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Recovery of Phenolic Acid and Enzyme Production from Corn Silage Biologically Treated by *Trametes versicolor*

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Abstract Corn silage is used as high-energy forage for dairy cows and more recently for biogas production in a process of anaerobic co-digestion with cow manure. In this work, fresh corn silage after the harvest was used as a substrate in solid-state fermentations with *T. versicolor* with the aim of phenolic acid recovery and enzyme (laccase and manganese peroxidase) production. During 20 days of fermentation, 10.4-, 3.4-, 3.0-, and 1.8-fold increments in extraction yield of syringic acid, vanillic acid, *p*-hydroxybenzoic acid, and caffeic acid, respectively, were reached when compared to biologically untreated corn silage. Maximal laccase activity was gained on the 4th day of fermentation (*V.A.* = 180.2 U/dm^3), and manganese peroxidase activity was obtained after the 3rd day of fermentation (*V.A.* = 30.1 U/dm^3). The addition of copper(II) sulfate as inducer during solid state fermentation resulted in 8.5- and 7-fold enhancement of laccase and manganese peroxidase activities, respectively. Furthermore, the influence of pH and temperature on enzyme activities was investigated. Maximal activity of laccase was obtained at T = 50 °C and pH = 3.0, while manganese peroxidase is active at temperature range T = 45–70 °C with the maximal activity at pH = 4.5.

Keywords Corn silage · Solid-state fermentation · *Trametes versicolor* · Phenolic acids · Laccase · Manganese peroxidase

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Introduction

Lignocellulose waste materials such as agricultural and forestry residues have been recently investigated for many purposes according to the local and global sustainable waste management requirements. Significant efforts are dedicated to the methods of biological treatment employing white rot fungi with the aim of lignocellulose material pretreatment for (a) biofuel production [1–4]; (b) production of value-added biotechnological products such as enzymes, organic acids, antibiotics, flavor, phenolic compounds, and aroma compounds [5, 6]; and (c) its use as livestock feed [7].

The main obstacle for lignocellulose degradation to useful compounds/products is lignin surrounding cellulose and hemicellulose, providing the plant cell wall structural rigidity and resistance to microbial attack. Degradation of lignin is difficult due to its structural complexity, high molecular weight, and insolubility [8]. It consists of phenylpropane units connected by different linkages. Three propionic alcohols exist as lignin monomers: coniferyl, coumaryl, and sinapyl alcohol. Lignin fractions of agricultural and forestry residues contain many phenolic components, mainly acids like ferulic, *p*-coumaric, syringic, vanillic, and *p*-hydroxybenzoic acids [9]. Maize bran is rich source of ferulic acid while *p*-CuA is predominant in cereal stems [10]. It is also known that ferulic acid content can vary with the corn grain maturity [11]. Phenols are bioactive compounds, known to be good antioxidants. They comprise flavonoids, phenolic acids, tannins, etc. Hydroxybenzoic and hydroxycinnamic acids are two subgroups of phenolic acids which differ in their structure [12]. Phenolic compounds have many beneficial health impacts including anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, anti-cancer, anti-aging, cardioprotective, and vasodilatory effects [13–16]. Some phenolic compounds also have industrial applications in production of paints, paper, cosmetics, and tanning agents and in the food industry as additives (as natural colorant and preservatives) [17].

Isolation of phenolic compounds from biological materials is usually performed by extraction with organic solvents [18]. This method is however only applicable for simple phenolic compounds, while the extraction of nonextractible highly polymerized proanthocyanidins and complexes of phenols with proteins, fibers, and polysaccharides is more difficult to perform by the aforementioned method [19]. Most high-molecular-mass polyphenols exist as condensed or hydrolyzable tannins and complexes with proteins, fibers and polysaccharides [19]. These compounds make the extraction of phenolic/polyphenolic compounds more difficult and therefore should be previously hydrolyzed or degraded. This is usually performed with acids (ecologically not acceptable) or enzymes (economically not acceptable). Recently, different microorganisms are used to make liberation and enhance extraction of low and high molecular phenolic antioxidants and other bioactive compounds [19]. For example, white-rot fungi have been so far cultivated in a solid-state culture using lignocellulose materials as substrates to produce various value-added products (enzymes, antibiotics, surfactants, etc.) [19–22].

