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Peroxidases from grass clippings for the removal of phenolic compounds from wastewater

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ABSTRACT

Green waste, especially of municipal origin, is currently used as a material only to a limited extent. However, the large material flows could also be used in a more economical way if they were integrated into biorefinery concepts. Besides the production of basic and fine chemicals, green waste could also be used as source of industrial relevant enzymes. Here, the purification and characterization of peroxidases from common lawn grass species *Lolium perenne* and *Festuca arundinacea* are reported. The purified peroxidase fractions as well as crude extracts were investigated for the removal of common wastewater pollutants such as phenol, *m*-cresol, and 2,4-dichlorophenol by oxidative polymerization. The grass-derived peroxidases showed the highest affinity towards 2,4-dichlorophenol, followed by *m*-cresol and phenol. A crude extract of real lawn grass was able to remove over 95 % of 0.5 mM 2,4-dichlorophenol within 20 min.

1. Introduction

In view of climate change, ongoing urbanization, and the depletion of fossil sources for fuels and chemicals, the use of bio-based resources is becoming more and more important. One approach is to optimize the (re-)use of organic waste as a raw material for different products. Especially in major cities, a large amount of organic waste is generated, which is still not recycled in an economical manner. A large share of organic waste that accumulates in urban areas is green waste. Herein, green waste is defined as plant material with a low lignin content, which accumulates primarily in urban areas from parks, gardens, and roadsides. It consists mainly of grass clippings, but may also include leaves, twigs, and smaller plants, the composition depending on the season and location. This results in highly heterogeneous waste material. So far, most of the green waste is composted or serves as raw material for energy production. However, the costs of collecting and transporting green waste exceed the value added by current reuse methods. Alternatively, green waste represents a promising biological feedstock (Langsdorf et al., 2021).

Recently, we explained efficient methods for the pretreatment of such materials (Varriale et al., 2022) and demonstrated the use of grass clippings as a substrate for the microbial production of the terpenoid α-humulene (Langsdorf et al., 2022). In continuation of this work, we hypothesized that green waste may also serve as a cost-effective source of enzymes. The extraction of technically relevant enzymes from waste materials is particularly interesting. The resource does not have to be cultivated but accumulates anyway. As a result, costs are reduced and acreage does not compete with food.

Peroxidases are enzymes in demand for a variety of industrial applications and are ubiquitously found in plants. They belong to the class of oxidoreductases (EC1.11.1.X) and are able to oxidize various substrates under the reduction of hydrogen peroxide (Pandey et al., 2017). Plant peroxidases are heme-dependent peroxidases and belong to the class III peroxidases of the peroxidase-catalase superfamily (Pandey et al., 2017). The numerous functions of plant peroxidases include germination, stress tolerance, cell wall metabolism, lignification, fruit ripening, and defense against pathogens (Pandey et al., 2017). Similarly diverse as the tasks of peroxidases in plants are the possible applications of (plant) peroxidases in industry, which have been summarized by several excellent reviews (Hamid and Khalil-ur-Rehman, 2009; Pandey et al., 2017; Sellami et al., 2022). Applications encompass biosensors, diagnostic and analytical kits, polymer synthesis, and synthetic dye

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degradation. Horseradish peroxidase (HRP) is the enzyme of choice in most of those applications. Nevertheless, alternative sources of peroxidases have been investigated. For example, peroxidases have been purified and characterized from turnip (Duarte-Vázquez et al., 2000), papaya fruit (Pandey et al., 2012), the fungus *Coprinus* (Ikehata et al., 2005), or the tree legume *Leucaena leucocephala* (Pandey and Dwivedi, 2011), amongst others. Peroxidases have also been isolated from different grasses such as Guinea grass (*Panicum maximum*) (Centeno et al., 2017), *Miscanthus x giganteus* (Dragana et al., 2017), or wheat grass (*Triticum aestivum*) (Lai et al., 2006).

Wastewater treatment applications of peroxidases do not necessitate highly purified enzymes (Feng et al., 2021), which is why crude enzyme extracts from commodity-scale biomass present a potential alternative. Target pollutants are mainly phenolic compounds, which must be removed from wastewater before it is released into the environment. Phenolic compounds are highly regulated, as many of them can be toxic or even carcinogenic and can accumulate in the food chain. Some major sources of industrial wastewater containing phenolic compounds are petroleum refining, coal refining, the textile and leather industry, and pulp and paper mills, amongst others (Kumaran and Paruchuri, 1997; Nicell et al., 1993). Conventional methods for removing phenolic contaminants from wastewater include distillation, adsorption, extraction, membrane processes, chemical as well as electrochemical oxidation, advanced oxidation processes, and biological treatment (Mohamad Said et al., 2021; Mohd, 2022; Villegas et al., 2016). Kilbanov et al. proposed enzymatic phenol removal as an alternative to bacterial degradation because it is less susceptible to factors such as substrate concentration, pH, temperature, and toxic contaminants (Klibanov et al., 1983). Peroxidases catalyze the formation of free radicals from various aromatic pollutants resulting in their polymerization and aggregation (Klibanov et al., 1983). In most cases, the oxidized products are less toxic and are more likely to be biodegradable compared to the phenolic starting compounds (Torres-Duarte and Vazquez-Duhalt, 2010). Due to the conversion of phenols into insoluble polymers, the products can be separated quite easily (Klibanov et al., 1983). While the advantages of enzymatic treatment are mainly the high transformation rate and high selectivity towards phenolic compounds (Mohamad Said et al., 2021), the enzymes are also highly dependent on the environmental conditions. In the case of oxidative polymerization of phenols, there is also enzyme inactivation by the end-product polymers, which adsorb to the enzymes and block access to the active site (Nakamoto and Machida, 1992).

The cost-effectiveness of enzymatic removal depends mainly on the properties and cost of the enzymes. To further optimize the process, different sources of plant peroxidases were investigated for wastewater treatment. Peroxidases from soybeans (Bódalo et al., 2006; Wright and Nicell, 1999; Steevensz et al., 2014) and turnips (Duarte-Vázquez et al., 2003) in particular have been suggested as promising alternatives to HRP for wastewater treatment. The application of peroxidases from alternative low-purity peroxidase sources was also already demonstrated as shown by Yadav et al. for the conversion of phenolic compounds with gourd (*Luffa aegyptiaca*) fruit juice (Yadav et al., 2017).

The aim of this study was to investigate grass clippings as the main component of green waste as a cheap and readily available source of peroxidases for phenol removal. Following the 12 principles of green chemistry according to Anastas and Warner, the use of enzymes from green waste for wastewater treatment can contribute especially in the form of using renewable raw materials and by using enzymes as "green" catalysts (Anastas and Warner, 2000). With regard to the UN Sustainable Development Goals (SDG), this will contribute in particular to SDG6 "Clean water and sanitation" as well as SDG12 "Responsible consumption and production". By removing pollutants from the environment, a contribution to the achievement of SDG14 "Life below water" and SDG15 "Life on land" can also be expected, while using waste materials for the production of enzymes contributes indirectly to SDG2 "Zero hunger".

