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# Evaluation of the Potential of Fungal and Plant Laccases for Active-Packaging Applications

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**ABSTRACT:** Laccases from *Trametes versicolor* (TvL), *Myceliophthora thermophila* (MtL), and *Rhus vernicifera* (RvL) were investigated with regard to their potential utilization as oxygen scavengers in active packages containing food susceptible to oxidation reactions. The substrate selectivity of the laccases was investigated with a set of 17 reducing substrates, mainly phenolic compounds. The temperature dependence of reactions performed at low temperatures (4−31 °C) was studied. Furthermore, the laccases were subjected to immobilization in a latex/clay matrix and drying procedures performed at temperatures up to 105 °C. The results show that it is possible to immobilize the laccases with retained activity after dispersion coating, drying at 75−105 °C, and subsequent storage of the enzyme-containing films at 4 °C. TvL and, to some extent, MtL were promiscuous with regard to their reducing substrate, in the sense that the difference in activity with the 17 substrates tested was relatively small. RvL, on the other hand, showed high selectivity, primarily toward substrates resembling its natural substrate urushiol. When tested at 7 °C, all three laccases retained >20% of the activity they had at 25 °C, which suggests that it would be possible to utilize the laccases also in refrigerated food packages. Coating and drying resulted in a remaining enzymatic activity ranging from 18 to 53%, depending on the drying conditions used. The results indicate that laccases are useful for active-packaging applications and that the selectivity for reducing substrates is an important characteristic of laccases from different sources.

**KEYWORDS:** laccase, *Trametes versicolor*, *Myceliophthora thermophila*, *Rhus vernicifera*, immobilization, active packaging

## INTRODUCTION

Food spoilage affects the texture, nutrition, and flavor of food. It destroys products and in time makes them unacceptable for consumption, which ultimately leads to the accumulation of waste. Food spoilage results in economical losses and can even be hazardous to human health, which have attracted increased attention to the area. Most of the spoilage of food is caused by bacteria, yeasts, and molds, but can also be due to chemical spoilage.[1] One of the most common chemical causes is the oxidation of unsaturated fats, which is an autoxidation reaction dependent on the amount of double bonds present in the fats of the product in question.[1] Products containing a high content of unsaturated fats, for example, fish, are thus susceptible to this deterioration of quality, which leads to shortened storage and processing possibilities.[2]

To minimize food spoilage reactions caused by oxidation, it is essential to remove molecular oxygen trapped within the package or that permeates the package. The problem can be controlled by the addition of oxygen-scavenging substances, such as iron powder or ascorbic acid.[3] In this study we have investigated the potential in using an enzyme, laccase, as an oxygen scavenger in active packages. Enzymes have the potential to make oxygen scavenging more effective and extend the range of reductants that can be used for this application, because enzymes will catalyze reactions that would not otherwise occur at practically reasonable rates. Furthermore, provided that the enzymes are stable enough to survive coating and drying, they could be immobilized as a part of the packaging material together with the reductant that serves as a substrate for the enzymes, rather than being enclosed in the package in a pouch or something similar.

Laccases (EC 1.10.3.2) are copper-containing oxidoreductases that catalyze the oxidation of a variety of substrates, primarily phenolic compounds, while reducing molecular oxygen to water. They can therefore tentatively be used to reduce the concentration of oxygen inside food packages. Enzymes have previously been rather expensive to produce, but the rapid development of genetic engineering and bioprocess technology has contributed to decreasing the production costs for enzymes and made them feasible alternatives in industrial applications, such as food packaging.

Previous results suggest that the two-enzyme system glucose oxidase (GOx) and catalase can be useful for active packaging.[4] The overall reaction leads to a decreased concentration of oxygen through an intermediate, hydrogen peroxide, which is produced by GOx and utilized by catalase. Laccases naturally reduce molecular oxygen into water without having hydrogen peroxide as an intermediate. By using laccase instead of GOx, the risk associated with reactive hydrogen peroxide would thereby be eliminated as well as the need to use a two-enzyme system. The use of laccase also broadens the substrate options, because the enzyme accepts a variety of reducing substrates including phenolic compounds derived from renewable raw materials.