In order to obtain desired products after biological treatment of lignocellulose using the method of solid-state fermentation, selection of microorganism is very important [12]. Many parameters can influence on the phenolic compound release, when treating lignocellulose with white-rot fungi, such as properties of substrate, particle size of substrate, addition of enzyme inducers, biodegradability, water absorption, water activity, mixing, temperature, pH, and aeration [23]. *Trametes versicolor* is a white-rot fungus, a

well-known producer of lignolytic enzymes (e.g., laccases, manganese peroxidases, lignin peroxidases, versatile peroxidases). In order to investigate the possibility to use *Trametes versicolor* cultivated under solid-state cultivation with the aim of lignolytic enzyme production (mainly laccase), different materials were explored so far, such as olive leaves [24], wheat straw, barley straw, wood shavings [25], barley bran [25, 26], steam-exploded corn stalk [27], wheat bran [26, 28], corn cobs [29], cone of *Pinus nigra*, sawdust, corn bran, oat, rice bran, canola, dried tea, ground clover straw, sunflower stalk, soybean bagasse [26], rapeseed meal [30], tomato pomace [31], and horticultural waste [32]. Different strategies were applied in the abovementioned researches, varying initial substrate moistures, addition of extra nitrogen or carbon sources, different initial inoculum concentration, different reactor designs, addition of inducers, etc. In this work, enhancement of laccase and manganese peroxidase activities is investigated by the addition of different organic and inorganic inducers during solid-state fermentation of *T. versicolor* on corn silage. Laccases are oxidative enzymes related to the degradation of phenolic compounds, including lignin units, with concomitant reduction of oxygen to water. They can be used in many biotechnological applications, in pharmaceutical and food industries, as biosensors, or in environmental applications [33]. Manganese peroxidases catalyze the oxidation of Mn^{2+} to Mn^{3+} which is a strong oxidant that can oxidize phenolic and aromatic amines to organic radicals [34, 35].

In our previous work [36], high lignin degradation (up to 71 %) was reached when corn silage was treated with *T. versicolor* in laboratory flasks and in a tray bioreactor. In this work, biological treatment of corn silage with white-rot fungus *Trametes versicolor* was performed with the aim (a) to enhance laccase and manganese peroxidase production and (b) to recover some phenolic acids, which are naturally incorporated in lignin structure. To the best our knowledge, this is the first attempt to produce caffeic acid, vanillic acid, *p*-hydroxybenzoic acid, and syringic acid from corn silage by solid-state fermentation of *T. versicolor*. This method could be applied for the materials that naturally have higher amounts of phenolic compounds.

Materials and Methods

Substrate and Microorganism

Corn silage (harvest August 2014, eastern Croatia) cultivated for forage consumption and biogas production were harvested, chopped, and delivered to the laboratory from Bovis Ltd., Ivankovo, Croatia. The samples were stored at −20 °C prior to use in biological treatment experiments. *T. versicolor* TV-6 (MZKI, Ljubljana, Slovenia) was cultivated on PDA medium for 7 days at 27 °C.

Experimental Setup 1: Biological Pretreatment of Corn Silage by *T. versicolor* with the Aim of Recovery of Phenolic Acids

Fifty grams of corn silage, particle size 1.0–2.0 cm, were mixed with 110 cm^3 of distilled water in 720-cm^3 laboratory jars autoclaved (121 °C/20 min), cooled overnight, and then inoculated with five mycelial plugs (diameter 1 cm) suspended in 10 cm^3 of sterile water. After inoculation, biological treatments were performed at 27 °C for 5, 9, 13, and 20 days in an

incubator (BINDER GmbH, KB 115, Germany) and all experiments were carried out in triplicate. After the treatment, the samples were dried at 45 °C during 48 h and milled by ultracentrifugal mill (Retsch ZM200, Germany) in order to obtain the 1-mm particle size, for further preparation of extracts.

Preparation of Extracts

About 1.0 g of milled dry substrate (experimental setup 1) was extracted by solvent (50:50, water/ethanol, *v*/*v*) with solid/liquid ratio 1:40. Extraction was performed in a water bath at 80 °C (Julabo, SW23, Germany) by shaking (200 rpm) during 120 min. Samples for extraction were withdrawn before fermentation, after the 5th, 9th, and 13th days of fermentation and at the end of fermentation (after the 20th day). After the extraction, samples were centrifuged at 10,000×*g* (Multifuge 3 L-R Centrifuge, Heraus, Germany) for 10 min in order to obtain liquid extracts for further UHPLC analysis of phenolic acids.