2. Materials and methods

2.1. Grass cultivation

To investigate peroxidases from grass clippings, the two common grass species *Lolium perenne* (perennial ryegrass) and *Festuca arundinacea* (tall fescue) were used as a season-independent and reproducible model substrate for the otherwise highly heterogeneous material. Grass seeds were obtained from Feldsaaten Freudenberger GmbH & Co. KG (Krefeld, Germany). The two types of grass were planted separately in planter boxes with a volume of 6 l (40 (L) x 18 (W) x 13.7 (H) cm). COMPO SANA® lawn soil (N 50–300 mg/l, P_2O_5 100–370 mg/l, K_2O 200–500 mg/l,) with a pH value of 5.0–6.5 from COMPO GmbH (Münster, Germany) was used for cultivation. To eliminate potential fungus gnat larvae and other biological contaminants, the soil was treated at 90 °C for 30 min. Before use, the soil was cooled to room temperature. The planter boxes were filled with soil to 2 cm below the rim, the seeds were distributed homogeneously and covered with a 1 cm layer of soil, which was then lightly pressed down. The planter boxes were placed in a KBW 240 growth chamber from BINDER GmbH (Tuttlingen, Germany). An image of the planter boxes in the growth chamber is shown in Fig. S1. The grass was cultivated in a day-night cycle with 20 °C and daylight for 14 h and 15 °C without light for 10 h. The soil was kept moist with dH_2O until the grass was fully grown. After that, the grass was watered once a week with 300 ml dH_2O per planter box. After reaching about 10 cm in length, the grass was cut to about 4 cm each time. The grass samples were stored at −80 °C. In addition, lawn grass was harvested from a semi-shaded meadow in the city of Giessen, Germany (50°35′24" N, 8°40′55″ E) during autumn in 2021.

2.2. Homogenization of grass

The enzymes were released by mechanical cell disruption of the grass clippings. The grass was homogenized with an Ultra-Turrax TP18/2N (20,000 rpm, 75 W, 50 Hz) from Janke & Kunkel KG. (Staufen im Breisgau, Germany). 2 g of grass were homogenized in 20 ml of 50 mM potassium phosphate buffer at the fixed speed of 20,000 rpm for 1 min while being cooled on ice. The homogenized grass was passed through a cotton cloth and wrung out to separate most of the solids. The homogenate was then centrifuged at 3000 ×*g* and 4 °C for 30 min. The supernatant was collected and stored at −80 °C.

2.3. Ammonium sulfate precipitation

Fractionation by precipitation was performed in two steps. In the first step, the crude extract was brought to an ammonium sulfate saturation of 40 % by adding solid ammonium sulfate. The mixtures were incubated at 4 °C for 30 min and then centrifuged at 4 °C and 16,000 ×g for 30 min. The supernatant was collected and the pellet was discarded. In the second step, the supernatant was brought to 80 % saturation by adding solid ammonium sulfate. Again, the mixtures were incubated at 4 °C for 30 min and then centrifuged at 4 °C and 16,000 ×g for 30 min. The supernatant was discarded and the pellet containing the peroxidases was resuspended in 0.5 ml of 50 mM sodium phosphate buffer (pH 8.0) with 2 M $(NH_4)_2SO_4$.

2.4. Hydrophobic interaction chromatography

Chromatography experiments were performed using 50 mM sodium phosphate buffer (pH 8.0) with 2 M $(NH_4)_2SO_4$ as buffer A and 50 mM sodium phosphate buffer (pH 8.0) as buffer B at room temperature and a constant flow rate of 1 ml/min. 1 ml of the precipitation product was loaded onto a HiTrap™ Phenyl HP 1 ml column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Before that, the column was equilibrated with 70 % buffer A and 30 % buffer B. After sample application, the column was washed with 8 CV of 30 % buffer B. The elution was

done with 100 % buffer B and fractions showing peroxidase activity were pooled. The peroxidase fractions were stored at −80 °C.

2.5. Desalting and concentration

After hydrophobic interaction chromatography, peroxidase fractions were desalted and concentrated with Amicon® Ultra-4 Ultracel® – 10 K (10 kDa MWCO) Centrifugal Filters (Merck Millipore Ltd., Ireland). The samples were concentrated 8-fold at 3000 ×g and 4 °C (4 ml of sample was concentrated to 0.5 ml) and then diluted 8-fold with 50 mM sodium phosphate buffer (pH 8.0). This was repeated three times to separate most of the salt. Finally, the sample was concentrated 20-fold from 4 ml to 0.2 ml. Desalted and concentrated peroxidase fractions were stored at −80 °C.

2.6. Enzyme activity assay

Peroxidase activity was measured via the oxidation of guaiacol to tetraguaiacol in the presence of H_2O_2. For this purpose, 20 mM guaiacol, 5 mM H_2O_2, and 5 µl enzyme sample were added to 50 mM potassium phosphate buffer (pH 7.0) to a final volume of 700 µl. The temperature was kept constant at 25 °C with a thermostat (Julabo F31-c, JULABO GmbH, Seelbach, Germany). The initial rate of tetraguaiacol production was recorded at 470 nm (Evolution 201 UV–Visible Spectrophotometer, Thermo Scientific, US-Waltham, Massachusetts, USA) as a triplicate. The concentration of tetraguaiacol was determined by the extinction coefficient of 26.6 mM^{-1} cm^{-1} at 470 nm (Maehly and Chance, 1954; Yadav et al., 2017). Enzyme activity is expressed as units, where 1 unit is defined as the formation of 1 µmol of tetraguaiacol per minute.

2.7. Determination of protein concentration

Protein concentration was determined via Bradford assay. A Bradford reagent was prepared from 109 ml 85 % phosphoric acid and 100 mg Coomassie® Brilliant Blue G-250 in 690 ml ddH_2O and stored under light exclusion at 4 °C. For the Bradford assay, 300 µl of Bradford reagent was added to 5 µl of the sample or standard solution. The mixture was incubated for 10 min at room temperature under light exclusion and measured at 595 nm in a plate reader (Infinite® M200 Pro NanoQuant, Tecan Group AG, Männerdorf, Switzerland). A standard curve with bovine serum albumin was used to calculate protein concentration from the measured absorption. All measurements for the determination of the protein concentration were performed in triplicates.