In this work, laccases from the basidiomycete *Trametes versicolor* (TvL), the ascomycete *Myceliophthora thermophila* (MtL), and the Japanese lacquer tree *Rhus vernicifera* (RvL)

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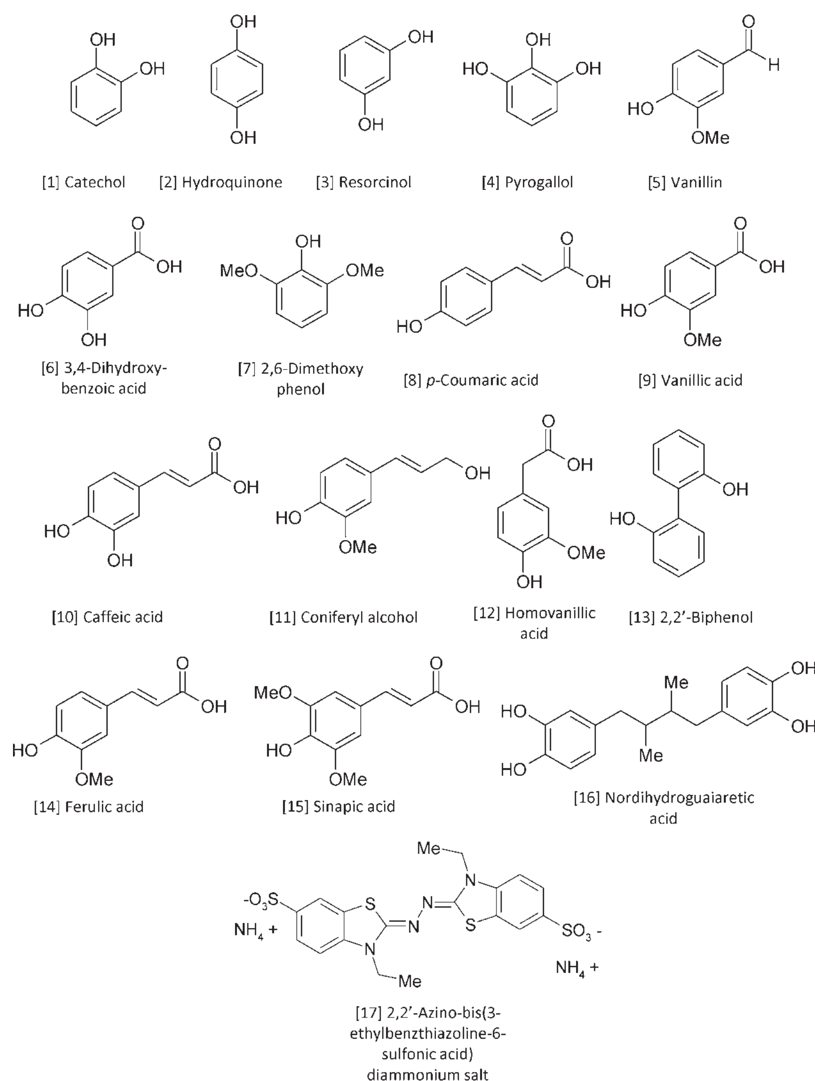


**Figure 1.** Compounds examined as substrates for laccases.

were investigated with regard to aptitude for inclusion in active packages. *Trametes* (syn. *Coriolus, Polyporus*) *versicolor* is a white-rot fungus that grows on decaying wood and produces laccase, lignin peroxidase, and other enzymes believed to be involved in the biodegradation of lignin.[5] *M. thermophila* is a thermophilic fungus, which produces a laccase that has been found to be relatively stable up to 60 °C.[6] Lacquer trees, such as *R. vernicifera*, produce a sap, which, among other components, contains urushiol and laccase, which are involved in the polymerization of a protective polymer that serves a function in wound healing.[7] Thus, laccases of widely different biological origin and derived from organisms inhabiting diverse environments were included in the investigation.