Determination of Phenolic Acids by Ultra-High Performance Liquid Chromatography

The identification of phenolic compounds in corn silage extracts was performed by high-performance liquid chromatography (HPLC) method according to Aliakbarian et al. [37] with injection volume modification (20 μl). Samples were analyzed by UHPLC (Nexera XR, Shimadzu, Japan), using a UV detector (at 280 nm). Separation was performed in a Shim-pack GIST C18 column (250 × 4.6 mm inner diameter, 5 μm, Shimadzu, Japan).

Experimental Setup 2: Biological Pretreatment of Corn Silage by *T. versicolor* with the Aim of Laccase and Manganese Peroxidase Production

For the measurements of laccase and manganese peroxidase activity, additional experiments with and without the addition of inducers were performed. Fifty grams of corn silage was moisturized with 100 cm^3 of water, sterilized, and cooled. Then, 10 cm^3 of sterile water with suspended spores of five mycelial plugs (diameter 1 cm) and 10 cm^3 of sterile inducer solution were added. Five different inducers in three different concentrations were used: inorganic inducer ($CuSO_4$, c = 0.05, 0.1, and 0.15 mol/dm^3) and organic inducers (ferulic acid, vanillic acid, syringic acid, and veratryl alcohol, c = 0.5, 1, and 1.5 mmol/dm^3). Two jars were prepared for each experiment, including experiment without addition of inducers. Incubations were performed in an incubator (BINDER GmbH, KB 115, Germany) at 27 °C during 7 days.

Extract Preparations

Samples (experimental setup 2) were withdrawn daily. One gram of the sample was extracted with 10 cm^3 of distilled water in a laboratory shaker at 27 °C and 150 rpm, during 30 min. After extraction, samples were centrifuged (10 min, 10,000×*g*) to obtain crude enzyme solution for activity measurements of enzymes.

Measurements of Enzyme Activities

Laccase and manganese peroxidase activities were measured according to previously published methods for laccase [38] and manganese peroxidase activity measurement

