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ORIGINAL ARTICLE

Effect of the taxonomic group of fungi and type of substrate on the antioxidant activity of a solid-state fermentation system

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Abstract

The enzymatic and non-enzymatic antioxidant activities of a solid-state fermentation system (SSFS) employing six basidiomycete and four ascomycete fungi on orange peel have been evaluated. Class comparisons revealed highly significant effect of fungal group on the antioxidant activity. Peroxidase activity appeared only in the basidiomycete fungi (particularly *Pleurotus columbinus*, *Ganoderma resinaceum*, and *Pleurotus floridanus*) whereas catalase activity appeared in the two fungal groups in favor of the ascomycetes (particularly *Paecilomyces variotii* and *Aspergillus fumigatus*). Maximal peroxidase and minimal catalase activities were found at moderate phenolic content, with extreme phenolic levels leading to low peroxidase activity but high catalase activity. Production of the non-enzymatic antioxidants (phenolics, flavonoids, reducing power, and DPPH scavenging) was in favor of the ascomycetes, which showed great native ability to synthesize flavonoids and also to release flavonoids from orange peel. The basidiomycete fungi, which have limited native ability to produce phenolics, had high ability to consume orange peel phenolics. By contrast, the ascomycete fungi exhibited great native ability for production of phenolics and low ability to consume exogenous phenolics.

Keywords Antioxidants · Ascomycetes · Basidiomycetes · Orange peel · Solid-state fermentation

Introduction

Free radicals, such as superoxide (O_2^{-}), nitric oxide (NO), peroxynitrite ($ONOO^{-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and alkyl peroxy radicals, are highly reactive species that can initiate degenerative oxidation reactions and cause peroxidative disintegration of cells. In particular, superoxide and hydroxyl radicals as well as hydrogen peroxide are reactive oxygen species (ROS) that can induce lipid peroxidation, decrease membrane fluidity, and attack DNA leading to the development of mutation (Cerutti 1994; Halliwell 1997; Pietta 2000) and several diseases including cancer, atherosclerosis, diabetes, and rheumatoid arthritis (Halliwell and Gutteridge 1984; Ranneh et al. 2017).

As a defense mechanism against oxidative stress, living cells have to manipulate multiple antioxidant systems to alleviate the toxic effects of ROS. Because the human body has a limited ability to synthesize antioxidants, intake of natural compounds with antioxidant activity is important to limit the generation of ROS or to ameliorate their deteriorative effects. In recent years, several undesirable disorders have been developed due to the side effects of the use of synthetic antioxidants, commonly applied in the food and flavoring industries (Juan and Chou 2010). Therefore, the search for new products—mainly of plant and microbial origins—with high ROS-scavenging activity is a growing concern. The benefit of this search will be highly appreciated if it is directed towards utilization of plant residues that are usually disposed as wastes rather than of valuable plant products which are customarily consumed in human and livestock nutrition. These criteria are fairly met in the agro-industrial byproducts which although of being wastes they represent a potential source of bioactive compounds of intimate relevance to food and pharmacological industries. Vegetables and fruits are important sources of phenolic compounds, anthocyanins, flavonols, catechins, and hydrolyzable or condensed tannins, among others (Martinez-Avila et al. 2014). In the food industry, after processing of fruits and vegetables, their solid wastes (seeds, peels, or whole pomace) contain soluble sugars, fibers, and other hydrolysable

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materials that can be metabolized by a wide range of microorganisms (mainly filamentous fungi) into value-added bioactive products. In recent years, bioconversion of lignocellulosic materials using solid-state fermentation (SSF) bioprocess has received much attention for their practical applications in several agro-industrial processes (Biswas and Biswas 2015).

These wastes, via fermentation by selected microorganisms, can yield a variety of useful products, some of which may exhibit pronounced antioxidant activities while some others can exert antibiotic as well as enzymatic activities. Generally, fermentation with appropriate microbes represents an environmentally safe technology for production of bioactive compounds, which can be manipulated in food and pharmaceutical industries. Solid-state fermentation system (SSFS)—a recent variant of the traditional fermentation technique—provides a controlled, more efficient, and economic method to produce several bioactive products from plant wastes. Until now, many enzymes, metabolites, and antibiotics have been successfully produced employing the SSF technique (Bind et al. 2014; Ibrahium 2010; Inácio et al. 2015; Schmidt et al. 2014).

Among several plant residues that can serve as substrates for SSFS, orange peel is one of the most promising candidates. Orange constitutes about 60% of the total world production of citrus. In Egypt, about 3.23 million tons of citrus fruits have been produced in 2008, including 2.14 million tons of orange (Hegazy and Ibrahium 2012). A large proportion of this production is addressed to the industrial extraction of citrus juice, which yields huge amounts of residues, including peels and segment membranes. Peels represent from 50 to 65% of the total weight of fruits and are, therefore, the primary byproduct of orange industry. If not appropriately processed, orange peel will produce odor, contribute to soil pollution, and provide harborage for insects which give rise to serious environmental risks. In Egypt, as well as in many southern Mediterranean countries, the major quantities of orange peel are not processed. Byproducts obtained from citrus juice production are rich in soluble and insoluble carbohydrates, making them an attractive raw material with great potential for biological conversion to value-added products (Elmastas et al. 2007). Some attempts were made to use these residues as livestock feed but their low nutritional value and unpalatable taste restricts the advance in this route (Bampidis and Robinson 2006); nevertheless, after subjecting to solid fermentation, the residual substrate of orange peel can be used as animal feed (Inácio et al. 2015). Furthermore, orange peel is rich in several secondary metabolites of appreciable industrial and medicinal values, among which are volatile oils and flavonoids (Lv et al. 2015).

Fungi, by themselves, can enormously participate in the production of antioxidants. For example, mushrooms can accumulate a variety of secondary metabolites, including phenolics, polyketides, terpenes, and steroids (Rodrigo and Bosco 2006). Oyster mushrooms, in particular, have several advantages over other mushrooms because of their ability to reduce body fats and blood sugar (El-Komy and El-Fallal 2006), delicate taste, mild chewy texture, and unique aroma. Expectedly, a combination of the appropriate fungal species and plant wastes in a SSFS will maximize the production of antioxidants. Considerable antioxidant activity of a SSFS employing different species of *Aspergillus* and *Penicillium* on agro-residues such as wheat straw, rice straw, corn cob, and sugarcane bagasse has been demonstrated (Chandra and Arora 2016). In the present work, the antioxidant activity of six basidiomycete and four ascomycete fungi have been investigated by manipulating the SSFS technique with orange (*Citrus sinensis* L.) peel as the substrate. The relative contribution of the system components (substrate and fungi) and the relative contribution of basidiomycete versus ascomycete fungi as well as the species variability among each fungal group in the antioxidant potentiality have also been evaluated. The hypothesis that the antioxidant activity of the SSFS is related to the taxonomic group of the employed fungi has been tested.