The selectivities of the three laccases with regard to their reducing substrate were investigated with a set of 17 aromatic, mainly phenolic, compounds (Figure 1). The activity at low temperatures (<10 °C) was studied because this is a crucial property if the enzyme should be used together with refrigerated foodstuff. The laccases were also assayed for their ability to be immobilized through entrapment[8] in a coating color, that is, a latex/clay matrix, and survive various drying conditions in terms of temperature and time.

## MATERIALS AND METHODS

**Enzymes.** *T. versicolor* laccase was obtained from Jülich Fine Chemicals GmbH (Jülich, Germany). The lyophilized enzyme was dissolved in deionized water (0.32 g of enzyme in 15 mL of water), aliquoted, and snap-frozen. *M. thermophila* laccase was kindly donated from Novozymes A/S (Bagsvaerd, Denmark). The enzyme comes as a liquid solution and was diluted with deionized water. *R. vernicifera* laccase was purified as described by Reinhammar.[9] All dilutions were made in deionized water. Enzyme dosages used in experiments are described below under Oxygen Electrode Activity Assay.

**Substrates.** The 17 substrates examined (Figure 1) were purchased from Sigma-Aldrich (Steinheim, Germany). Substrate solutions were freshly prepared by dissolving the substrate in deionized water to a stock concentration of 10 mM prior to the activity assay.

**Oxygen Electrode Activity Assay.** The activity assay was performed with an oxygen electrode (Hansatech Instruments Ltd., Norfolk, U.K.). The screening assay was conducted inside the tempered chamber (25 °C) controlled by a thermostatic bath, Multitemp III thermostatic circulator (GE Healthcare, Uppsala, Sweden). First, 850 $\mu$L of 50 mM MOPS buffer (pH 6.5) and 100 $\mu$L of substrate were added to the reaction chamber. The enzyme, 50 $\mu$L of the desired diluted enzyme solution (diluted to obtain the dosages indicated below), was added after