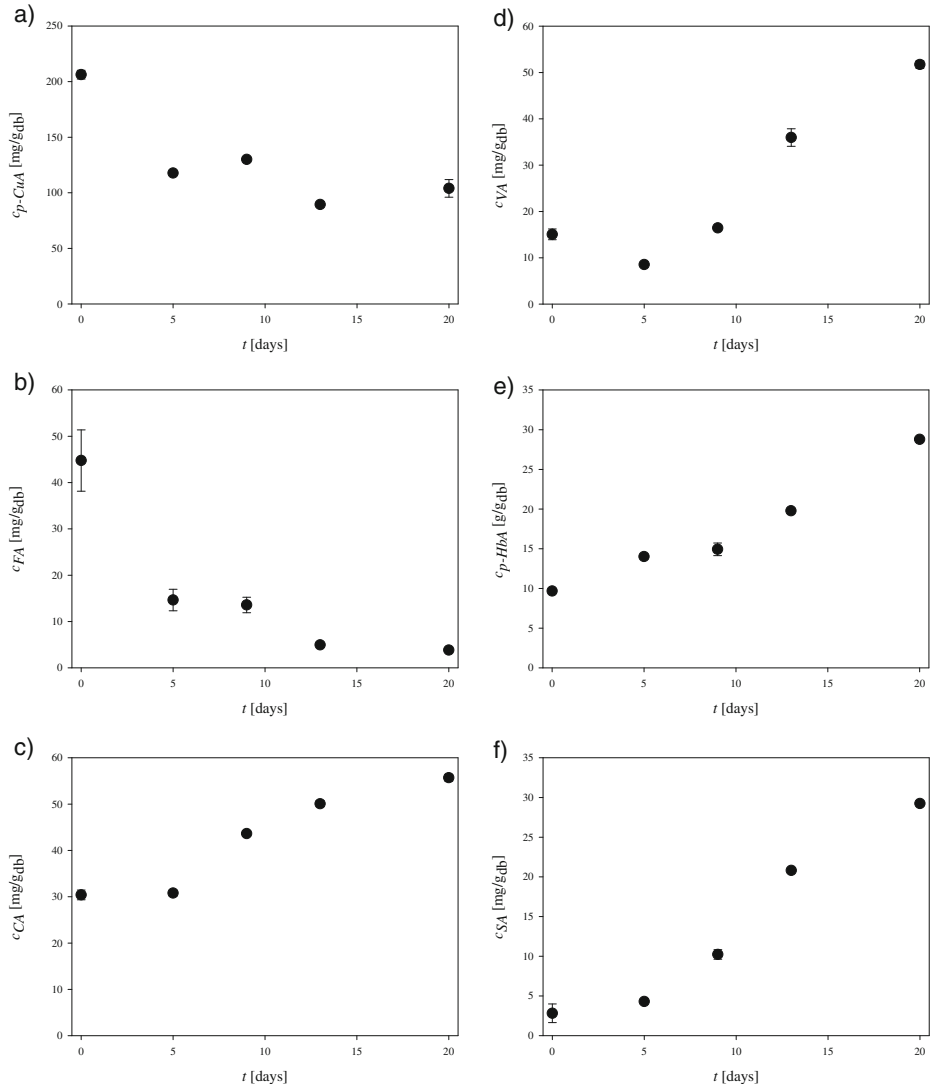

Fig. 1 Content of *p*-coumaric acid, *p*-CuA (**a**); ferulic acid, FA (**b**); caffeic acid, CA (**c**); vanillic acid, VA (**d**); *p*-hydroxybenzoic acid, *p*-HbA (**e**); and syringic acid, SA (**f**) of corn silage extracts obtained during biological treatment with *T. versicolor*

[39]. The influence of pH and temperature on crude laccase activity was measured as described in our previous work [40]. The influence of pH on laccase activity was investigated in 0.05 mol/dm^3 citrate-phosphate buffer (pH 3.0–5.4) and 0.2 mol/dm^3 glycin-HCl buffer (pH 2.4–3.6) by measuring the activity after incubation of the crude enzyme solution for 5 min at 27 °C. The influence of pH on manganese peroxidase activity was investigated in 0.05 mol/dm^3 sodium-malonate buffer in pH range 2.0–7.0 by the same method as used for laccase. The effect of temperature on enzyme activity was determined from 25 to 75 °C in 0.2 mol/dm^3 glycin-HCl buffer, pH 3.5 (for laccase) and in 0.05 mol/dm^3 sodium-malonate buffer, pH 4.5 (for manganese -peroxidase). All

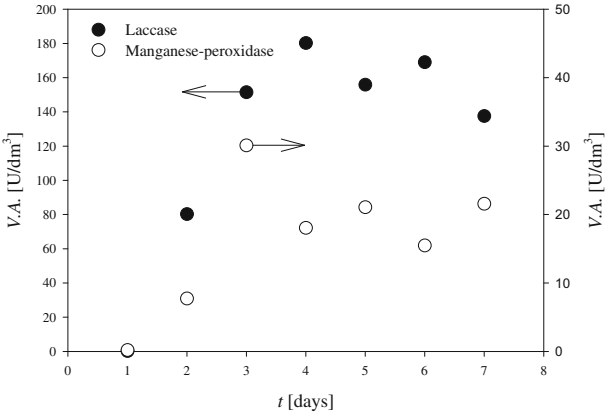

Fig. 2 Activities of laccase and manganese peroxidase during *T. versicolor* growth on corn silage (T = 27 °C)

measurements are done in triplicate. The results are expressed as relative values of an average with the standard deviation.

Results and Discussion

Recovery of Phenolic Acids

The aim of this experiment was to investigate the possibility to increase the amount of phenolic acids present in corn silage after its treatment with *T. versicolor*. Figure 1 presents the changes of phenolic acid content during 20 days of *T. versicolor* cultivation of corn silage. According to the obtained results, it was shown that among three hydroxybenzoic acids (vanillic acid, VA; syringic acid, SA; *p*-hydroxybenzoic acid, *p*-HBA) and three hydroxycinnamic acids (*p*-coumaric acid, *p*-CuA; ferulic acid, FA; caffeic acid, CA), untreated corn silage (on a dry basis, db) possessed the highest amount of *p*-CuA (206.3 $\mu g/g_{db}$) and FA (44.8 $\mu g/g_{db}$). Decrease of *p*-CuA and FA was obtained during 20 days and ranged from 206.3 to 103.0 $\mu g/g_{db}$ and from 44.8 to 3.8 $\mu g/g_{db}$, respectively (Fig. 1a, b). It can be calculated that the conversion of *p*-CuA was X = 50.1 % while the conversion of FA was X = 91.5 %. However, the aim was not to decrease, but to increase content of phenolic acids presented in corn silage. It was successfully performed with four other investigated phenolic acids. The increase of CA, VA, *p*-HBA, and SA was gained and was in the range 30.4–55.7, 15.1–51.7, 9.7–28.8, and 2.8–29.2 $\mu g/g_{db}$, respectively (Fig. 1c–f).