Materials and methods

Fungal species and substrates

Ten fungal species were used in this study: six basidiomycetes and four ascomycetes. The basidiomycete fungi were *Pleurotus sajor-caju* (Fr.) Sing, *Pleurotus pulmonarius* (Fr.), *Pleurotus columbinus* (Quel. Ap. Bres), *Pleurotus floridanus* Sing (38539), *Ganoderma resinaceum* EGM (AC:LN774970), and *Emmia latemarginata* EM26 (AC:KX428467). The ascomycete fungi were *Aspergillus fumigatus*, *Aspergillus ochraceus*, *Penicillium oxalicum*, and *Paecilomyces variotii*. The basidiomycete fungi *P. sajor-caju*, *P. pulmonarius*, and *P. columbinus* were supplied from Consultative Comet Company of Mushroom cultivation (CCCM) at Giza, Egypt and *P. floridanus* from the American Type Culture Collection (ATCC). *G. resinaceum* and *E. latemarginata* were isolated from several trees at Damietta province (Egypt) and have been identified by traditional and molecular techniques (El-Fallal et al. 2015; El-Gharabawy et al. 2016). The ascomycete fungi were isolated from the soil at Damietta province (Egypt) and have been identified in the International Mycological Institute (IMI). The fungal cultures were regenerated and maintained on potato dextrose agar (PDA) medium at 25 °C for 5–7 days until sufficient growth was achieved. The PDA medium was prepared by mixing 200 g of potato extract with 20 g of glucose; after complete dissolution of glucose, the volume was made to 1 l with distilled water, 20 g agar were added, and the mixture was autoclaved at 121 °C for 15 min. Orange peel was obtained from the Washington navel orange (*Citrus sinensis* L.)

fruits. The peel was washed twice with distilled water, blotted with filter papers, and dried in a hot air oven at 50 ± 5 °C. Dried peels were grinded into a fine powder, passed through 1-mm mesh, and kept in an airtight container until used in solid-state fermentation.

Solid-state fermentation

The antioxidant activity of the studied fungi was investigated using the powdered orange peel as the substrate in a SSFS in comparison with the standard PDA medium. According to Kheng et al. (2006) and Patel and Gupte (2016), the peel powder: water ratio (*w*/*v*) was 1:4 for the basidiomycete fungi and 1:3 for the ascomycete fungi. The substrates were inoculated with two discs, each of 2-cm diameter from an established culture of the tested fungi and the cultures were incubated at 25 ± 2 °C for 14 days for basidiomycete fungi and 7 days for ascomycete fungi. In addition, the contents of total phenolics, total flavonoids as well the reducing power, and the DPPH scavenging activity of orange peel alone were also assayed.

Extraction of enzymes and antioxidants

For enzyme extraction, the whole culture was mixed thoroughly with 50 ml of phosphate buffer (0.1 M, pH 7.0) and the mixture was incubated in a rotary shaker at 180 rpm and 30 °C for 2 h. The mixture was then squeezed through a wet muslin cloth, followed by centrifugation at 10,000×*g* for 15 min at 4 °C and the clear supernatant (crude extract) was used for enzyme assay. For estimation of non-enzymatic antioxidants, 50 ml of 95% ethanol were added to the culture, and the mixture was incubated for 2 h at room temperature in a rotary shaker at 250 rpm. The mixture was then squeezed through wet muslin cloth and the filtrate was centrifuged at 2800×*g* for 10 min at room temperature.

Estimation of protein content

Protein content was determined by the method of Bradford (1976). An aliquot of the buffer extract (up to 1 ml) was completed to 1 ml with the extraction buffer, followed by addition of 5 ml of the Coomassie brilliant blue reagent and absorbance was read at 595 nm after 2 min. The protein content was estimated with reference to a standard curve of bovine serum albumin in the range of 0–100 μg ml^{-1}.

Assay of peroxidase activity

Peroxidase (EC 1.11.1.x) activity was assayed according to the method described by Ramachandra et al. (1988), using 2,4-dichlorophenol (2,4-DCP) as the substrate. The final reaction mixture (3 ml) contained 0.6 ml of potassium phosphate buffer (0.1 M, pH 7.0), 0.6 ml of 4-aminoantipyrine (16 mM), 0.6 ml of 2,4-dichlorophenol (25 mM), and 0.6 ml of the culture supernatant. The reaction was initiated by the addition of 0.6 ml of hydrogen peroxide (50 mM). The mixture was then incubated at 50 °C for 1 min. The increase in absorbance as a result of the oxidation of 4-aminoantipyrine was measured at 510 nm. One unit of enzyme activity was defined as the amount of enzyme required to increase absorbance by 1 unit ml^{-1}.

Assay of catalase activity

Catalase (EC 1.11.1.6) activity was assayed according to the procedure described by Teranishi et al. (1974). An aliquot of 0.1 ml the culture supernatant was added to 1 ml of 2 mM H_2O_2 and 1.9 ml of the phosphate buffer (0.1 M, pH 7.5). The mixture was incubated at 30 °C for 5 min, then 4 ml of the titanium reagent were added to stop the reaction and the mixture was centrifuged at 3000×*g* for 10 min. Absorbance was measured at 415 nm. The residual H_2O_2 of the sample was calculated by reference to a standard curve in the range of 0–200 μmol H_2O_2. Enzyme activity was expressed as micromole H_2O_2 per milligram protein per minute. Titanium reagent was prepared by dissolving 1 g of titanium oxide and 10 g of K_2SO_4 in 150 ml conc. H_2SO_4 with heating for 2–3 h on a hot plate; the mixture was cooled and made up to 1.5 l with water.

Assay of total phenolics

The total phenolics content of the culture was assayed according to the method described by Luque-Rodríguez et al. (2007). An aliquot of 0.4 ml of the ethanolic extract was added to a mixture of 2 ml of 0.25 N Folin–Ciocalteu phenol reagents and 1.6 ml of 7.5% Na_2CO_3. The mixture was heated in water bath at 50 °C for 5 min, cooled to room temperature in darkness and the absorbance was read at 760 nm by a Pye Unicam SP6-550, UK spectrophotometer. The content of total phenolics was estimated with reference to a standard curve using gallic acid in the range of 0–100 μg ml^{-1}; and the concentration was expressed as micrograms gallic acid equivalent (μg GAE) per gram culture.

Assay of total flavonoids

The total flavonoids content of the culture was assayed according to the method described by Yang et al. (2009). An aliquot of 0.25 ml of the ethanolic extract was mixed with 1.25 ml of distilled water and 75 μl of 5% $NaNO_2$. After 6 min, 150 μl of 10% $AlCl_3$ were added and the mixture was left for 5 min prior to addition of 0.5 ml of 1 M NaOH and 775 μl of distilled water. Absorbance was measured at 510 nm. The content of total flavonoids was estimated with

reference to a standard curve using quercetin in the range of 0–500 μg ml^{-1}, and the concentration was expressed as microgram quercetin equivalent (μg QE) per gram culture.