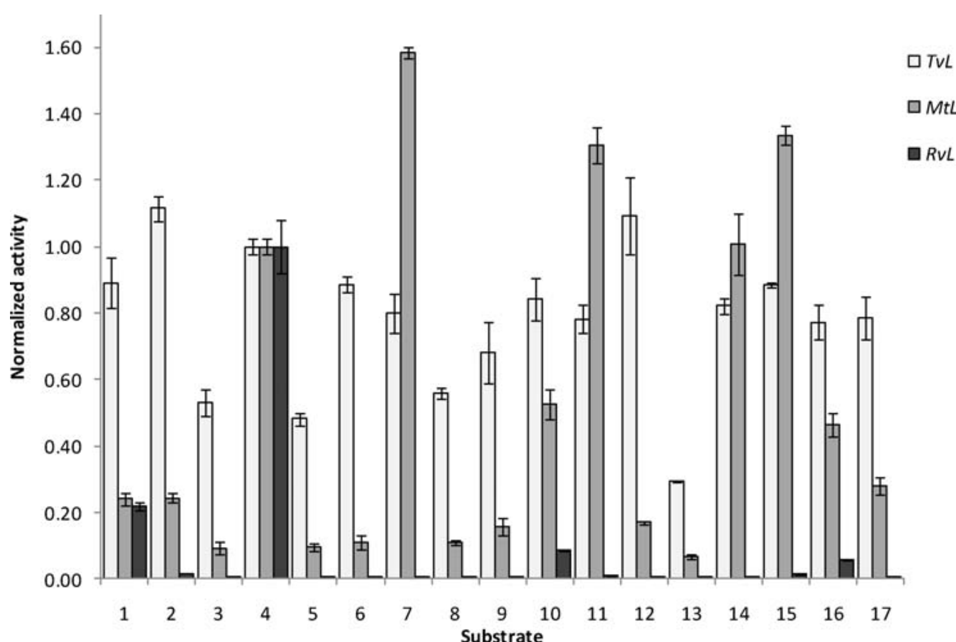


**Figure 2.** Mean activity and standard deviations for the 17 substrates in Figure 1 obtained with the laccases from *T. versicolor* (TvL), *M. thermophila* (MtL), and *R. vernicifera* (RvL). "Normalized activity" refers to the activity of an enzyme in relation to the activity obtained with the same enzyme and the reference substrate pyrogallol (pyrogallol, substrate 4, therefore per definition has a normalized activity of 1.00).

the signal from the electrode was stable. The enzyme dosages were different for the different laccases: TvL, 0.83 U/mL; MtL 0.95 U/mL; RvL, 27.7 U/mL (where 1 U equals the amount of enzyme needed to catalyze the reduction of 1 $\mu$mol of molecular oxygen per minute with pyrogallol as the substrate in a reaction performed at pH 6.5 and at a temperature of 25 °C). For each enzyme, the activity obtained with substrates other than pyrogallol was divided by the pyrogallol activity to obtain a normalized activity, where the activity with pyrogallol per definition is set to 1.00. The oxygen depletion, with each of the 17 substrates, was followed on the monitor, and the initial enzyme velocity was calculated from the graph and finally converted into the normalized activity value.

The temperature dependence in the range of 4−31 °C was investigated using pyrogallol as substrate. The chamber was left to stabilize at each temperature for at least 15 min, in accordance with instructions from the manufacturer of the oxygen electrode. The electrode was thereafter recalibrated before the activity was measured. The activity shown by the enzymes at 25 °C was set to 100%.

**Immobilization of the Enzymes.** *Preparation of Enzyme-Containing Films.* The enzymes were immobilized through entrapment in a coating formulation based on a latex polymer and a barrier kaolin clay as described by Johansson et al.[10] The styrene−butadiene latex (SB-latex) was kindly supplied by Styron Europe GmbH (Horgen, Switzerland). According to the manufacturer, the SB-latex has a glass transition temperature ($T_g$) of 6 °C. The dry solids content (SCAN-P 39:80) and pH were measured prior to use and found to be 50% and 5.5, respectively. The clay, Barrisurf LX from Imerys Minerals Ltd. (Cornwall, U.K.), has an aspect ratio (i.e., length over thickness) of 60 according to the manufacturer. It was dispersed in deionized water with a conductivity of 1 $\mu$S/cm to a final solids content of 63%. The coating color was prepared by mixing the latex with the clay in a pigment volume concentration of 16% at 4 °C, and the pH was adjusted to 6.5 (using a 4.5 M solution of NaOH). Laccase was added in a concentration of 0.7 U/g wet coating color, resulting in a theoretical maximum activity of the dry films of 1.4 U/g for TvL, 1.3 U/g for RvL, and 1.14 U/g for MtL. The variations in theoretical maximum activity are due to differences between the enzyme preparations, which affect the weight of the dry films. The mixtures were subsequently left to stir for 15 min prior to coating.

Enzyme-containing films were prepared by coating the backside of a silicon-treated paper mounted onto a smooth-surfaced cardboard with a bench coater, K202 Control Coater (RK Print-Coat Instruments Ltd., Royston, U.K.), using a wire-wound rod giving a nominal wet deposit of 60 $\mu$m. The tests included six different drying conditions based on three different temperatures, namely, (i) 75 °C for 30, 40, or 50 s; (ii) 90 °C for 30 or 40 s; and (iii) 105 °C for 30 s. The short drying steps performed at 75−105 °C were followed by an additional drying step performed at 30 °C and 50% relative humidity (RH) for 24 h to ensure complete film formation of the latex. The films were subsequently stored at 4 °C until the activity was measured.