2.8. SDS-PAGE of the purified peroxidases

SDS-PAGE according to Laemmli was performed with the purified peroxidase fractions of both grass species to compare and evaluate the purification. For this, 5 µl of the peroxidase fractions (10 µl reduced sample) were applied to a 4–15 % Mini-PROTEAN TGX Gel (Bio-Rad Laboratories, USA). As molecular weight marker, the Precision Plus Protein™ All Blue Standards (Bio-Rad Laboratories, USA) was used. Protein bands were stained with Coomassie brilliant blue.

2.9. Characterization of the purified peroxidases

The temperature and pH optimum were determined for the peroxidase fractions of both types of grass. The determination was performed analogously to the enzyme activity assay. For the determination of optimal temperature, 20 mM guaiacol, 5 mM H_2O_2, and 5 µl enzyme sample were added to 50 mM potassium phosphate buffer (pH 7.0) to a final volume of 700 µl. The temperature was varied from 15 to 60 °C in increments of 5 °C. For the determination of optimal pH value, 20 mM guaiacol, 5 mM H_2O_2, and 5 µl enzyme sample were added to 50 mM sodium citrate buffer for the pH range from 3.0 to 5.5, 50 mM potassium phosphate buffer for the pH range from 6.0 to 7.5, or 50 mM TRIS buffer for the pH range from 8.0 to 9.0 to a final volume of 700 µl. The pH was varied in increments of 0.5 while keeping the temperature constant at 25 °C. The determination of the kinetics parameters was performed at the previously determined optimal temperatures and pH values. The initial reaction rate of the peroxidases was measured at different guaiacol concentrations. All other parameters were kept analogous to the enzyme activity assay. All measurements for the determination of the optimal reaction conditions and the determination of the kinetics parameters were performed in triplicates.

2.10. Conversion of phenolic compounds

To investigate the suitability of the grass peroxidases for wastewater treatment, the purified peroxidase fractions were used for the conversion of the phenolic compounds phenol, *m*-cresol, and 2,4-dichlorophenol. 20 mM stock solutions were prepared for the phenolic compounds in 50 mM potassium phosphate buffer (pH 6.0). 0.5 mM phenolic substrate, 2.5 mM H_2O_2, and 50 µl of the enzyme sample were added to 50 mM potassium phosphate buffer (pH 6.0) to a final volume of 1400 µl. The mixture was incubated on a shaker at 800 rpm and 30 °C and samples of 50 µl were taken every 5 min for 1 h. To stop the reaction, 10 µl of a 2860 U/ml catalase solution was added to the sample. The conversion of phenolic compounds was performed in triplicates in each case.

To optimize the conversion of the phenolic compounds, polyethylene glycol (PEG) was added to the reaction batch in some experiments. For this purpose, a stock solution of 1 g/l PEG with an average molecular weight of 20,000 g/mol was prepared in the reaction buffer. 100 mg/l PEG was added to the reaction batch.

The concentration of phenolic compounds was determined photometrically (Wu et al., 1997). For this purpose, two reagents, 83.4 mM $K_3Fe(CN)_6$ in 0.25 M $NaHCO_3$ (pH 10.0) and 20.8 mM 4-aminoantipyrine (AAP) in 0.25 M $NaHCO_3$ (pH 10.0), were prepared. The sample/catalase mixture (60 µl) was mixed with 100 µl of each of the two reagents and 740 µl of ddH_2O. The absorbance was measured at 510 nm against the reagent blank (without sample).

In addition to the purified peroxidase fractions, crude extracts of the grasses were prepared for phenol conversion. As described above in the chapter “2.2 Homogenization”, crude extracts of *L. perenne* and *F. arundinacea* were prepared by homogenization, filtration, and centrifugation. In addition, a crude extract of grass from the “real” lawn grass was prepared in the same way. The crude extracts were stored at −80 °C until use.

3. Results and discussion

3.1. Purification of peroxidases from Lolium perenne and Festuca arundinacea

A purification scheme was developed for the peroxidases from the two grass species *Lolium perenne* and *Festuca arundinacea*, which could be applied equally to both types of grass. The use of individual grass species as a model substrate is especially important to obtain reproducible, season-independent results on the otherwise highly heterogeneous material. The two types of grass were incubated in a growth chamber in the laboratory and harvested regularly (see Fig. S1). After the homogenization of the grass samples and separation of the solids, the peroxidases were fractionated with ammonium sulfate in two steps. Subsequently, the peroxidases were further purified by hydrophobic interaction chromatography (HIC) and finally simultaneously desalted and concentrated. In addition to the final concentration step, the peroxidases were already concentrated by the second precipitation step, as the precipitate was dissolved in a smaller volume than before. In contrast, the samples were diluted by the HIC. A compromise of optimal conditions for both grass species was selected for the purification. Fortunately, the peroxidase fractions of the two model types of grass showed similar properties, enabling a common purification process. By optimizing the

process steps to the individual grasses, it is most likely that higher purification can be achieved. However, the purification process should be universally applicable to real grass clippings. To evaluate the quality of the purification, the specific activity was determined from the protein concentration and the enzyme activity. Table 1 shows the resulting total protein concentration, total activity, and specific activity after each purification step. The crude extract of *F. arundinacea* showed a three times higher specific activity compared to L. *perenne*. A similar ratio was also observed after the entire purification process. Based on the increase in specific activity, 3.8-fold purification was obtained for peroxidases from L. *perenne*. In comparison, a 2.8-fold purification could be achieved for *F. arundinacea*. An SDS gel showed three major bands at approximately 27, 37, and 55 kD in the peroxidase fractions of both species in addition to minor impurities (see Fig. S2). According to UniProt, *L. perenne* has a peroxidase with a mass of 36.5 kD (entry A2BCZ0). Thus, it can be assumed that the band around 37 kD is a peroxidase. For *F. arundinacea* no information exists in this regard. The lower protein concentration of the sample from *F. arundinacea* resulted in weaker bands when the same sample volume was applied. The similar band pattern further demonstrates the similarity of the enzyme fractions of both types of grass. A comparable crude extract from horseradish showed a specific activity of 2.65 U/mg (Lavery et al., 2010). With values of 3.7 and 12.3 U/mg the specific activity of the grasses after homogenization is in a comparable range. However, after extensive purification, a much higher specific activity of 772 U/mg was obtained for the highly purified HRP (Lavery et al., 2010).