This is the consequence of the complex enzymatic system of *T. versicolor* which successfully degrades lignin, as was shown in our previous work [36]. Ferulic acid is a component having great potential to be further processed in order to obtain products like vanillic acid, vanillin, and 4-vinyl guaiacol [10, 41, 42]. Vanillin is a main flavoring and aroma compound used in a wide range of foods and also in some fragrances [10, 43]. During this research, a strong negative correlation between FA and VA was gained (−0.96) and the concentration of vanillic acid increased for 3.3-fold. This strategy could be applied by using substrates with higher initial concentration of phenolic compounds. The results obtained in this study are in accordance to literature data. Dinis et al. [44] employed four white-rot fungi (*Trametes versicolor*, *Bjerkandera adusta*, *Ganoderma applanatum*, and *Phlebia rufa*) in wheat straw bioconversion. During 28 days of SSF, constant degradation of esterified hydroxycinnamic acid occurred. Cai et al. [45] carried out solid-state fermentation of oat

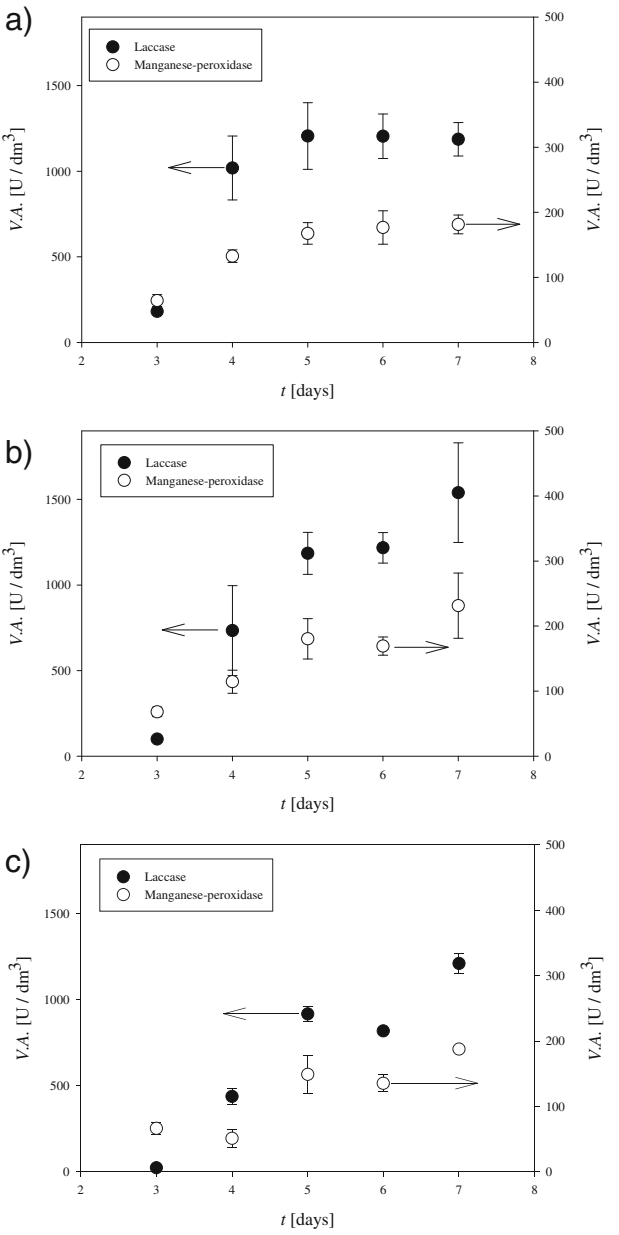

Fig. 3 The effect of different $CuSO_4$ concentrations (**a**) 0.05 mol/dm^3 $CuSO_4$, (**b**) 0.10 mol/dm^3 $CuSO_4$, and (**c**) 0.15 mol/dm^3 $CuSO_4$ on production of laccase and manganese peroxidase during solid-state fermentation of *T. versicolor* on corn silage (T = 27 °C)

(*Avena sativa* L.) with three filamentous fungi (*Aspergillus oryzae* var. *effuses*, *Aspergillus oryzae*, and *Aspergillus niger*) during 3 days and achieved increment of hydroxycinnamic acid content. During both researches, phenolic acid content varied depending on the applied microorganism. Results given by Dueñas et al. [46], where solid-state fermentation was conducted on soybean seeds with three fungi (*Aspergillus oryzae*, *Rhizopus oryzae*, and *Bacillus subtilis*), showed increment in *p*-HBA, VA, and SA concentration with exception of SA where a slight reduction was observed in bioconversion with *B. subtilis*. However, FA disappeared after fermentation process.