*Enzyme Activity Measurements.* Prior to testing, the films had been stored for 1−6 days at 4 °C. The enzymatic activity of the films was measured by means of an oxygen electrode (Hansatech Instruments Ltd.). A small piece of film (approximately 0.005 g) was added to the reaction chamber containing 2 mL of pretempered (25 °C) substrate solution (10 mM pyrogallol and 50 mM MOPS, pH 6.5). The decrease in signal was monitored during 6 min. All tests were performed in triplicate. The enzyme activity was calculated as micromoles of oxygen per minute per gram of film. The remaining activity after immobilization was calculated in percentage according to eq 1, where the activity has the units of micromoles of oxygen per minute per gram of film.

$$\frac{\text{measured activity}}{\text{theoretical maximum activity}} \times 100 \quad (1)$$

**Statistical Analysis.** Student's $t$ test was applied to calculate 95% probability values ($p$ values) for the differences in remaining activity between different enzymes at the same drying conditions and between the same enzyme at different drying conditions. In this way, the calculated $p$ values provided an overview of the significance of the variation in the data, which was further used in the data interpretation.

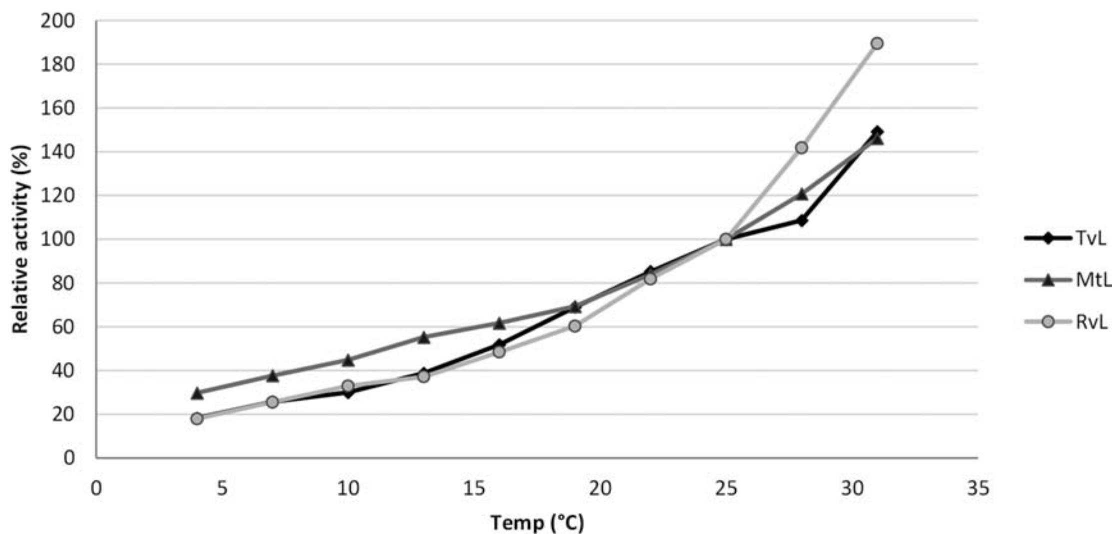


**Figure 3.** Activity of laccases from *T. versicolor* (TvL), *M. thermophila* (MtL), and *R. vernicifera* (RvL) in the temperature interval 4−31 °C. Pyrogallol was used as substrate, and the activity at 25 °C was set to 100%.