Estimation of the reducing power

The reduction potential of the culture was estimated according to the method of Oyaizu (1986). An aliquot of 1 ml of the ethanolic extract was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50 °C for 20 min, and 2.5 ml of 10% trichloroacetic acid were added. The mixture was centrifuged for 10 min at 1000×*g*. An aliquot of 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$. The absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates greater reduction potential.

DPPH scavenging activity

The capacity of the fermentation system to scavenge the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was monitored according to the method of Hatano et al. (1988). An aliquot of 0.3 ml of the ethanolic extract was mixed with 2.7 ml of DPPH solution (6×10^{-5} mol l^{-1}). The mixture was shaken vigorously and left to stand for 60 min in the dark to attain stable absorbance. The reduction of the DPPH radical was measured by monitoring continuously the decrease in absorbance at 517 nm. The DPPH scavenging activity was calculated as a percentage of DPPH discoloration using the equation:

$$\text{DPPH scavenging activity} = \frac{(A_d - A_c)}{A_d} \times 100$$

where A_c is the absorbance of the sample and A_d the absorbance of the DPPH solution.

Alignment and phylogenetic analyses

The ITS DNA sequences of the examined fungal species were aligned using CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The phylogenetic tree analysis was viewed and analyzed using MEGA version 4 software. The neighbor joining was performed using the maximum composite likelihood methods with confidence levels estimated by 1000 bootstrap replicates (Tamura and Nei 1993).

Statistical analysis

The experiment was factorial with two factors and three replications in a completely randomized design. The two factors were (1) type of substrate with two levels: the standard culture medium (PDA) and orange peel and (2) species of fungi with ten levels (fungal species). Data were subjected to ANOVA using the SPSS program, version 22. Since the DPPH scavenging activity was expressed as percentage, the data were arcsine transformed before performing ANOVA. Likewise, because of the wide variability in the magnitudes of phenolics and flavonoids contents among the different treatments, their data were log transformed before performing ANOVA. ANOVA and mean separation were applied to the transformed data to ensure homogeneity of variance. Mean separation, to verify the effect of the system components (substrate and fungi) and the fungal group as well as their interaction on the antioxidant activity of the SSFS, was performed using class comparisons according to the planned *F* test adopted by Little and Hills (1978). The means of the six basidiomycete fungi as well as those of the four ascomycete fungi were separated using the Duncan's multiple range test.

Results

Based on ITS-5.85 DNA sequencing, the four *Pleurotus* species were segregated into two intimate groups: *P. floridanus* and *P. columbinus* in one group versus *P. sajor-caju* and *P. pulmonarius* in another group, while *Emmia latemarginata* and *Ganoderma resinaceum* were clustered away from these two groups. The tested ascomycete fungi were clustered in one clade, where *Aspergillus fumigatus* was more related to *Penicillium oxalicum* than to *A. ochraceus,* and *Paecilomyces variotii* was mildly distinct from the other ascomycetes (Fig. 1).

The planned *F* test (Table 1) revealed very highly significant effect of substrate, fungi, fungal group, and the coexistence of system components on the antioxidant activity of the SSFS. Among the different measures of antioxidant activity, the most reliable measure (according to the *F* ratio) was peroxidase activity and phenolics content. Likewise, the most effective factors controlling the antioxidant activity of the

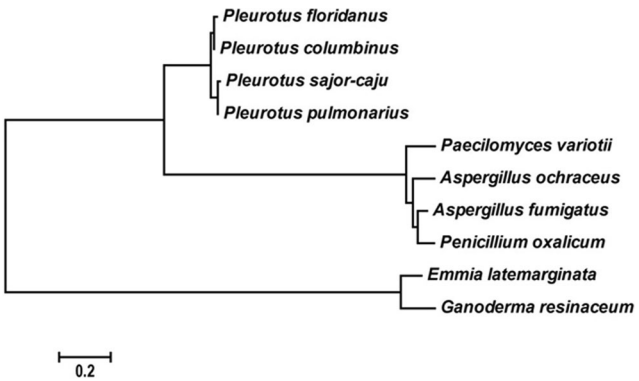

Fig. 1 Phylogenetic tree of the tested fungal species based on ITS-5.85 DNA sequencing

Table 1 Planned *F* test to evaluate the antioxidant activity of six basidiomycete and four ascomycete fungi on a solid-state fermentation system using orange peel. The comparisons—with one DF each—were the effect of (1) substrate (fungi on orange vs. fungi on PDA), (2) fungi (fungi on orange vs. orange), (3) fungal group (basidiomycetes vs. ascomycetes), (4) components of fermentation system (fungi on PDA vs. orange), (5) the interaction of substrate and fungal group (basidiomycetes vs. ascomycetes on PDA and on orange), and (6) the coexistence of system components (fungi on orange vs. fungi on PDA + orange)

Effect of	*F*	*P*	*F*	*P*
	Peroxidase		Total flavonoids	
Substrate (fungi on orange vs. fungi on PDA)	45,485	0.000	6085.2	0.000
Fungi (fungi on orange vs. orange)	8512.4	0.000	468.84	0.000
Fungal group (Basidio. vs. Asco.)	32,113	0.000	684.77	0.000
System components (fungi on PDA vs. orange)	1.7500	0.193	134.79	0.000
Substrate × fungal group	30,323	0.000	42.850	0.000
Coexistence of system components (fungi on orange vs. fungi on PDA + orange)	47,777	0.000	5976.8	0.000
	Catalase		Reducing power	
Substrate (fungi on orange vs. fungi on PDA)	606.33	0.000	6730.2	0.000
Fungi (fungi on orange vs. orange)	182.59	0.000	2.8200	0.101
Fungal group (Asco. vs. Basidio.)	140.97	0.000	1831.1	0.000
System components (fungi on PDA vs. orange)	576.58	0.000	1109.05	0.000
Substrate × fungal group	2202.9	0.000	949.36	0.000
Coexistence of system components (fungi on orange vs. fungi on PDA + orange)	398.53	0.000	5883.0	0.000
	Total phenolics		DPPH scavenging activity	
Substrate (fungi on orange vs. fungi on PDA)	28,989	0.000	3940.4	0.000
Fungi (fungi on orange vs. orange)	27.99	0.000	5.9300	0.019
Fungal group (Asco. vs. Basidio.)	1319.1	0.000	31.310	0.000
System components (fungi on PDA vs. orange)	6067.1	0.000	592.03	0.000
Substrate × fungal group	162.30	0.000	290.92	0.000
Coexistence of system components (fungi on orange vs. fungi on PDA + orange)	24,734	0.000	3473.9	0.000

system were the type of substrate and coexistence of system components (fungi and substrate), followed by the fungal group.