3.2. Characterization of the purified peroxidases

The purified peroxidase fractions were compared with respect to their optimal reaction conditions, mainly temperature and pH. Fig. 1 shows the relative specific activity of the peroxidase fractions of *F. arundinacea* and L. *perenne* as a function of temperature and pH. The peroxidases from both types of grass exhibited an apparent temperature optimum around 30 °C with maximum specific activities of 17.7 U/mg for L. *perenne* and 36.0 U/mg for *F. arundinacea*. Regarding pH, the peroxidases from L. *perenne* displayed the maximal specific activity of approximately 17.0 U/mg in the range from pH 5.0 to pH 6.0, while the peroxidases from *F. arundinacea* showed a clear maximum of 40.1 U/mg at pH 6.0. Both grass species peroxidases share a pH optimum at pH 6.0. Pandey et al. summarized the properties of a variety of plant-derived peroxidases (Pandey et al., 2017). In most cases, the pH optimum is in the slightly acidic range around pH 5 to 6. Thus, the optima of grass peroxidases fit well within this range. The optimal temperature of most peroxidases, on the other hand, is somewhat higher than the grass peroxidases with temperatures around 40 °C. However, this is more of an advantage for the grass peroxidases, as they require a lower temperature and thereby lower energy input with an optimum of around 30 °C.

With the optimal reaction parameters of 30 °C and a pH of 6.0, experiments were conducted with different guaiacol concentrations to determine kinetics parameters according to Michaelis and Menten (see Fig. S3). For the peroxidase fraction of *F. arundinacea*, this results in a maximum reaction rate V_{max} of 41.4 µmol min^{-1} mg^{-1} and a Km value of 5.3 mM. The peroxidase fraction of L. *perenne* shows a lower maximum reaction rate V_{max} of 25.8 µmol min^{-1} mg^{-1} and a lower substrate affinity with a Km value of 7.5 mM. Most Km values of plant peroxidases for guaiacol range from about 0.3 mM to 9.5 mM (Pandey et al., 2017).

3.3. Application of grass peroxidases for the conversion of phenolic compounds

3.3.1. Conversion of phenolic compounds by purified peroxidase fractions

In the first step, the purified peroxidases from the two types of grass were investigated for the conversion of the common phenolic pollutants phenol, *m*-cresol, and 2,4-dichlorophenol. All reactions were carried out at the previously determined reaction conditions of 30 °C and pH of 6.0. In addition, it was investigated whether the conversion of the substrates could be improved by the addition of 100 mg/l PEG. It has been previously shown that 100 mg/l PEG is a sufficient dose to enhance the conversion of different phenolic compounds by protecting the peroxidases from inactivation (Quintanilla-Guerrero et al., 2008; Wu et al., 1997). In general, PEG with a higher molecular weight causes a stronger enhancement of phenol conversion (Kinsley and Nicell, 2000; Nakamoto and Machida, 1992), which is why a molecular weight of 20,000 was chosen for the PEG in our experiments.

Fig. 2 shows the removal of phenolic compounds by the peroxidase fractions of the two types of grass with and without the addition of 100 mg/l PEG. After one hour, over 70 % of phenol, over 80 % of *m*-cresol, and over 95 % of 2,4-dichlorophenol were converted by the purified peroxidases of *Lolium perenne*. After the same time, almost 70 % of phenol, almost 85 % of *m*-cresol, and over 95 % of 2,4-dichlorophenol were converted by the crude extract of *Festuca arundinacea*. Thus, the degree of conversion after one hour was similar with both extracts, although the fractions show clear differences in specific activity towards guaiacol. Accordingly, the enzyme activity applied in the experiment was 1.04 U/ml for L. *perenne* and 1.55 U/ml for *F. arundinacea*. However, this higher activity becomes apparent after the first 5 min of the reaction. The peroxidases from L. *perenne* converted about 35 % of the phenol and about 45 % of the *m*-cresol after 5 min, while the peroxidases from *F. arundinacea* had already converted more than 45 % of the phenol and about 55 % of the *m*-cresol during the period. In the case of 2,4-dichlorophenol, a conversion of over 95 % was already achieved after 5 min in both cases. The addition of 100 mg/l PEG increased both, the initial reaction rate and conversion of phenol and *m*-cresol after 1 h. Due to the already rapid conversion of 2,4-dichlorophenol, no improvement could be observed by the addition of PEG for the substrate. In the case of phenol and *m*-cresol, about 10 to 15 % were additionally converted after one hour by both grass extracts in the presence of PEG. In the first 5 min, the conversion could also be increased by approximately another 5 to 20 % depending on substrate and grass species. For a more accurate comparison of the conversion, a quotient was formed from converted phenolic compounds after 1 h and enzyme activity used. A higher value indicates a more efficient conversion of the phenolic compounds. For the substrates phenol, *m*-cresol, and 2,4-dichlorophenol, values of 0.24,

Table 1
Degree of peroxidase purification from *Lolium perenne* and *Festuca arundinacea*.

Purification step	Total volume [ml]		Protein concentration [mg ml^{-1}]		Total activity [U]		Specific activity [U $mg^{-1}_{protein}$]	
	Lolium perenne	*Festuca arundinacea*	*Lolium perenne*	*Festuca arundinacea*	*Lolium perenne*	*Festuca arundinacea*	*Lolium perenne*	*Festuca arundinacea*
Homogenization	310.0	298.0	0.400	0.187	466.1	685.3	3.756	12.312
Precipitation (40 %)	339.0	328.0	0.172	0.132	351.3	614.8	6.025	14.249
Precipitation (80 %)	8.5	8.2	3.922	2.329	284.9	424.5	8.570	22.230
HIC	118.7	114.8	0.174	0.119	207.6	295.6	10.050	21.668
Desalting and concentration	5.9	5.7	2.033	1.265	172.6	248.4	14.313	34.205