Laccase and Manganese Peroxidase Production

The aim of these experiments was to improve the production of manganese peroxidase and laccase with the addition of different inducers into nutrient media. The experiments lasted 7 days since in preliminary experiments it was shown that both enzyme activities decrease after 8 days of fermentation under investigated process conditions. Laccase and manganese peroxidase activities measured in the crude enzyme solution obtained during 7 days of cultivation without the addition of inducer were presented in Fig. 2.

Maximal laccase activity was gained on the 4th day (*V.A.* = 180.2 U/dm^3) and did not significantly decrease until the end of experiments. Maximal manganese peroxidase activity was obtained after the 3rd day, and it was *V.A.* = 30.1 U/dm^3 after what decreased on the 4th day to the value of 18.0 U/dm^3 and remained stable until the end of experiment.

In order to obtain higher laccase or manganese peroxidase activities, different inorganic inducers, such as $CuSO_4$, and organic inducers, such as ferulic acid, vanillic acid, syringic acid, and veratryl alcohol, were added into the corn silage at three different concentrations. Among all investigated inducers, $CuSO_4$ was shown to be the best inducer for both enzymes. The results are presented in Fig. 3. The maximal volumetric activities of laccase were up to 8.5 times higher in comparison to the experiment performed without the addition of the inducer. The maximal activities were gained after the 7th day of cultivation and were in the range from 1187.2 to 1539.4 U/dm^3. However, for the production of laccase of activities up to 1539.4 U/dm^3, up to 7 days of cultivation of *T. versicolor* on corn silage with the addition of $CuSO_4$ were needed. In our previous work, by cultivating *T. versicolor* in a submerge culture, 15 different chemicals were used to prepare nutrient media to produce laccase during 11 days (3 days for inoculum production and 8 days for enzyme production) with volumetric activities of 2378 U/dm^3 [38]. This is additional confirmation on the benefit of solid-state fermentation over submerge cultivation of white-rot fungi for enzyme production.

The maximal activities of manganese peroxidase were up to seven times higher in comparison to the experiment performed without the addition of the inducer. The maximal volumetric activities were gained after the 7th day of cultivation and were 181.4, 231.5, and 187.6 U/dm^3 for c ($CuSO_4$) = 0.05 mol/dm^3, c ($CuSO_4$) = 0.1 mol/dm^3, and c ($CuSO_4$) = 0.15 mol/dm^3, respectively.

In order to better emphasize the influence of different inducers on laccase and manganese activity, only the experiments with the highest obtained activities were presented on Fig. 4.

Based on the obtained results, it can be confirmed that the choice and concentration of the inducer have influence on enzyme activity, as given in numerous researches and reviews during the last few decades and recently [33, 47–49]. An extensive work of Knežević et al. [48] on the influence of the addition of inducers (*p*-anisidine and veratryl alcohol) on laccase and manganese peroxidase activities of different *Trametes* species on delignification properties was done. They confirmed previous researches that the extent of delignification is not in correlation with the enzyme activity.

When veratryl alcohol and ferulic acid were used, maximal laccase activities were obtained when 1 mmol/dm^3 solution of inducer was added into nutrient media, while when syringic acid was used, the addition of the lowest investigated concentration (0.05 mmol/dm^3) led to the highest laccase activities. When ferulic acid was applied, maximal laccase activities were gained on the 4th day (*V.A.* = 283.5 U/dm^3, c (FA) = 1 mmol/dm^3)). When veratryl alcohol was applied, maximal activities were