Table 2 shows the relative contribution of the system components to the antioxidant activity. Expectedly, the dried orange peel had no enzymatic activity, in contrast to the fungi which expressed variable enzyme activity according to the fungal group and presence of substrate. The activity of fungal peroxidase was 66 folds higher on orange peel than on PDA and was expressed only in the basidiomycete fungi with absolute absence in the ascomycete fungi. On the other hand, the activity of catalase was expressed in the two fungal groups with higher activity in the basidiomycetes than ascomycetes on PDA but the reverse was true on orange peel. The differential genotypic variability in catalase activity was particularly evident on orange peel, in which the enzyme activity of the ascomycetes was about nine times that of the basidiomycetes in contrast to only two times advantage of basidiomycetes on PDA.

Table 2 Interaction of fungal group and substrate (the antioxidant activities of the basidiomycete and the ascomycete fungi on orange peel and on the PDA medium). Each value is the mean of *n* replicates ± SE. Replication (*n*) was 18 for the basidiomycetes and 12 for the ascomycetes

Antioxidant activity	Orange peel	Fungi on PDA		Fungi on orange peel	
		Basidiomycetes	Ascomycetes	Basidiomycetes	Ascomycetes
Peroxidase (U ml^{-1})	0.000	0.016 ± 0.006	0.000	1.11 ± 0.15	0.000
Catalase (μmol H_2O_2 ml^{-1})	0.000	6.74 ± 0.69	3.32 ± 0.48	0.73 ± 0.40	6.46 ± 0.33
Phenolics (μg gallic acid ml^{-1})	548 ± 2.0	16.0 ± 0.91	38.9 ± 3.43	383 ± 22.2	568 ± 5.21
Flavonoids (μg quercetin ml^{-1})	137 ± 5.1	37.8 ± 5.04	108 ± 8.79	709 ± 50.1	1446 ± 75
Reducing power (A_{700})	0.48 ± 0.1	0.17 ± 0.02	0.39 ± 0.04	0.48 ± 0.03	0.51 ± 0.06
DPPH scavenging activity (%)	72.1 ± 0.42	30.2 ± 1.91	44.8 ± 4.19	77.9 ± 0.68	70.4 ± 5.09

The phenolic content of orange peel alone was 20% higher than that of the SSFS (the average of the ten fungi on orange peel), and the latter was in turn 17-folds higher than that of fungi alone (the average of the ten fungi on PDA). The effect of fungal group on phenolic content of the SSFS was evident in favor of the ascomycetes, which produced 50% higher phenolic content than the basidiomycetes. The overall low phenolic content of the PDA culture was associated with 150% higher phenolic content of the ascomycetes over the basidiomycetes. By contrast, the by far higher phenolic content of the SSFS was associated with 48% higher content in the basidiomycetes over the ascomycetes. The flavonoid content of orange peel alone was about twice that of fungi alone (the average of the ten fungi on PDA), but the action of fungi on orange peel increased flavonoid content of the SSFS by an average of 14-folds above that of fungi on PDA and by an average of sixfolds above that of orange peel alone. The flavonoid content of the culture was in general higher with the ascomycetes than with the basidiomycetes, and the magnitude of increase amounted to 185% and 100% on PDA and orange peel, respectively (Table 2).

The reducing power and the DPPH scavenging activity of orange peel alone were about twice those of the ten fungi on PDA, but the action of fungi on orange peel restored the reducing power and DPPH scavenging activity of the SSFS to a level comparable to that of orange peel alone. The reducing power of the ascomycete fungi was generally higher than that of the basidiomycetes, and the increase amounted to 125% on PDA and only 7% on orange peel. The DPPH scavenging activity of fungi on PDA was 48% higher with the ascomycetes than with the basidiomycetes but was comparable in the two fungal groups on orange peel (Table 2).

The differential antioxidant activity of the six basidiomycete fungi and the four ascomycete fungi in relation of the nature of substrate was specified in Tables 3 and 4. The peroxidase activity was very weak in the fungi grown on PDA compared with those grown on orange peel. On PDA, only two out of the six investigated basidiomycete fungi, viz. *P. columbinus* and *P. floridanus*, exhibited limited comparable peroxidase activity. By contrast, on orange peel, all six basidiomycete fungi exhibited marked and variable peroxidase activity, with the highest activity for *P. floridanus*, followed by

Table 3 The activity of peroxidase and catalase in the solid-state fermentation system involving ten fungal species grown either on PDA or orange peel. Each value is the mean of three replicates ± SE. Within each fungal group, means with common letters are not significantly different at $P < 0.05$

Fungus	Peroxidase activity (U ml^{-1})	Catalase activity (μmol H_2O_2 ml^{-1})
Fungi on PDA medium		
Basidiomycetes		
Emmia latemarginata	0.000	5.79 ± 0.06^{b}
Ganoderma resinaceum	0.000	4.99 ± 0.40^{a}
Pleurotus columbinus	0.04 ± 0.01^{a}	12.1 ± 0.17^{d}
P. floridanus	0.05 ± 0.01^{ab}	8.67 ± 0.11^{c}
P. pulmonarius	0.000	4.43 ± 0.24^{a}
P. sajor-caju	0.000	4.40 ± 0.63^{a}
Ascomycetes		
Aspergillus fumigatus	0.000	2.57 ± 0.07^{b}
A. ochraceus	0.000	5.58 ± 0.19^{d}
Paecilomyces variotii	0.000	3.84 ± 0.13^{c}
Penicillium oxalicum	0.000	1.31 ± 0.05^{a}
Fungi incubated with orange peel		
Basidiomycetes		
Emmia latemarginata	0.39 ± 0.003^{a}	0.000
Ganoderma resinaceum	1.32 ± 0.014^{e}	0.000
Pleurotus columbinus	1.15 ± 0.013^{d}	4.40 ± 0.29
P. floridanus	2.29 ± 0.02^{f}	0.000
P. pulmonarius	0.62 ± 0.01^{b}	0.000
P. sajor-caju	0.87 ± 0.001^{c}	0.000
Ascomycetes		
Aspergillus fumigatus	0.000	6.88 ± 0.11^{b}
A. ochraceus	0.000	5.29 ± 0.11^{a}
Paecilomyces variotii	0.000	7.97 ± 0.06^{c}
Penicillium oxalicum	0.000	5.69 ± 0.34^{a}

Table 4 The antioxidant activity, in terms of the of phenolics content, flavonoids content, reducing power, and DPPH scavenging activity, of the solid-state fermentation system involving ten fungal species grown either on PDA or orange peel. Each value is the mean of three replicates ± SE. Within each fungal group, means with common letters are not significantly different at $P < 0.05$