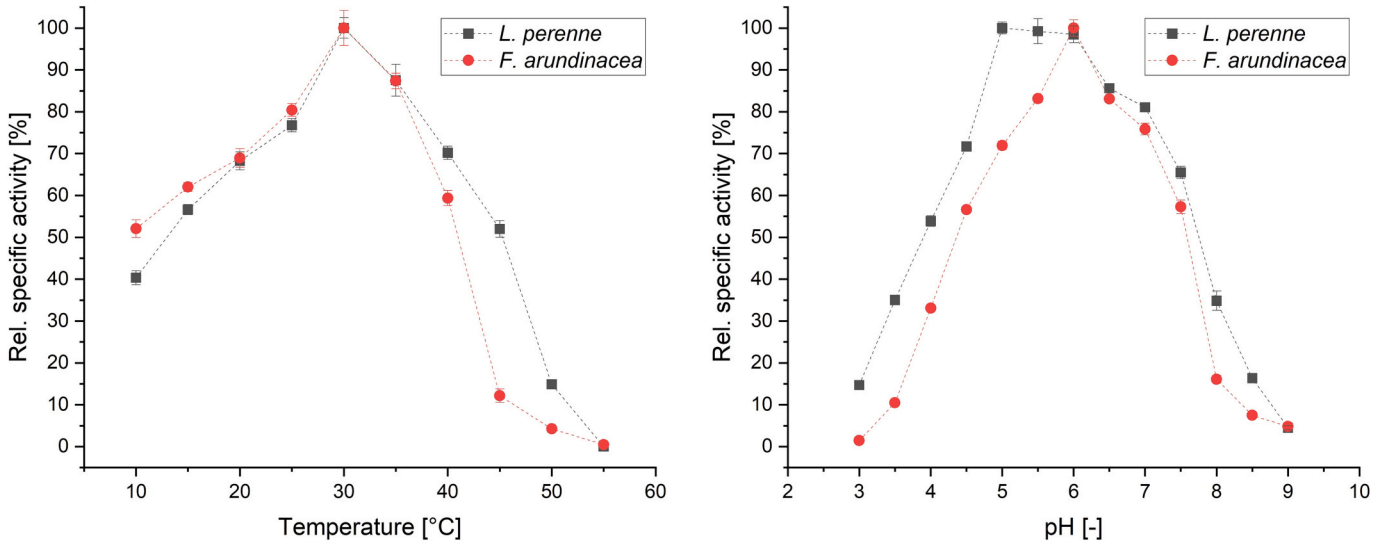

Fig. 1. Relative specific activity of the purified peroxidase fractions from *Lolium perenne* and *Festuca arundinacea* as a function of temperature (left) and pH (right). Maximum specific activity at optimal temperature is 17.7 U/mg for L. *perenne* and 36.0 U/mg for *F. arundinacea*, while at optimal pH it is 17.0 U/mg for *L. perenne* and 40.1 U/mg for *F. arundinacea*. All reactions were carried out with 20 mM guaiacol and 5 mM H_2O_2. For the determination of the optimum temperature, the pH was kept constant at 7.0, while the temperature was kept constant at 25 °C for the determination of the optimum pH ($n = 3$).

0.28, and 0.33 mM/U were obtained for L. *perenne* and 0.16, 0.19, and 0.22 mM/U for *F. arundinacea*, respectively. Thus, for the enzyme activity used, *L. perenne* was more effective. A possible explanation could be that the preparations contain different amounts of contaminants, which exhibit radical scavenging activity. By adding PEG, these values can be slightly increased to 0.28, 0.31, and 0.34 mM/U for *L. perenne* and 0.20, 0.22, and 0.23 mM/U for *F. arundinacea*, respectively.

Since the composition of the reaction mixture, especially enzyme and substrate concentration, as well as reaction conditions are different in each publication, an accurate comparison is difficult. For example, it was shown that 9.5 U/ml HRP can convert 5.2 mM 2,4-dichlorophenol in wastewater within 5 to 10 min under optimal conditions (Dec and Bollag, 1994). Wagner and Nicell demonstrated the removal of at least 95 % of 1 mM phenol and more than 96 % of 0.6 mM 2,4-dichlorophenol by 4.5 U/ml HRP after 3 h (Wagner and Nicell, 2002). The different efficiencies of removal of each substrate in our experiments may also be due to varying reaction optima. Caza et al. have shown that optimal pH values vary depending on the phenolic substrate (Caza et al., 1999). In the case of soybean peroxidase, the optimum pH for phenol is 6.0, while for both *m*-cresol and 2,4-dichlorophenol, it is 7.0. Interestingly, soybean peroxidase shows a similar affinity for the three substrates studied as in our case. Phenol requires the highest enzyme dose at 0.90 U/ml, followed by *m*-cresol at 0.75 U/ml and by a considerable margin 2,4-dichlorophenol at 0.08 U/ml the least (1 mM of each substrate) (Caza et al., 1999). Akhtar and Husain isolated peroxidase from bitter gourd (*Momordica charantia*) and applied the soluble and immobilized enzyme in the treatment of single phenolic compounds and model wastewaters (Akhtar and Husain, 2006). Contrary to the results with the grass peroxidases, the soluble and immobilized bitter gourd peroxidase showed the highest conversion towards phenol, followed by 2,4-dichlorophenol and *m*-cresol. However, a pH of 6 has also proven to be optimal with respect to all substrates, whereas the optimal temperature was again 40 °C as described previously.

3.3.2. *Conversion of phenolic compounds by crude grass extracts*

For the detailed characterization of the peroxidases, the enzymes had to be purified. However, this is probably too expensive for a real application. Dec and Bollag were the first to show that raw plant material can also be used for the conversion of phenols (Dec and Bollag, 1994). We wanted to test the conversion of phenolic compounds with a crude grass homogenate. The crude extracts of L. *perenne* and *F. arundinacea* exhibited a volumetric activity for guaiacol of 1315 U/l and 1375 U/l, respectively. This results in an activity of 9.9 U per gram of grass for L. *perenne* and 10.3 U per gram of grass for *F. arundinacea*. The conversion of the phenolic compounds with the crude extracts was carried out in the same way as in the experiments before. Since the positive effect of adding PEG was shown in the experiments with the purified peroxidases, PEG was also added in the experiments with the crude extracts. The reduction in reaction time as well as the increase in residual enzyme activity by the addition of PEG has already been confirmed for wastewater treatment with crude extracts from plant sources (Diao et al., 2011; Duarte-Vázquez et al., 2003). Fig. 3 shows the removal of phenolic compounds by the crude extracts of the two types of grass in the presence of 100 mg/l PEG. After one hour, approx. 10 % of phenol, 20 % of *m*-cresol, and almost 80 % of 2,4-dichlorophenol were converted by the crude extract of L. *perenne*. After the same time, approx. 10 % of phenol, over 15 % of *m*-cresol, and almost 75 % of 2,4-dichlorophenol were converted by the crude extract of *F. arundinacea*. Thus, in contrast to the purified peroxidases, the conversion of all substrates with both types of grass was poorer probably due to the lower volumetric activity of the crude extracts. Overall, the conversion by both crude extracts was very similar, as confirmed by the similar specific activity towards guaiacol. The results show that the peroxidases of the two types of grass do not differ significantly in their affinity towards the substrates. Instead, the rate and extent of conversion depended on the enzyme concentration indicated by the specific activity.

Again, a quotient of the converted phenolic compound and enzyme used was formed. The applied enzyme activities of 0.047 U/ml and 0.049 U/ml for L. *perenne* and *F. arundinacea*, respectively, were about 20- to 30-fold lower than that of the purified peroxidases. For the substrates phenol, *m*-cresol, and 2,4-dichlorophenol, values of 0.83, 1.71, and 6.01 mM/U were obtained for L. *perenne* and 0.61, 1.15, and 5.42 mM/U for *F. arundinacea*, respectively. As before with the purified peroxidases, the quotients of both grass species are similar due to similar enzyme activity used. The quotient should not be used as the only evaluation criterion, since higher quotients are generated here despite more incomplete conversion compared to the purified peroxidases.