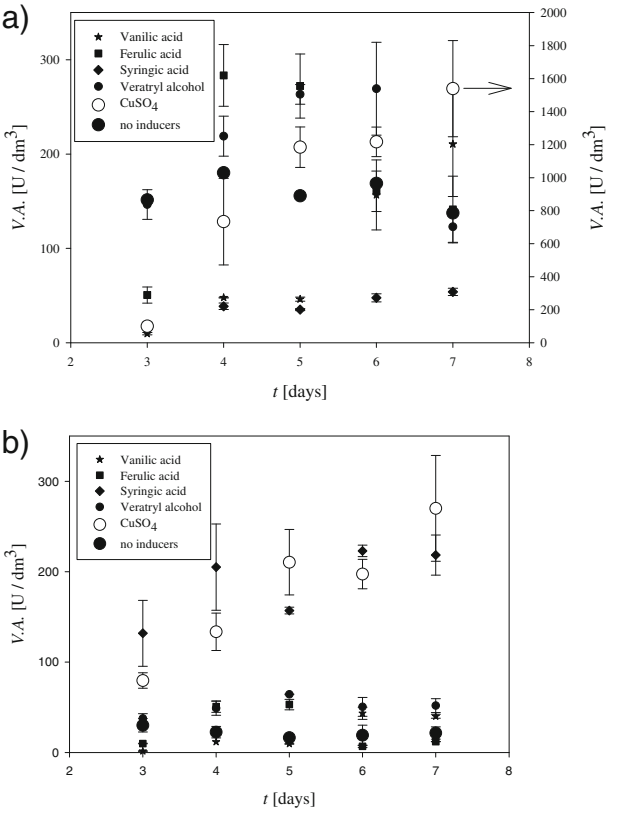

Fig. 4 The influence of inducers on laccase (**a**) and manganese peroxidase (**b**) activities during *T. versicolor* growth on corn silage (*m* (substrate) = 50 g; inoculum, five plugs; initial moisture, 75 %, *T* = 27 °C, *c* (inducers) = 0.1 mol/dm^3 ($CuSO_4$); *c* (inducers) = 1 mmol/dm^3 (ferulic acid, veratryl alcohol); *c* (inducers) = 0.05 mmol/dm^3 (syringic acid); *c* (inducers) = 1.5 mmol/dm^3 (vanillic acid))

obtained on the 6th day (*V.A.* = 269.3 U/dm^3, *c* (VA) = 1 mmol/dm^3). When syringic acid was used as inducer, maximal activities were obtained on the 6th day (*V.A.* = 223.0 U/dm^3, *c* (SA) = 0.5 mmol/dm^3). In an experiment performed with vanillic acid, laccase activity was very low in the first 5 days, after which it started to increase. The maximal activity was gained on the 7th day (*V.A.* = 210.6 U/dm^3, *c* (VA) = 1.5 mmol/dm^3). It can be concluded that the use of inducers postponed the laccase induction, since in the experiment without the addition of inducers maximal laccase activity was obtained after the 4th day of cultivation (*V.A.* = 180.2 U/dm^3). Maximal manganese peroxidase activities of 64.3 and 53.1 U/dm^3 for veratryl alcohol and ferulic acid, respectively, were obtained when 1.0 mmol/dm^3 solution of inducer was added during cultivation of *T. versicolor*. Vanillic acid led to the highest manganese peroxidase activity (43.4 U/dm^3) when added into nutrient media in a concentration of 1.5 mmol/dm^3 while when syringic acid was used, the addition of the lowest investigated concentration (0.05 mmol/dm^3) caused the highest manganese peroxidase activities (53.9 U/dm^3).

After crude enzyme suspension was produced, dependence of laccase and manganese-peroxidase activities on pH value and temperature were measured. The results are presented in Figs. 5 and 6.

According to the obtained results, it is clear that laccase shows its maximal activity at *T* = 50 °C and pH = 3.0, while manganese peroxidase is most active at temperature range *T* = 45–70 °C and pH = 4.5.