Fungus	Phenolics (μg gallic acid ml^{-1})	Flavonoids (μg quercetin ml^{-1})	Reducing power (A_{700})	DPPH scavenging activity %
Fungi on PDA medium				
Basidiomycetes				
Emmia latemarginata	16.0 ± 0.54^{cd}	26.5 ± 2.2^{b}	0.30 ± 0.017^{e}	29.3 ± 0.63^{c}
Ganoderma resinaceum	18.3 ± 0.07^{e}	65.6 ± 6.6^{ef}	0.16 ± 0.002^{c}	36.0 ± 0.56^{d}
Pleurotus columbinus	16.0 ± 0.86^{c}	59.2 ± 0.7^{e}	0.17 ± 0.001^{d}	32.0 ± 0.80^{cd}
P. floridanus	13.5 ± 0.59^{b}	27.8 ± 4.4^{bc}	0.08 ± 0.001^{a}	18.7 ± 1.95^{a}
P. pulmonarius	10.5 ± 0.42^{a}	6.73 ± 0.8^{a}	0.14 ± 0.009^{b}	24.2 ± 3.81^{b}
P. sajor-caju	21.4 ± 2.35^{f}	41.2 ± 3.0^{d}	0.17 ± 0.002^{cd}	41.0 ± 1.92^{e}
Ascomycetes				
Aspergillus fumigatus	28.7 ± 0.70^{a}	83.2 ± 2.9^{a}	0.32 ± 0.003^{ab}	56.0 ± 0.42^{c}
A. ochraceus	36.9 ± 1.57^{c}	155 ± 6.5^{d}	0.32 ± 0.015^{b}	40.5 ± 0.49^{b}
Paecilomyces variotii	57.8 ± 0.24^{d}	90.8 ± 5.3^{ab}	0.59 ± 0.012^{c}	58.8 ± 1.28^{cd}
Penicillium oxalicum	32.1 ± 1.29^{ab}	103 ± 6.6^{abc}	0.30 ± 0.003^{a}	24.1 ± 1.18^{a}
Fungi incubated with orange peel				
Basidiomycetes				
Emmia latemarginata	265 ± 12^{a}	630 ± 29^{bc}	0.55 ± 0.002^{d}	76.7 ± 1.8^{ab}
Ganoderma resinaceum	290 ± 8.4^{ab}	554 ± 14^{b}	0.27 ± 0.003^{a}	75.6 ± 0.40^{a}
Pleurotus columbinus	350 ± 5.5^{c}	971 ± 36^{e}	0.46 ± 0.002^{c}	79.5 ± 0.24^{b}
P. floridanus	512 ± 20.6^{ef}	412 ± 84^{a}	0.58 ± 0.001^{e}	83.0 ± 0.32^{c}
P. pulmonarius	469 ± 6.73^{e}	769 ± 21^{cd}	0.58 ± 0.004^{e}	76.8 ± 0.61^{ab}
P. sajor-caju	412 ± 14.6^{d}	920 ± 22^{de}	0.41 ± 0.002^{b}	76.2 ± 0.09^{ab}
Ascomycetes				
Aspergillus fumigatus	560 ± 5.4^{a}	1219 ± 22^{a}	0.58 ± 0.002^{c}	83.7 ± 0.56^{d}
A. ochraceus	588 ± 6.3^{a}	1345 ± 46^{ab}	0.16 ± 0.001^{a}	41.5 ± 1.05^{a}
Paecilomyces variotii	570 ± 13^{a}	1690 ± 195^{c}	0.54 ± 0.001^{b}	76.9 ± 0.70^{b}
Penicillium oxalicum	555 ± 4.4^{a}	1530 ± 146^{abc}	0.76 ± 0.002^{d}	79.5 ± 0.91^{bc}
Orange peel	548 ± 2.0	137 ± 5.1	0.48 ± 0.001	72.1 ± 0.42

Ganoderma resinaceum and *P. columbinus* and the lowest activity for *Emmia latemarginata.* In contrast to the basidiomycete fungi, peroxidase activity was absolutely absent in the four investigated ascomycete fungi, neither on PDA nor on orange peel. In contrast to peroxidase, the fungal catalase was characterized with higher activity on PDA than on orange peel, and this was associated with more frequent occurrence only within the basidiomycetes. On PDA, *P. columbinus* exhibited the highest catalase activity among the six investigated basidiomycete fungi, followed by *P. floridanus* with comparably lower activity in the remaining four basidiomycetes. On orange peel, only *P. columbinus* among the basidiomycetes and the four investigated ascomycetes exhibited moderate catalase activity with limited interspecies variability.

The low phenolic content of the PDA culture as well as the high phenolic content of the SSFS was accompanied with marked interspecies diversity. On PDA, the phenolic content among the six basidiomycetes was highest in *P. sajor-caju*, followed by *Ganoderma resinaceum* but was least in *P. pulmonarius*. Among the four ascomycetes, phenolic content was highest in *Paecilomyces variotii* but lowest in *Aspergillus fumigatus*. However, the ranking of species differed on orange peel; on which, *P. floridanus* exhibited the highest phenolic content among the basidiomycetes, followed by *P. pulmonarius* while *Emmia latemarginata* and *Ganoderma resinaceum* exhibited the least content. The interspecies diversity in phenolic content was distinct among the four ascomycetes grown on PDA but was very weak among those grown on orange peel.

On PDA, *Ganoderma resinaceum* and *P. columbinus* exhibited the highest flavonoid content among the basidiomycetes while *P. pulmonarius* exhibited the lowest content. The interspecies diversity among the ascomycetes on PDA was relatively weak compared to that among the basidiomycetes, with relatively high flavonoid content in *Aspergillus ochraceus* but low content in *A. fumigatus*. On orange peel,

P. columbinus and *P. sajor-caju* exhibited the highest flavonoid content among the basidiomycete fungi, while *P. floridanus* exhibited the least content. The interspecies diversity in flavonoid content was less evident in the ascomycetes than in the basidiomycetes, with relatively high content in *Paecilomyces variotii* and low content in the two *Aspergillus* species.

The higher reducing power of the ascomycete fungi over the basidiomycete fungi was more evident on PDA than on orange peel. On PDA, *Emmia latemarginata* exhibited the highest reducing power among the basidiomycete fungi while *P. floridanus* exhibited the least reducing power. Among the ascomycete fungi, *Paecilomyces variotii* exhibited the highest reducing power which was almost twice that of the other three species. The genotypic difference in the reducing power between the two fungal groups was less evident on orange peel than on PDA. Among the basidiomycete fungi, *P. floridanus*, *P. pulmonarius*, and *Emmia latemarginata* exhibited a reducing power 30% higher than that of *P. columbinus* and *P. sajor-caju* and about twice that of *Ganoderma resinaceum*. Among the ascomycete fungi, *Penicillium oxalicum* exhibited reducing power about 34% higher than those of *A. fumigatus* and *Paecilomyces variotii* and 4.5 times that of *A. ochraceus*.

The genotypic variability in the antioxidant activity among the two fungal groups was least expressed in terms of DPPH scavenging activity, with marked superiority of ascomycetes on PDA, but marginal superiority of basidiomycetes on orange peel. Among the basidiomycetes grown on PDA, *P. sajor-caju* exhibited the highest DPPH scavenging activity, followed by *Ganoderma resinaceum* and *P. columbinus*, while the least activity was exhibited by *P. floridanus*. Among the ascomycete fungi, *Paecilomyces variotii* and *A. fumigatus* exhibited the highest DPPH scavenging activity while the least activity was for *P. oxalicum*. On orange peel, the interspecies diversity in DPPH scavenging activity was limited among the basidiomycetes, where *P. floridanus* exhibited 7% higher activity than the remaining five species. Among the ascomycetes, *A. fumigatus*, *Penicillium oxalicum*, and *Paecilomyces variotii* exhibited about twice the DPPH scavenging activity of *A. ochraceus*.