Finally, we also wanted to test the conversion of phenolic compounds with "real" grass from a lawn in the next step. We prepared a crude extract exactly as described above. The volume activity of the homogenate from the lawn grass was 4080 U/l. Furthermore, the crude extract of the lawn grass showed an activity of 30.6 U per gram of grass. Again,

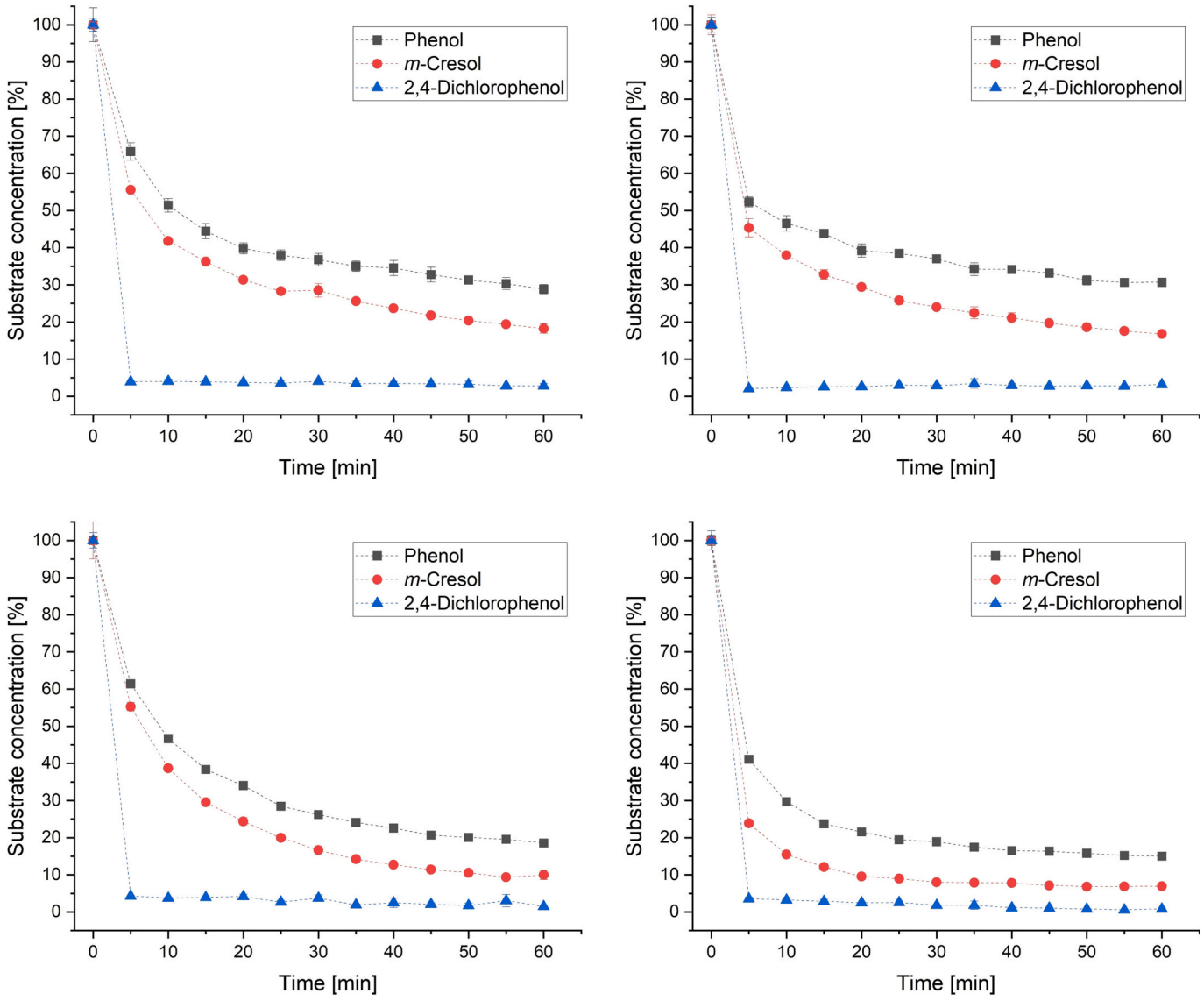

Fig. 2. Removal of the phenolic compounds phenol, *m*-cresol, and 2,4-dichlorophenol (each 0.5 mM) by purified peroxidase fractions of the cultivated grasses *Lolium perenne* (left) and *Festuca arundinacea* (right) without (top) and with the addition of 100 mg/l PEG (bottom) resulting in an applied enzyme activity of 1.039 U/ml for *Lolium perenne* and 1.546 U/ml for *Festuca arundinacea*. All reactions were carried out with 5 mM H_2O_2 at 30 °C and 800 rpm in a 50 mM potassium phosphate buffer with a pH of 6.0 (n = 3).

the conversion of the phenolic compounds with the crude extract was carried out in the same way as before. With 0.146 U/ml, the enzyme activity used was approx. three times higher in comparison to the laboratory grown grasses. Fig. 4 shows the removal of phenolic compounds by the crude extract of lawn grass with the addition of 100 mg/l PEG. After one hour, approx. 20 % of phenol, 40 % of *m*-cresol, and more than 95 % of 2,4-dichlorophenol were converted by the crude extract of lawn grass. Here, all three substrates were converted faster than with the crude extracts of the two types of grass grown in the laboratory. This may be attributed to the approx. three times higher volume activity of the lawn grass extract in comparison to the crude extracts of L. *perenne* and *F. arundinacea*. Again, *m*-cresol was converted somewhat faster than phenol, and 2,4-dichlorophenol was again clearly converted fastest. The quotients of phenol removal and enzyme activity used were 0.52, 1.03, and 2.38 mM/U for the substrates phenol, *m*-cresol, and 2,4-dichlorophenol, respectively. Similar values have been obtained for the other crude extracts. These results confirm that the conversion of phenolic compounds with peroxidases from grasses also works with real raw materials.

