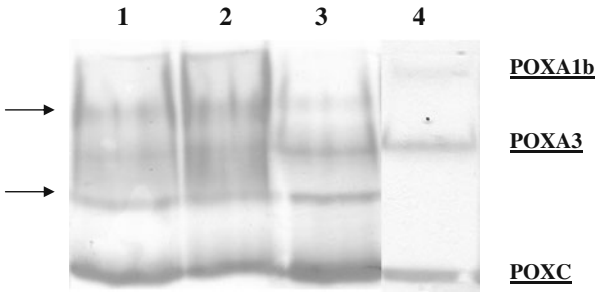

Fig. 6 Native gel for revelation of laccase isoenzymes produced at the second (lane 1), third (lane 2), and fourth (lane 3) of *P. ostreatus* SSF on tomato pomace, compared to those produced in liquid culture in PDY medium supplemented with 150 μM copper sulfate (lane 4). *Arrows* point to the new bands

tomato wastes as a source of laccases, taking into account that no optimization of culture conditions was performed in this study.

Furthermore, it is worth noting that the laccase activity levels produced by SSF fermentation with the *P. ostreatus* strain used in this study appear significant even when compared with its laccase production in liquid culture. As shown in Fig. 5, laccase activity produced by *P. ostreatus* during SSF and reported as U mg^{-1} of total secreted protein is almost threefold higher than that obtained in liquid culture (PDY). Moreover, as shown in Fig. 6, native PAGE analysis of the laccase isoenzymes produced by *P. ostreatus* SSF revealed two more bands besides the three isoenzymes normally secreted in liquid cultures [18, 24, 36–38]. The existence of these new isoenzymes is consistent with the multiplicity of *P. ostreatus* laccase genes [39, 40].

Among the assayed glycosyl hydrolytic enzymes, only xylanase activity was detected, with maximum values (around 10 and 50 U g^{-1} of d.m. with *P. ostreatus* and *T. versicolor*, respectively) representing lower levels than those previously reported in other studies on fungal SSF. Gawande and Kamat [41] have reported a maximum xylanase activity of 344.5 U g^{-1} by *A. terreus* SSF on wheat bran (supplemented with tryptone or yeast extract). A maximum xylanase production of around 540 U g^{-1} has been achieved by *P. dryinus* and *P. ostreatus* on tree leaves in SSF experiments developed by Elisashvili et al. [28].

The enormous potential of the developed *P. ostreatus* and *T. versicolor* SSF processes on tomato pomace for protease production (Fig. 4) was proved in comparison with data previously reported for other fungi. Even if several SSF processes have been developed for protease production, data for *P. ostreatus* and *T. versicolor* SSF have not been available, so far. On the other hand, many surveys have highlighted the potential of other fungi for the

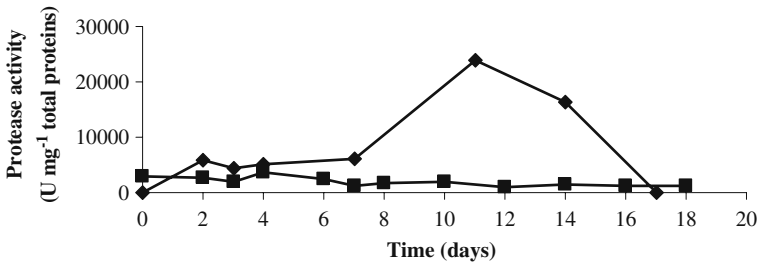

Fig. 7 Production of protease activity referred to mg of total secreted proteins by *P. ostreatus* in SSF on tomato pomace (*filled square*) and in PDY containing liquid culture (*filled diamond*)

production of proteases by SSF, e.g., *Aspergillus* strains. Villegas et al. [42] have reported a maximum value of 52 U g^{-1} for protease production by *A. niger* SSF on wheat bran after 36 h. When another strain of the same fungus was grown on rice mill wastes, a protease production of 67.7 U g^{-1} has been achieved in the best conditions [43]. Chutmanop et al. [44] have reported a protease production of 1,200 U g^{-1} by *Aspergillusoryzae* SSF on a mixture of rice and wheat brans (75:25, respectively).