The peroxidase and catalase activities of the investigated fungal species exhibited distinct curvilinear relationships with the phenolic content of the fermentation system. Whereas peroxidase activity increased with the increase in phenolic content of the medium up to an optimal concentration followed by marked decline with further increase in phenolic content, catalase activity exhibited the opposite trend, with a phenolic content of 300 μg gallic acid ml^{-1} corresponding to the maximum activity of peroxidase but to the minimum activity of catalase (Fig. 2a). The contrasting behavior of catalase and peroxidase activities towards phenolics content of the medium was expressed clearly in the inverse relationship between catalase and peroxidase activities of the investigated fungal species (Fig. 2b).

A close positive correlation was observed between the non-enzymatic antioxidant activities of the SSFS, with correlation coefficient (R) of 0.910 and 0.888 between phenolic content of the medium in one hand and both of the flavonoids content and the DPPH scavenging activity, respectively on the other hand. Similarly, the value of R between the reducing power and the DPPH scavenging activity approached 0.818 (Fig. 3).

Discussion

Catalase and peroxidase exhibited contrasting response to the cultural conditions in the two fungal groups. Whereas incubation with orange peel led to radical enhancement of fungal peroxides and inhibition of catalase activity in the basidiomycetes, it led to marked promotion of catalase activity in the ascomycetes with absolute absence of peroxidase in this group either on PDA or orange peel. This differential behavior of the two enzymes can be related to the phenolic content of the fermentation medium. The contrasting behavior of the two enzymes towards phenolics is evident in Fig. 2, where the relationship between enzymes activity and the phenolic content of the medium approached a quadratic function with maximal peroxidase but minimal catalase activity at a moderate phenolic content and a tendency towards low peroxidase activity but high catalase activity at extreme phenolic levels. It seems probable that phenolics—at moderate concentrations—might serve as the substrate for peroxidase but at the same time might inhibit catalase activity; whereas, high levels of phenolics seem to inhibit peroxidase but to promote catalase activity. The differential response of fungal enzymes towards substrate phenolics has been reported (Schmidt et al. 2014), where phenolic extracts showed an inhibition potential for peroxidase but not for polyphenol oxidase.

Furthermore, the high phenolic content of native orange peel was subjected to reduction upon incubation with fungi, particularly the basidiomycetes. This might mean that fungi which have limited native ability to produce phenolics (on PDA) can at the same time consume significant proportions of the surplus phenolics of orange peel and that this consumption is accomplished by virtue of the fungal peroxidase. This effect was particularly evident for basidiomycetes which might possess limited native ability to produce phenolics but at the same time have greater ability to consume phenolics of orange peel, compared with the ascomycetes. The low phenolics content of the SSFS can be attributed to consumption of substrate phenolics by microorganisms in growth (Kawai et al. 1994). The strong positive linear correlation ($R = 0.910$) between phenolics and flavonoids contents of the fermentation system implies that the contrasting behavior of the two enzymes can also be extended to flavonoids where increasing flavonoid content—released from orange peel—within limits

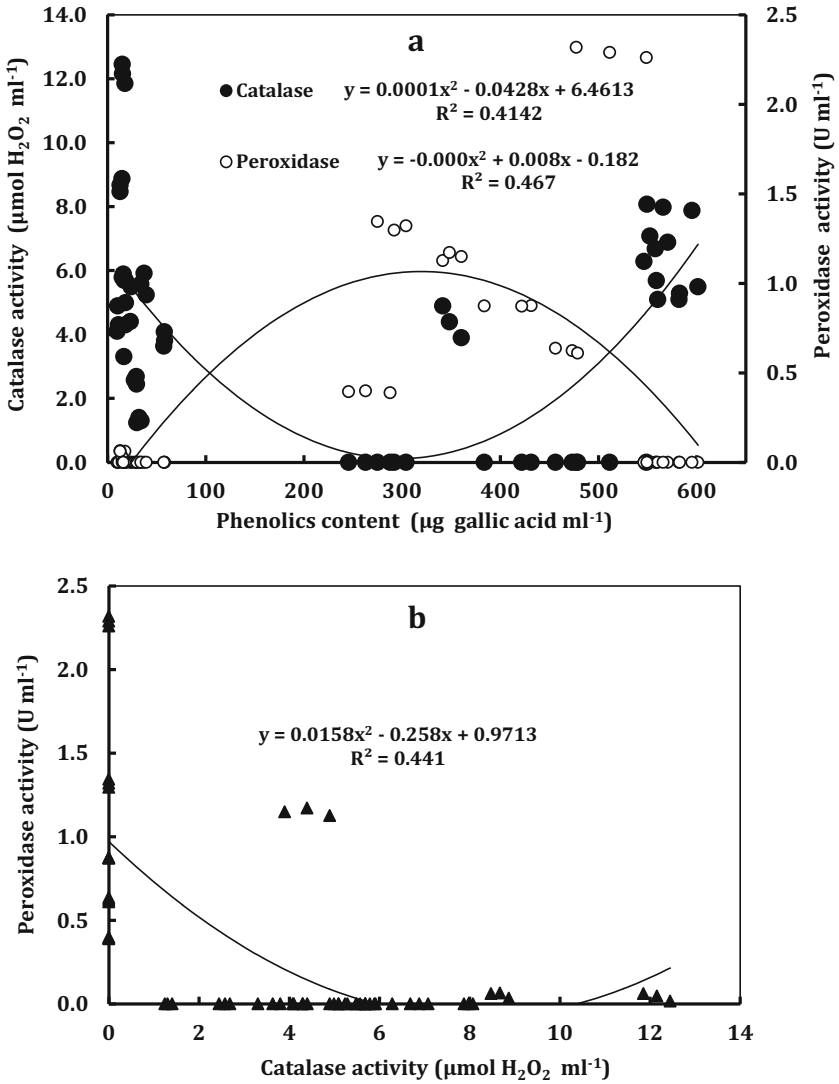

Fig. 2 The contrasting behavior of catalase and peroxidase activities towards phenolics content of the solid-state fermentation system (**a**) and the inverse relationship between catalase and peroxidase activities (**b**)

can promote peroxidase but inhibit catalase activity, with the reverse being evident beyond a certain flavonoid content.