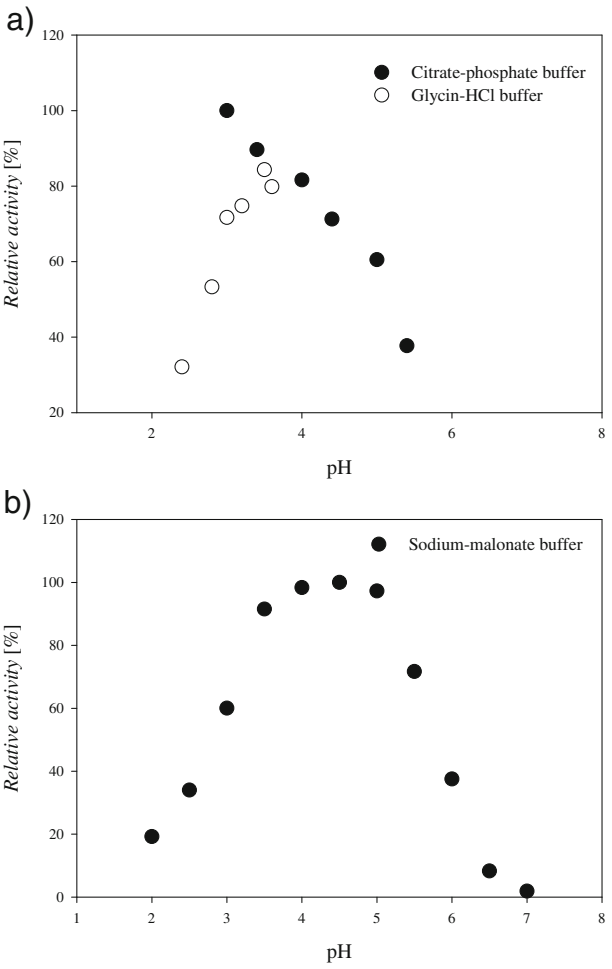

Fig. 5 Dependence of laccase (**a**) and manganese peroxidase activity (**b**) on pH

Conclusion

Corn silage can be used as a substrate for the production of caffeic acid, vanillic acid, *p*-hydroxybenzoic acid, and syringic acid by solid-state fermentation of *T. versicolor.* After 20 days of corn silage treatment with *T. versicolor*, 10.4-, 3.4-, 3.0-, and 1.8-fold increments in extraction yield of syringic acid, vanillic acid, *p*-hydroxybenzoic acid, and caffeic acid, respectively, were reached.

It was proved that the choice and the concentration of the inducer have influence on enzyme activity. Among all tested inducers ($CuSO_4$, ferulic acid, vanillic acid, syringic acid, and veratryl alcohol), $CuSO_4$ greatly induced the laccase and manganese peroxidase production ability of *T. versicolor*. The maximal obtained laccase activity was 1539.4 U/dm^3, while the maximal manganese peroxidase activity was 209.1 U/dm^3. It was shown that the optimal temperature for laccase activity is T = 50 °C while the optimal pH is 3.0. Manganese peroxidase is active in a broad temperature range from 45 to 70 °C with the maximal activity at pH = 4.5.

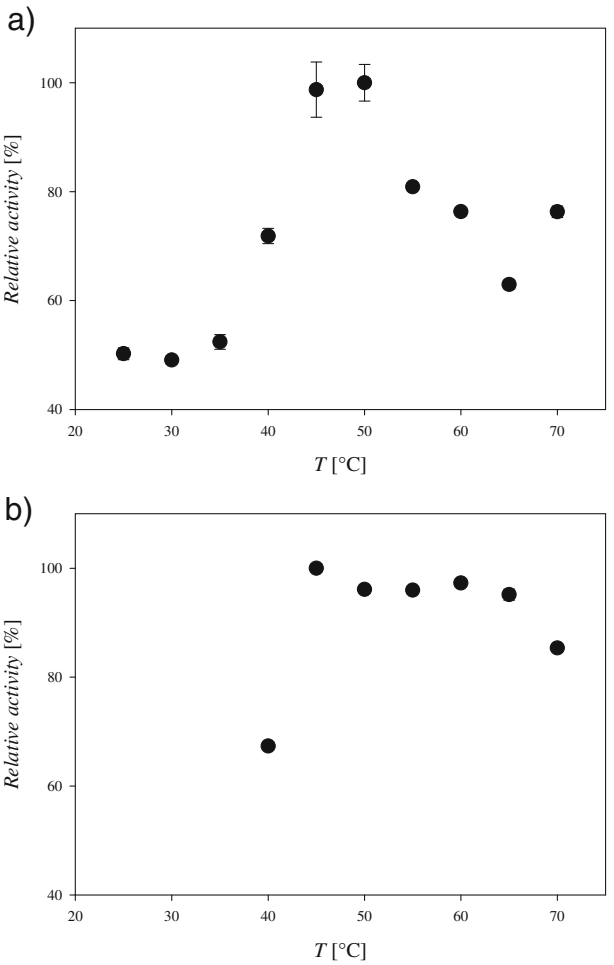

Fig. 6 Dependence of laccase (**a**) and manganese peroxidase activity (**b**) on temperature

Results of this study indicate that the solid-state fermentation using *Trametes versicolor* is a promising environmentally friendly method for the recovery of target phenolic compounds and enzyme productions, and it will be further tested with the substrates that are richer in phenolic compounds, such as agro-food waste.

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