When protease production by *P. ostreatus* SSF was compared with that achieved in liquid culture (PDY) with the same strain, similar levels (protease activity ratio to total protein content) were obtained until the seventh day, beginning from which the production in liquid culture increased until it got to values which are sevenfold higher than SSF (Fig. 7).

Conclusions

The developed processes of *P. ostreatus* and *T. versicolor* SSF on tomato pomace can represent efficient and cost effective systems for enzyme production, requiring low technical and economical inputs. This study showed the good potential of tomato pomace as substrate to produce laccases by *P. ostreatus* and *T. versicolor* SSF, considering that significant enzyme activity levels were achieved without any optimization of culture conditions, neither by nutrient addition nor by O_2 enrichment. Furthermore, the developed processes hold enormous potential for protease production, giving activity levels higher than those reported for the fungi typically considered as the best protease producers such as *Aspergillus* strains.

Moreover, as one of the most significant results of this study, the developed *P. ostreatus* SSF process provides the production of two laccase isoforms not detected in any other liquid culture conditions which have been analyzed so far.

Acknowledgments This work was supported by grants from the Ministero dell'Università e della Ricerca Scientifica (Progetti di Rilevante Interesse Nazionale, PRIN), and from the Ministero Degli Affari Esteri di Intesa con il Ministero dell'Università e della Ricerca (Progetti di ricerca di base e tecnologica approvati nei protocolli di cooperazione scientifica e tecnologica bilaterale come previsto dal protocollo bilaterale tra Italia e Turchia).

References

1. Viniegra-González, G., Favela-Torres, E., Aguilar, C. N., Rómero-Gomeza, S. J., Díaz-Godínez, G., & Augur, C. (2003). *Biochemical Engineering Journal, 13*, 157–167.
2. Hölker, U., Höfer, M., & Lenz, J. (2004). *Applied Microbiology and Biotechnology, 64*, 175–186.
3. Pandey, A. (2003). *Biochemical Engineering Journal, 13*, 81–84.
4. Pandey, A., Soccol, C. R., & Mitchell, D. (2000). *Process Biochemistry, 35*, 1153–1169.
5. Rodriguez Couto, S. (2008). *Biotechnology Journal, 3*, 859–870.
6. Laufenberg, G., Kunz, B., & Nystroem, M. (2003). *Bioresource Technology, 87*, 167–198.
7. Pandey, A., Selvakumar, P., Soccol, C. R., & Nigam, P. (1999). *Current Science, 77*, 149–162.
8. Rodriguez Couto, S., & Sanromán, M. A. (2005). *Biochemical Engineering Journal, 22*, 211–219.
9. Rodriguez Couto, S., Gundin, M., Lorenzo, M., & Sanromán, M. A. (2002). *Process Biochemistry, 8*, 249–255.
10. Jaszek, M., Malarezyk, E., & Leonowicz, A. (1998) Proceedings of the 7th International Conference on Biotechnology in the Pulp and Paper industry, Vancouver, 16-18 June.
11. Mamma, D., Kourtoglou, E., & Christakopoulos, P. (2008). *Bioresource Technology, 99*, 2373–2383.