In addition, the difference in enzyme activity between the two fungal groups was also distinct. Whereas peroxidase activity was expressed only in the basidiomycetes, particularly on orange peel, with absolute absence in the ascomycetes, the activity of catalase, on the other hand, was expressed in the two fungal groups but in favor of the ascomycetes, which expressed higher catalase activity on orange peel than on PDA. In this respect, *Aspergillus fumigatus* has been reported to produce three active catalases: one from conidia and two from mycelia (Paris et al. 2003). By contrast, basidiomycetes as wood-decomposing fungi excrete extracellular oxidoreductases, including peroxidases and laccases which are directly involved in the degradation of lignin in their natural lignocellulosic substrates (Dashtban et al. 2010; Gao et al. 2011; Martínková et al. 2016). The ability of *Pleurotus pulmonarius* to grow on orange residue has been attributed to its capability to produce phenol oxidases (Inácio et al. 2015). The presence of fungal catalase inhibited laccase activity of several basidiomycete fungi (Ballaminut et al. 2009).

Orange peel thus might provide the substrate for peroxidase, which might aid in resolution of the rather vague genotypic variability among the investigated basidiomycete fungi in peroxidase activity. Among the six basidiomycete fungi investigated, *P. columbinus*, *Ganoderma resinaceum*, and *P. floridanus* exhibited high peroxidase activity whereas *Emmia latemarginata* was the least efficient species. Likewise, *P. columbinus* and *P. floridanus* exhibited the highest catalase activity among the basidiomycete fungi while *P. pulmonarius* and *P. sajor-caju* were the least effective. The genotypic variability in catalase activity was rather weak among the ascomycete fungi which, in contrast to the basidiomycetes, exhibited higher catalase activity on orange peel. Among the four investigated ascomycete fungi grown on

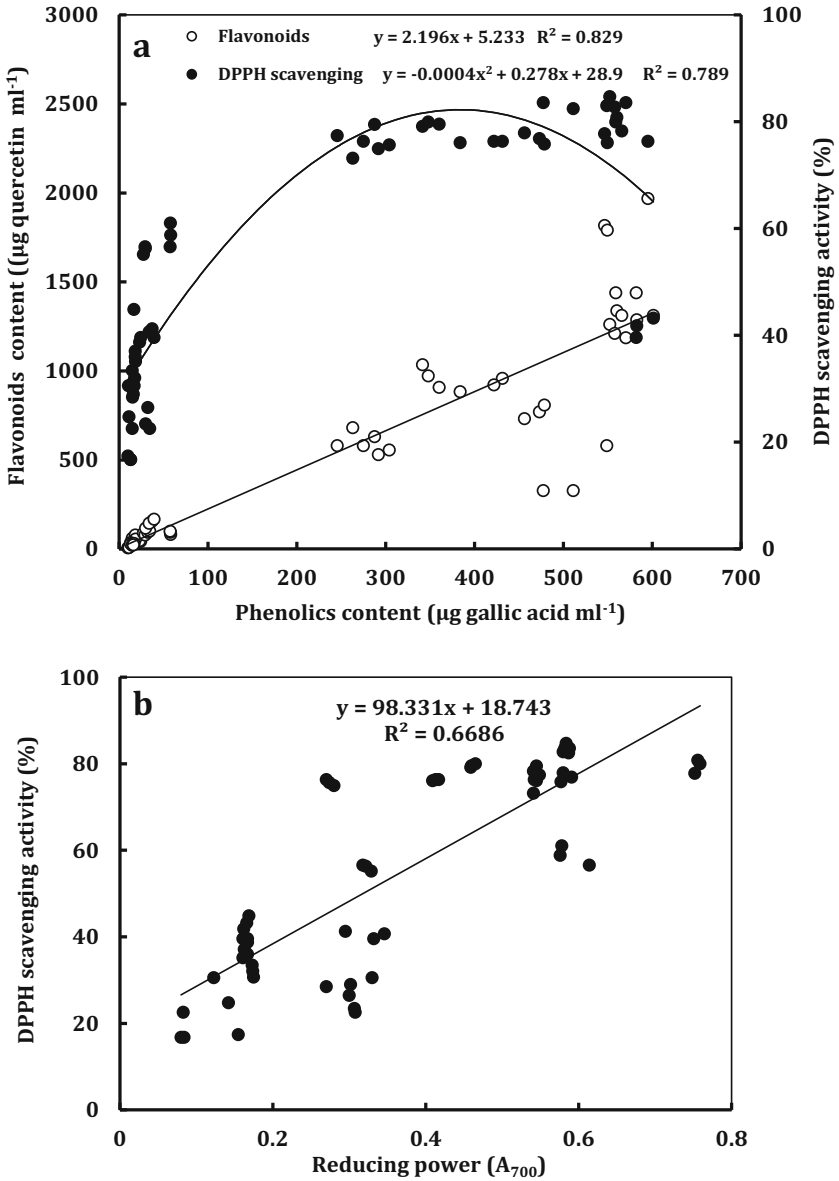

Fig. 3 Correlation between the non-enzymatic antioxidant activities of the solid-state fermentation system: flavonoids content and DPPH scavenging activity against phenolics content (**a**) and DPPH scavenging activity against reducing power (**b**)

orange peel, *Paecilomyces variotii* and *A. fumigatus* exhibited higher catalase activity than did *A. ochraceus* and *Penicillium oxalicum*. In this respect, the biodegradation potentiality of Basidiomycota has been attributed to their ability to produce laccases, tyrosinases, and various types of peroxidases (Kües 2015). The present work suggests that orange peel with its high phenolic content promotes fungal catalase but inhibits peroxidase activity, and this is in agreement with the findings of Schmidt et al. (2014) that application of phenolic extracts inhibited peroxidase activity in *Rhizopus oryzae* without effect on polyphenol oxidase activity. However, it seems that the effect of phenolics on enzyme activity seems to be dose dependent since, in the present work, maximum peroxidase activity and minimum catalase activity was found at moderate phenolics concentration of the SSFS with extreme levels leading to the opposite effect.

The difference between the two fungal groups was also evident in the non-enzymatic antioxidant activity (phenolic and flavonoid contents as well as the reducing power and DPPH scavenging activity) which were all higher in the ascomycete fungi than in the basidiomycetes. The tested fungal species might have an inherent limited ability to produce phenolics from the PDA medium that was by far lower than the phenolic content of orange peel. The lower phenolic content of the basidiomycete culture either on PDA or on orange peel below that of ascomycetes, along with an overall lower phenolic content of the SSFS than that of orange peel alone refers to lower native capacity of the basidiomycetes to produce phenolics but to a greater ability to consume the phenolics of orange peel compared with the ascomycetes. This behavior varied greatly among species and it seems that among the tested basidiomycete fungi some species of high inherent

ability to produce phenolics (for example *Emmia latemarginata* and *Ganoderma resinaceum*) also exhibit high ability to consume the orange peel–phenolics and vice versa for the less efficient phenolics-producing species (*P. pulmonarius* and *P. floridanus*). *Ganoderma* spp. exhibited high antioxidant activity, reducing power, scavenging and chelating abilities, and total phenol content (Mau et al. 2002). Ascomycetes reflected a different pattern of greater inherent ability for production of phenolics, associated with lower ability to consume exogenous phenolics. Furthermore, the interspecies diversity, among the investigated ascomycete fungi, was evident only in the phenolics-producing ability (on PDA; being high for *Paecilomyces variotii* but low for *Aspergillus fumigatus*) with an overall limited ability, along with faint interspecies diversity, in consuming exogenous phenolics of orange peel. In agreement with our findings, it has been claimed that Basidiomycota can degrade phenolics employing the phenol oxidases such as laccase and tyrosinase enzymes and peroxidases (Martínková et al. 2016). In particular, *Pleurotus pulmonarius* has the ability to grow on orange residue (Inácio et al. 2015), by virtue of its capability to produce phenol oxidases.