## RESULTS AND DISCUSSION

**Substrate Selectivity of Laccases.** A set of 17 aromatic compounds (Figure 1) revealed important differences between the substrate selectivities of three laccases and exposed their potential with regard to active-packaging applications. By including as many substrates as 17, it was possible to achieve a more comprehensive view of the selectivity of the laccases examined. Furthermore, for technical applications it would be important to have many substrates to choose between, because not only is reactivity important but also issues such as availability, effect on film formation, migration, and compatibility with food products. Whereas many laccases prefer acidic reaction conditions, a near neutral pH, 6.5, was chosen to represent the typical environment in cardboard-packaging materials. Most substrates investigated were phenolic compounds (Figure 1) and previous studies of TvL have indicated an optimal pH for phenolic substrates of 4.[11] Nonphenolic substrates, such as ABTS (structure 17 in Figure 1), typically have lower pH optima.[11]

TvL showed least selectivity for the different substrates (Figure 2). The poorest substrate for TvL was 2,2′-biphenol, which had 29% of the activity of the reference substrate pyrogallol. MtL was more selective than TvL and showed especially high activity for phenols in which methoxyl substituents surrounded the phenolic group, such as 2,6-dimethoxyphenol and sinapic acid (Figure 2). RvL was the most selective enzyme (Figure 2) and, apart from pyrogallol, only catechol, caffeic acid, and nordihydroguaiaretic acid gave any appreciable activity. These substrates all have phenolic hydroxyl groups in the *ortho*-position.

The substrate selectivity patterns (Figure 2) exhibited by the three laccases can be discussed in relation to their redox potential. TvL has a relatively high redox potential (785 mV),[12] which explains its low selectivity for the 17 different substrates studied. The higher selectivities of MtL and RvL can be explained by their relatively low redox potentials (between 450 and 480 mV[13] and 410[12] mV, respectively). The preference of RvL for substrates with *ortho*-positioned phenolic groups can be related to their resemblance to urushiols, which are the natural substrates of RvL.[7]

**Activity at Low Temperatures.** The temperature investigation shows that all of the enzymes were active at low temperatures (Figure 3). Even below 10 °C, all three laccases maintained >20% of the activity shown at 25 °C. As expected with regard to the Q10 value, the activity approximately redoubled with every 10 °C rise in temperature.

Many organisms, psychrophiles, have evolved enzymes that are active and even have their optima at low temperatures (<10 °C). These enzymes are characterized by their conformational flexibility, instead of the thermal stability demanded of enzymes from thermophiles.[14] The results in Figure 3 show that the enzyme originating from the thermophilic organism (MtL) appears to retain more activity under 10 °C compared to the other two. This result is, however, probably because 25 °C is used as reference and the curves have different shapes in the temperature interval studied. The enzyme with the highest temperature optimum would be expected to exhibit a flatter curve, as MtL does. The steeper curves observed for the other two laccases, TvL and RvL, are probably related to their having lower temperature optima than MtL. Therefore, despite the performance of the three laccases in the temperature study (Figure 3), it is still probable that TvL and RvL retain more of their maximal activity at low temperatures than MtL does.

**Immobilization of Enzymes in Films.** When enzymes are immobilized through entrapment in coating materials, they may be exposed to high temperatures in the drying process. The latex forms a polymer film when it is applied to a carrier material and is dried above its minimum film-forming temperature (MFFT).[15] An efficient and fast drying process is achieved by using elevated temperatures for a short period of time. For improved film properties (water resistance and strength), the short drying can be followed by a longer period of time at lower temperatures, still above the MFFT of the latex.[16] High temperatures can cause the enzymes to denature, and it is therefore of great importance to optimize the drying process so that the latex film exhibits good film properties and that the loss of enzymatic activity is as low as possible.