It was shown before that crude plant material shows similar results to isolated peroxidases in terms of phenol conversion (Dec and Bollag, 1994). Logically, high costs can be saved due to the low purification. Cooper and Nicell compared the removal of phenols from foundry wastewater using purified HRP and a crude HRP extract (Cooper and Nicell, 1996). In both cases, with the purified enzyme and with the crude extract, more than 95 % of the 3.5 mM phenol mixture was removed from wastewater. Duarte-Vázquez et al. produced a crude extract from turnip for the removal of phenolic compounds (Duarte-Vázquez et al., 2003). They were able to remove over 95 % of phenol, *m*-cresol, and 2,4-dichlorophenol with the addition of PEG within 10 min. Interestingly, they found decreased removal efficiency with increasing concentration of phenolic compounds for all compounds studied except 2,4-dichlorophenol. A similar effect can be seen here, in which the conversion of 2,4-dichlorophenol was hardly affected by the changed enzyme concentration in comparison to phenol and *m*-cresol. Yadav et al. demonstrated the use of gourd (*Luffa aegyptiaca*) fruit juice as a low-purity peroxidase source for the removal of guaiacol, phenol, and *m*-cresol amongst other substrates (Yadav et al., 2017). According to the Km

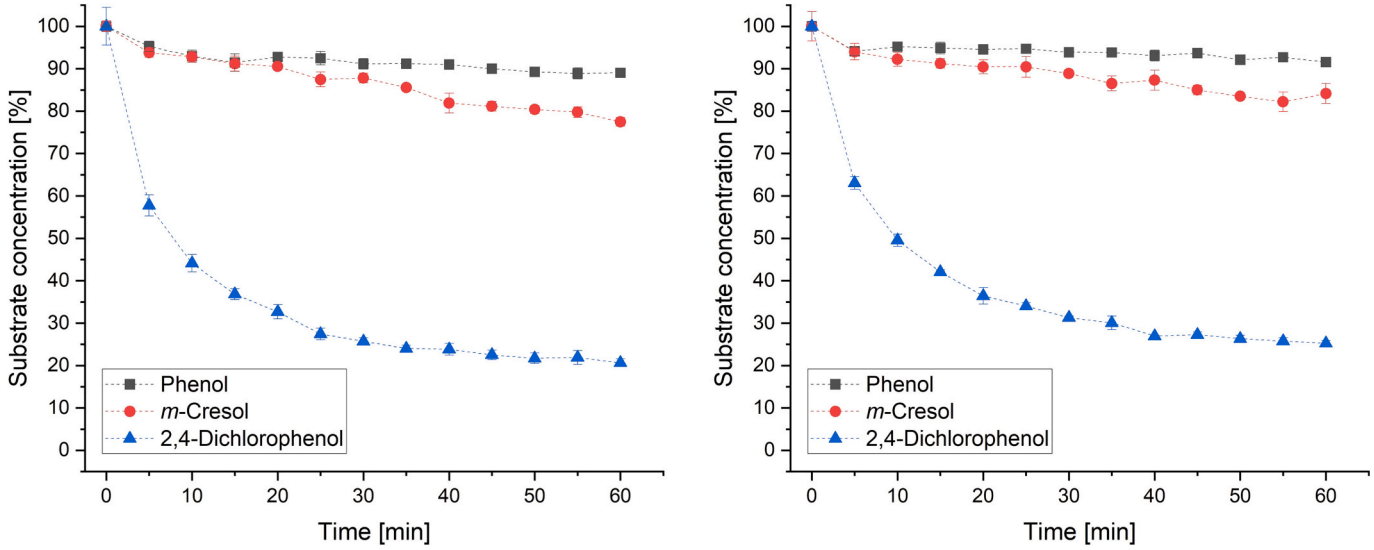

Fig. 3. Removal of the phenolic compounds phenol, *m*-cresol, and 2,4-dichlorophenol (each 0.5 mM) by crude extract of the cultivated grasses *Lolium perenne* (left) and *Festuca arundinacea* (right) with the addition of 100 mg/l PEG resulting in an applied enzyme activity of 0.047 U/ml and 0.049 U/ml, respectively. All reactions were carried out with 5 mM H_2O_2 at 30 °C and 800 rpm in a 50 mM potassium phosphate buffer with a pH of 6.0 (n = 3).