Among the antioxidant compounds, polyphenols have gained special importance due to their large array of biological actions that include free radical scavenging, metal chelation, enzyme modulation activities, and inhibition of low density lipoprotein (LDL) oxidation, among others (Rodrigo and Bosco 2006). A strong positive correlation has been demonstrated between the total phenolics content (TPC) and the antioxidant activity of SSFS using different fungal species on different types of plant residues (Cai et al. 2011; Elmastas et al. 2007; Ibrahium 2010; Mansuri et al. 2018). Furthermore, this trend has been demonstrated in higher plants, where the antioxidant activity and the concentrations of phenolics and flavonoids of *Lawsonia inermis* seeds are proportionate to each other (Philip et al. 2011).

The flavonoids, reducing power and DPPH scavenging activity followed, but to a lesser extent, a pattern similar to that of phenolics; in that, their levels were higher in orange peel than in fungi alone (on PDA). Nevertheless, the activity of fungi on orange peel yielded either higher (flavonoids and reducing power) or comparable (DPPH scavenging activity) levels to that of orange peel alone. Thus, the flavonoid content, as well as the reducing power and the DPPH scavenging activity of the SSFS, can be attributed mainly to the substrate (orange peel) with minor participation of fungi, which might have a limited inherent ability to synthesize flavonoids on PDA. This ability of fungi to synthesize flavonoids was further augmented on orange peel, which points to the ability of fungi to utilize flavonoid precursors in orange peel to release free flavonoids. In this respect, isoflavonoids of *A. niger*-soybean SSFS were thought to be derived from isoflavone glucosides in soybean (Kawai et al. 1994). Similarly, most natural phenolics of apple pomace were in the bound form, conjugated with protein, carbohydrates, lipids, terpenes, chlorophyll, and other organic compounds and only a relatively small part was in the free or soluble form (Hamdipour et al. 2014). The phenolic content of fermented rice bran is mainly caused by the cleavage of compounds complexed with lignin (Schmidt and Furlong 2012). The lignin fraction of agro-industrial residues of vegetables and cereals contains numerous phenolic compounds, which can also be recovered by SSF. Since fungi grow on these residues, they use the polysaccharides after lignin degradation in order to grow and reproduce (Martins et al. 2011). The extract of pomegranate peel, which contains high number of flavonoids and polyphenolic compounds, exhibited great antioxidant activity, and this might be due to hydroxyl groups existing in the phenolic compounds that can provide the necessary component as a radical scavenger (Pourmorad et al. 2006).

Furthermore, the inherent fungal ability to synthesize flavonoids as well as the fungal ability to release flavonoids from orange peel was in favor of the ascomycete fungi, and this was associated with limited interspecies diversity. The interspecies diversity in producing flavonoids was, however, marked in the basidiomycetes with different trends on PDA and orange peel. Whereas the inherent ability to synthesize flavonoids (on PDA) was highest in *Ganoderma resinaceum* and *P. columbinus* and least in *P. pulmonarius*, the ability to release flavonoids from orange peel was highest in *P. columbinus* and *P. sajor-caju* and lowest in *P. floridanus* and *Ganoderma resinaceum*. Likewise, the marked superiority of the ascomycetes over basidiomycetes regarding the reducing power and DPPH scavenging activity on PDA points to greater inherent ability for reducing power and DPPH scavenging activity of the ascomycetes over basidiomycetes. Among the basidiomycetes, *Emmia latemarginata* exhibited the highest reducing power on PDA, and *E. latemarginata* along with *P. floridanus* and *P. pulmonarius* exhibited the highest reducing power on orange peel. Among the ascomycete fungi, *Paecilomyces variotii* exhibited the highest reducing power on PDA but *Penicillium oxalicum* exhibited the highest reducing power on orange peel.

The present work suggests that the reducing power and DPPH scavenging activity were the least sensitive measures of non-enzymatic antioxidant activity, exhibiting limited variability among the SSFS components (fungi on PDA versus orange peel), among the two fungal groups (ascomycetes versus basidiomycetes) and among individual fungal species within each group. This agreement in behavior between those less sensitive measures is manifested as a strong curvilinear correlation ($R^2 = 0.694$) between reducing power and DPPH scavenging activity. Nevertheless, the fungal group × substrate interaction was evident, with marked superiority of the ascomycetes on PDA but comparable activity of the two fungal groups on orange peel. The interspecies diversity in DPPH

scavenging activity was relatively evident on PDA, on which *P. sajor-caju* exhibited the highest activity and *P. floridanus* the least activity among the basidiomycetes. Likewise, *Paecilomyces variotii* and *A. fumigatus* exhibited the highest DPPH scavenging activity among the ascomycetes while *P. oxalicum* exhibited about half such activity. On orange peel, the interspecies diversity in DPPH scavenging activity was limited among the basidiomycetes compared with the ascomycetes, in which *A. fumigatus* exhibited twice the activity of *A. ochraceus*. The interspecies diversity in DPPH scavenging activity has been reported among several mushrooms (Barros et al. 2007). The high antioxidant levels, estimated as DPPH scavenging activity, coincided with high total phenolics content of apple pomace (Hamdipour et al. 2014).

Conclusions

Phenolics and flavonoids at moderate concentrations might promote peroxidase and inhibit catalase activity, but the reverse was evident at high levels. Fungi, particularly the basidiomycetes, have limited native ability to produce phenolics but can consume exogenous phenolics, through the action of peroxidase, which was expressed only in the basidiomycetes. The activity of catalase, on the other hand, was expressed in the two fungal groups in favor of the ascomycetes. Fungi can utilize flavonoid precursors in orange peel to release free flavonoids. Furthermore, the inherent ability to synthesize flavonoids as well as the ability to release flavonoids from orange peel was in favor of the ascomycete fungi. The interspecies diversity in producing flavonoids was marked in the basidiomycetes with different trends on PDA and orange peel. The reducing power and DPPH scavenging activity were the least sensitive measures of non-enzymatic antioxidant activity, with limited variability among the SSFS components (fungi versus orange peel), among the two fungal groups (ascomycetes versus basidiomycetes) and among individual fungal species within each group. The matching between the segregation patterns of the ten investigated fungal species based on DNA sequencing and the differential antioxidant activity of the SSFS is not consistently evident. However, this similarity emerged quite convincingly in PDA, compared with orange peel, in terms of catalase activity and contents of phenolics and flavonoids.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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