To exploit laccases as oxygen scavengers in active packaging, they need to be incorporated into the packaging material and survive the process. To test dispersion coating of laccases, TvL,

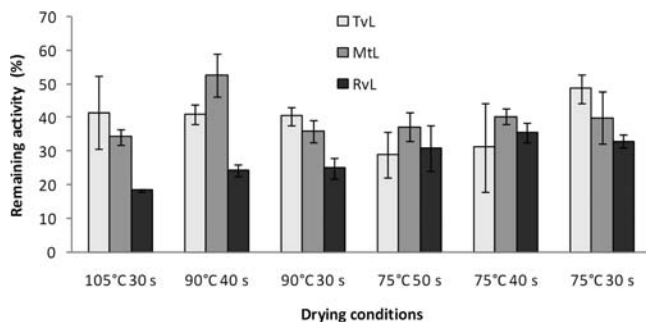


**Figure 4.** Remaining activity of laccases from *T. versicolor* (TvL), *M. thermophila* (MtL), and *R. vernicifera* (RvL) after entrapment in latex/clay matrix and drying. The average remaining activity of three replicates is shown together with the standard deviation.

MtL, and RvL were immobilized into a latex/clay matrix and dried under various conditions. The enzyme activity in the films was compared to the enzyme activity for the free enzymes at pH 6.5 and 25 °C. The results from the activity measurements are presented in Figure 4 as the remaining activity. Data were analyzed statistically, and $p$ values of <0.05 were considered to indicate significant differences (see Materials and Methods). The remaining activity (expressed in percent as means and standard deviations of triplicates) ranged from 18 ± 1 (for RvL after drying at 105 °C for 30 s) to 53 ± 6 (for MtL after drying at 90 °C for 40 s). Irrespective of the conditions used, drying of MtL and TvL resulted in significantly higher remaining activity than drying of RvL. Comparison of drying at different temperatures during the same period of time (30 or 40 s) indicates that RvL exhibited significantly lower remaining activity at high temperature, whereas the differences for TvL and MtL were not significant. When drying was performed at 75 °C during different periods of time (30 or 50 s), TvL exhibited significantly higher remaining activity when the drying time was short. Although enzymes are sensitive to exposure to high temperatures during long periods of time, it is a well-known phenomenon that most of them exhibit a better temperature stability upon immobilization.[17] This may possibly explain the relatively low activity seen for TvL at 75 °C and 50 s. At the higher temperatures, 90 and 105 °C, the film formation of the latex is faster and, as a consequence, the enzymes are more rapidly immobilized.

Our results indicate that all three laccases can be successfully entrapped in the latex/clay matrix with a substantial part of the activity remaining (i.e., 18−53%). This can be compared to the study performed on GOx by Nestorson et al., in which only 6% of the enzymatic activity remained after immobilization in pure latex films.[4] In both studies no substrate was added to the coating color but was present in the reaction mixture used for determination of the enzyme activity. The difference in activity can therefore be partially explained by the log $P_{oct/wat}$ values (partition coefficient between $n$-octanol and water) for the substrates pyrogallol and glucose. Glucose is a very hydrophilic substrate (log $P_{oct/wat} \approx -3$),[18] whereas pyrogallol is more soluble in organic solvents compared to aqueous solutions (log $P_{oct/wat} \approx 1$).[19] The large difference, about 10000 times, suggests that glucose has poorer capacity to diffuse in the film, which would contribute to the large activity dissimilarity seen.

**Conclusions.** The results show that TvL and MtL are less selective than RvL with regard to their reducing substrate and have higher potential to be useful as oxygen scavengers in active-packaging applications. It is possible to produce coatings containing laccases with retained catalytic activity even after drying at temperatures as high as 105 °C. Furthermore, the relatively high activities observed at 4 °C suggest that laccases can serve as oxygen scavengers in packages with refrigerated food. Several factors including the high remaining activity after coating and drying, a wide variety of phenolic substrates to choose between, and no problem with hydrogen peroxide as byproduct contribute to make laccases attractive catalysts in future research on oxygen scavengers.

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## ABBREVIATIONS USED

TvL, *Trametes versicolor* laccase; MtL, *Myceliophthora thermophila* laccase; RvL, *Rhus vernicifera* laccase; GOx, glucose oxidase; MFFT, minimum film forming temperature.

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