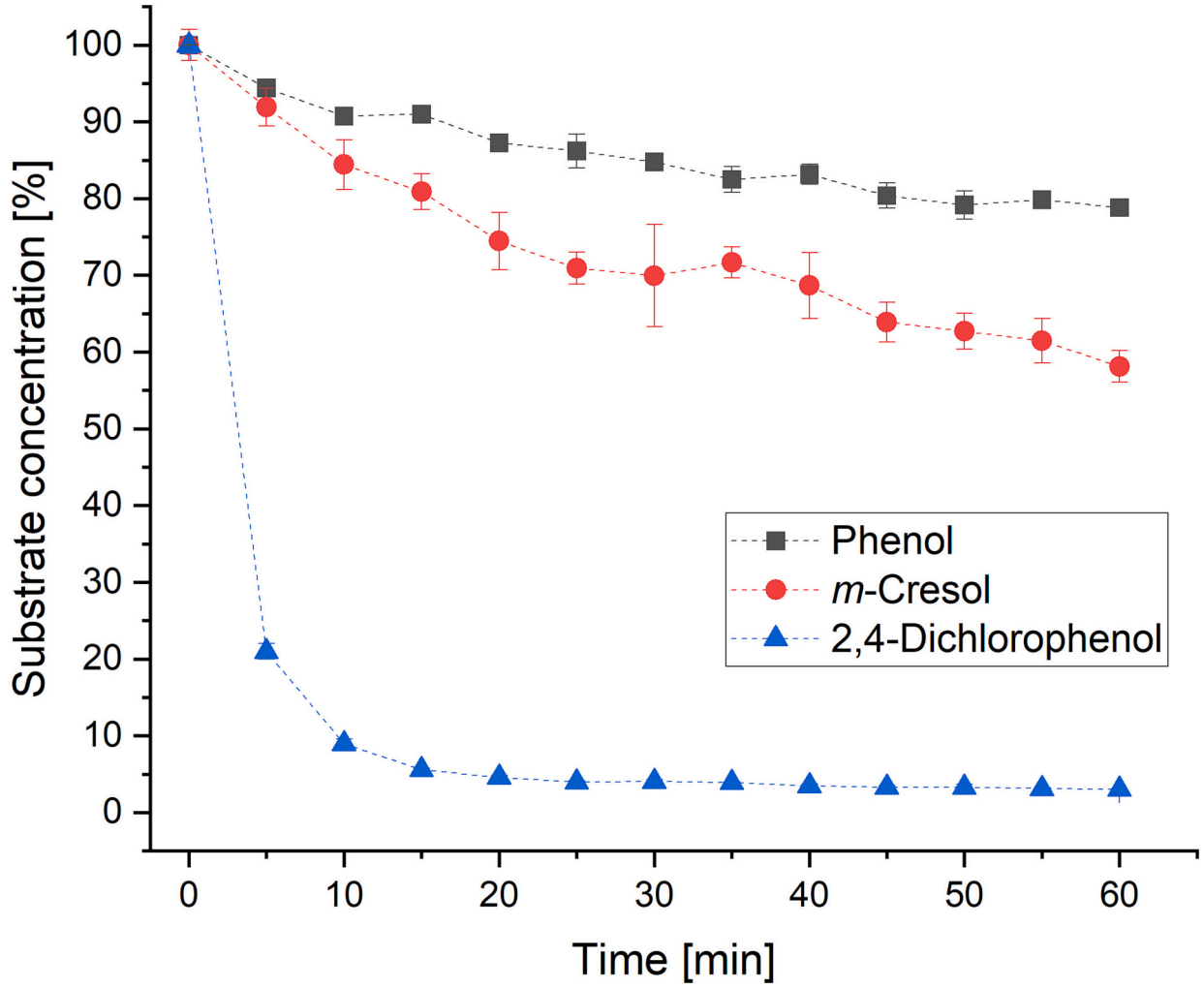

Fig. 4. Removal of the phenolic compounds phenol, *m*-cresol, and 2,4-dichlorophenol (each 0.5 mM) by crude extract of lawn grass with the addition of 100 mg/l PEG resulting in an applied enzyme activity of 0.146 U/ml. All reactions were carried out with 5 mM H_2O_2 at 30 °C and 800 rpm in a 50 mM potassium phosphate buffer with a pH of 6.0 (n = 3).

values, the fruit juice peroxidases have a higher affinity for phenol, rather than *m*-cresol in contrast to the grass peroxidases. Again, purity did not affect the effectiveness of enzymatic conversion. Kurnik et al. demonstrated the phenol removal with potato pulp peroxidases (Kurnik et al., 2015). They were able to achieve a phenol removal efficiency of over 95 % for up to 2 mM phenol after 2 h from synthetic wastewater with the potato pulp. With industrial effluent with much lower phenol concentrations of 0.02 to 0.1 mM, over 80 % of the phenol was removed by the addition of the potato pulp even without the addition of H_2O_2. This was explained by the adsorption of phenol to the pulp solids, as was also previously described for other crude plant materials (Duarte-Vázquez et al., 2003; Dec and Bollag, 1994). In the future, the efficiency of phenol removal with grass peroxidases should be compared to conventional peroxidases like HRP to help assess the performance of the crude extract from grass clippings.

In general, the use of a crude extract is the more economical option for the conversion of phenolic compounds. However, the removal of phenolic compounds can be further optimized by selecting the optimal parameters for the conversion of the respective substrates. Optimization of these parameters such as enzyme concentration, pH, H_2O_2 to substrate ratio, and PEG concentration was demonstrated by Caza et al. using soybean peroxidase (Caza et al., 1999). During our experiments, we observed that the removal of phenolic compounds is progressively worse at higher initial substrate concentrations, while the conversion was increased when a larger volume of enzyme extract was added to the reaction batch. Caza et al. showed that the degree of phenol conversion is strongly dependent on the enzyme concentration used (Caza et al., 1999). Quintanilla-Guerrero et al. have also confirmed that the removal efficiency decreases with increasing concentration of phenolic compounds (Quintanilla-Guerrero et al., 2008). Duarte-Vázquez et al. concentrated the crude extract from turnip roots 10-fold before use (Duarte-Vázquez et al., 2003). In the future, a concentration step should follow in the preparation of the crude extract to achieve higher enzyme concentrations. Additionally, the amount of enzyme required can be reduced by the addition of PEG (Caza et al., 1999; Cooper and Nicell, 1996; Wagner and Nicell, 2001). Furthermore, the optimal pH values and temperatures must be determined for the enzyme specific to the phenolic substrates. For example, the optimal conditions of the gourd fruit juice for the conversion of phenol were 28 °C and pH 7.0, while for the conversion of *m*-cresol, the optimum conditions were 35 °C and pH 6.0 (Yadav et al., 2017). It is promising that the two grass species show an equal pH and temperature optimum as far as the industrial use of the enzyme extract from grass mixtures is concerned. The substrate/H_2O_2 ratio can also be improved since it can have a great influence on the conversion as it has already been shown in numerous publications (Akhtar and Husain, 2006; Caza et al., 1999; Dahili et al., 2015; Wang et al., 2015; Wu et al., 1997). Wagner and Nicell were able to save 20 % of the enzyme by optimizing the H_2O_2 concentration (Wagner and Nicell, 2001). Hydrogen peroxide-mediated inactivation of peroxidases can be minimized by enzyme immobilization or *in situ* generation of H_2O_2 (Burek et al., 2019). Immobilization of enzymes is commonly used to improve their performance or properties (Quintanilla-Guerrero et al., 2008). Dahili et al. demonstrated the immobilization of peroxidase from a crude extract of horseradish (Dahili et al., 2015). However, enzymes in crude plant extracts may have a higher stability due to the fact that they are already immobilized in the plant material (Dec and Bollag, 1994).

Furthermore, there may be other phenolic substrates such as 2,4-dichlorophenol that are effectively converted by the peroxidases from grass clippings. It has been demonstrated that HRP can also be used for the removal of azo dyes (Mohan et al., 2005) or endocrine-disrupting compounds (Auriol et al., 2007). Using peroxidases from the perennial grass *Miscanthus x giganteus*, Dragana et al. have already demonstrated the degradation of dyes with a type of *Poaceae* (Dragana et al., 2017). In addition, the application with a model or real industrial wastewater has to be tested further on. Garg et al. summarized and compared different examples of phenol removal from industrial wastewater using various crude and purified plant peroxidases (Garg et al., 2020).

Apart from considering the enzymatic process, the acquisition and condition of the raw material should not be neglected. Several challenges still exist that make it difficult to effectively process green waste. When collecting green waste from public areas, contamination by waste is unavoidable and must be separated beforehand. Another major problem is the strong heterogeneity of green waste. The composition of green waste varies greatly depending on location and season. Even when harvesting the grasses planted in the laboratory, which consist of only one plant species, differences in peroxidase activity and protein concentration could be detected depending on when the grass samples were cut. This demonstrates the challenges of using heterogeneous plant feedstocks. For these reasons, the robustness of the process should also be studied depending on the characteristics of the raw material.

4. Conclusions

Peroxidases from grass clippings show potential for application in wastewater treatment. In our opinion, the enzymes need to be used as a crude extract because intensive purification would again entail high costs. These crude extracts can be used as a "green" supplementary part of peroxidases for the conversion of phenolic compounds since the plant material probably cannot meet the demand for peroxidases alone. Finally, the extraction of industrially relevant enzymes can contribute to a higher added value of green waste. However, a holistic ecological and economic utilization to close the material cycle as far as possible can only be realized by a cascade-like green waste-biorefinery.

CRediT authorship contribution statement

Alexander Langsdorf: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Visualization. **Marianne Volkmar:** Conceptualization, Methodology, Writing – review & editing. **Roland Ulber:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Frank Hollmann:** Conceptualization, Methodology, Writing – review & editing. **Dirk Holtmann:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2023.101471.

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