12. Sabu, A., Pandey, A., Daud, M., & Szakacs, G. (2005). *Bioresource Technology, 96*, 1223–1228.
13. The website for disseminating to small and medium enterprises the strategies for the extraction of bioactive compounds from vegetables processing residues, developed by the Bioactive Project. www.bioactive-net.com. Accessed 15 January 2010.
14. National Food Farming Association. www.anicav.it. Accessed 15 January 2010.
15. Rossi, L., Piccinini, S., Soldano, M., & Labartino, N. (2006). *Centro Ricerche Produzioni Animali CRPA SpA*. ECOMONDO: Reggio Emilia.
16. Assi, J. A., & King, A. J. (2007). *Journal of Agricultural and Food Chemistry, 55*, 9095–9098.
17. Miller, G. L. (1959). *Analytical Chemistry, 31*, 426–428.
18. Giardina, P., Aurilia, V., Cannio, R., Marzullo, L., Amoresano, A., Siciliano, R., et al. (1996). *European Journal of Biochemistry, 235*, 508–515.
19. Giardina, P., Calmieri, G., Fontanella, B., Rivieccio, V., & Sannia, G. (2000). *Archives of Biochemistry and Biophysics, 376*, 171–179.
20. Tien, M., & Kirk, T. K. (1984). *Proceedings of the National Academy of Science, 81*, 2280–2284.
21. Ghose, T. K. (1987). *Pure and Applied Chemistry, 59*, 257–268.
22. Bailey, M. J., Biely, P., & Poutanen, K. (1992). *Journal of Biotechnology, 23*, 257–270.
23. Bradford, M. M. (1976). *Analytical Biochemistry, 72*, 248–254.
24. Palmieri, G., Giardina, P., Bianco, C., Fontanella, B., & Sannia, G. (2000). *Applied and Environmental Microbiology, 66*, 920–924.
25. Stajic, M., Persky, L., Friesem, D., Hadar, Y., Wasser, S. P., Nevo, E., et al. (2006). *Enzyme and Microbial Technology, 38*, 65–73.
26. Kerem, Z., Friesem, D., & Hadar, Y. (1992). *Applied and Environmental Microbiology, 58*, 1121–1127.
27. Membrillo, I., Sanchez, C., Meneses, M., Favela, E., & Loera, O. (2008). *Bioresource Technology, 99*, 7842–7847.
28. Elisashvili, V., Penninckx, M., Kachlishvili, E., Tsiklauri, N., Metreveli, E., Kharziani, T., et al. (2008). *Bioresource Technology, 99*, 457–462.
29. Rosales, E., Rodriguez Couto, S., & Sanroman, A. (2002). *Biotechnological Letters, 24*, 701–704.
30. Moldes, D., Gallego, P. P., Rodriguez Couto, S., & Sanroman, M. A. (2003). *Biotechnological Letters, 25*, 491–495.
31. Rodriguez Couto, S., Lopez, E., & Sanroman, M. A. (2006). *Journal of Food Engineering, 76*, 291–302.
32. Rosales, E., Rodriguez Couto, S., & Sanroman, M. A. (2005). *Journal of Food Engineering, 66*, 419–423.
33. Osma, J. F. T., Herrera, J. L., & Rodriguez Couto, S. (2007). *Dyes and Pigments, 75*, 32–37.
34. Winquist, E., Moilanen, U., Mettälä, A., Leisola, M., & Hatakka, A. (2008). *Biochemical Engineering Journal, 42*, 128–132.
35. Vikineswary, S., Abdullah, N., Renuvathani, M., Sekaran, M., Pandey, A., & Jones, E. B. G. (2006). *Bioresource Technology, 97*, 171–177.
36. Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Papasso, A., & Sannia, G. (1997). *The Journal of Biological Chemistry, 272*, 31301–31307.
37. Palmieri, G., Cennamo, G., Faraco, V., Amoresano, A., Sannia, G., & Giardina, P. (2003). *Enzyme and Microbial Technology, 33*, 220–230.
38. Giardina, P., Palmieri, G., Scaloni, A., Fontanella, B., Faraco, V., Cennamo, G., et al. (1999). *The Biochemical Journal, 341*, 655–663.
39. Pezzella, C., Autore, F., Giardina, P., Piscitelli, A., Sannia, G., & Faraco, V. (2009). *Current Genetics, 55* (1), 45–57.
40. *Pleurotus ostreatus* genome databank (http://genome.jgi-psf.org/PleosPC15_1. Accessed 15 January 2010)
41. Gawande, P. V., & Kamat, M. Y. (1999). *Journal of Applied Microbiology, 87*, 511–519.
42. Villegas, E., Aubague, S., Alcantara, L., Auria, R., & Revah, S. (1993). *Biotechnology Advances, 11*, 387–397.
43. Paranthaman, R., Alagusundaram, K., & Indhumathi, J. (2009). *World Journal of Agricultural Sciences, 5*, 308–312.
44. Chutmanop, J., Chuichulcherm, S., Chisti, Y., & Srinophakun, P. (2008). *Journal of Chemical Technology and Biotechnology, 83*, 1012–1018.