Evaluation of the Laccase from *Myceliophthora thermophila* as Industrial Biocatalyst for Polymerization Reactions

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ABSTRACT: The commercially available laccase from *Myceliophthora thermophila* was evaluated as catalyst for the polymerization of acrylamides. Using the so-called laccase mediator system (LMS), comprising laccase and β-diketones, polymerization reactions can be performed using molecular oxygen as terminal electron acceptor. Thus, the LMS can substitute for diazo- or peroxocompounds as radical starters. Factors influencing the efficiency of the LMS, polymer properties, and the stability of the biocatalyst were investigated. Optimal reaction conditions were slightly acidic reaction media at elevated temperatures (around 50 °C). The enzyme is active and stable in the presence of high concentrations of water-soluble and water-insoluble cosolvents but is inactivated by acrylates. The average polymer weight can efficiently be controlled via the ratio of monomer to enzyme. The mediator (β-diketone) concentration had no significant influence on the polymer properties. Laccase-catalyzed oxidation appears to be rate-limiting step of the overall reaction. The ambivalent role of molecular oxygen for reaction initiation as well as inhibitor of the polymerization reaction was investigated. Current limitations of the LMS are analyzed and an improved setup comprising physical separation of the enzymatic initiation reaction from the polymerization via immobilized enzymes is proposed. Thus, not only the biocatalyst is highly stabilized but also the product properties can be controlled.

Introduction

Polymers play a central role in our today's life. It is practically impossible to numerate all sorts and applications of polymeric substances used today. Also the personal care market is leavened by oligomeric and polymeric substances, especially by poly(acrylates) and their derivatives. This substance class is extremely versatile as product properties can be controlled manifold by choice of monomer mixtures and chemical modification of the product. Applications range from superabsorbing in diapers with poly(acrylates) and rheology modification with poly(acrylamides) to advanced, high-performance polymeric surfactants with fine-tuned mixed polymers.

Poly(acrylates) are produced every year on a multithousand ton scale preferably via radical chain polymerization. Polymerization initiators are chosen from organic peroxides and diazo compounds such as benzoylperoxide or AIBN. Such compounds are, however, somewhat questionable from an environmental- and work-safety point-of-view.

Therefore, we became interested in environmentally more benign polymerization approaches. Suitable enzyme systems capable of initiating radical reactions comprise peroxidases and laccases.[1] The two enzyme classes differ in their preference for the terminal electron acceptor. While peroxidases rely on hydrogen peroxide, laccases utilize molecular oxygen. Thus, no additional cofactor or cosubstrate other than H_2O_2 or O_2 is necessary to sustain the catalytic cycle.

Laccases are found in all kingdoms of life but they are especially produced by wood-degrading fungi and excreted in the extracellular environment to initiate lignin degradation. Besides phenols, laccases accept various other substrates.[2] In recent years, laccases have gained increased interest as catalyst for organic synthesis. Reported applications of laccases include delignification and decolorization within the pulp and paper industry and for textile care within food- and beverage processing and cosmetics.[3−5] Environmentally benign oxidations utilizing laccases as cocatalysts have also been reported[2,6−10] as well as polymerization of phenolic substrates.[11,12] Previous works by Kobayashi and co-workers have demonstrated the principal feasibility of laccase-initiated polymerization of acrylic acid derivates such as acrylamide and acrylic acid esters.[13,14]

The goal of the present study was to evaluate the scope of this reaction for industrial production of poly(acrylates).

Materials and Methods

The laccase (originating from *Myceliophthora thermophila*, M_W = 85 kDa) was purchased from Novozymes under the trade name Flavorstar. The enzyme concentration was determined to be 17 mg × mL^{-1} (0.2 mM). Any other chemicals were purchased from Sigma-Aldrich and used without further purification.

Activity Assay. Laccase activity was determined UV-spectrophotometrically using ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) as probe. Typically, an activity assay mixture contained a final volume of 1 mL of 100 mM potassium phosphate buffer (pH 6) and 18.8 μM ABTS, being equilibrated to the assay temperature for 5 min. The assay was started by addition of 10 μL of laccase solution. ABTS oxidation was followed at 405 nm for 30 s to 10 min. Rates were determined from the linear part of the ABTS curve.

Polymerization Reaction Including GPC Analysis. Acrylamide polymerization was performed in aqueous phosphate buffer (100 mM, pH 6) with addition of 2,4-pentandione as mediator and laccase as catalyst. Unless indicated, the reaction was carried out with 0.7 M acrylamide, 50 mM 2,4-pentandione and 5 μM laccase at 65 °C for 4 h. After enzyme inactivation (either thermally at 85 °C for 10 min or via acidification with 1 M HCl) the polymers formed were directly analyzed with size exclusion chromatography (SEC) analysis: Agilent HP 1100 using a refractive index detector at 30

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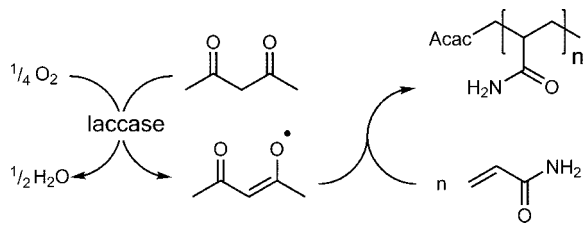
Scheme 1. Laccase Mediator System (LMS) for the Mediated Polymerization of α,β-Unsaturated Carboxylic Acid Derivates



°C under following conditions: Suprema 100 column (length 30 cm, internal diameter 0.8 cm) containing 0.1 M NaCl/10% acetonitrile as eluent at a flow rate 1.0 mL/min. The calibration curve was obtained using pullulan (180 − 380 000 g/mol) as standard (Polymer Standard Service GmbH). For polyacrylamide and acrylamide quantification commercial standards were used, it should be noted that quantification can be considered to be only semiquantitative as even with commercial standard an average deviation of approximately 10−20% was observed.

Results and Discussion

As a model reaction we chose the laccase-initiated polymerization of acrylamide in the so-called laccase mediator system (LMS, Scheme 1) using 2,4-pentanedione (acetylacetone, Acac) as mediator. Feasibility of this reaction has been demonstrated[13] but a thorough evaluation of its practical feasibility is missing so far.

Therefore, the aim of this study was on the one hand to evaluate the scope of the LMS for industrial application and, on the other hand, to evaluate to which extend the LMS is suitable to control polymer properties.

Enzyme Stability. The biocatalyst represents the biggest cost-factor in the envisaged chemo-enzymatic polymerization reaction. Thus, optimal conditions for activity and stability were evaluated first in order to minimize the enzyme amount necessary for catalysis.

pH dependence of laccase activity was determined using syringaldazine as photometric probe. Thus, a pH maximum around pH 6 was observed with more than 75% of the maximal activity between pH 5 and 7.5. A maximal specific activity of 141 U mg^{-1} was estimated corresponding to a turnover frequency of the biocatalyst of 200 s^{-1}. pH 6 was found to be most favorable in respect to laccase stability (Figure 1). Simultaneously, we determined the thermal stability of laccase at different pH values. At pH 6 and 50 °C, laccase showed a half-life of approximately 180 min under storing conditions. Stability increased exponentially with decreasing temperature.

Envisaging also the polymerization of water immiscible substrates necessitating organic cosolvents, we also evaluated the stability of laccase toward various solvents. As a rule of thumb, up to 10% (v/v) of the water-miscible substrates were tolerated without significant loss of catalytic activity (as determined after 1 h incubation time) (Figure 2). For example, laccase stability did not decrease significantly after 24 h incubation in the presence of 10% (v/v) of polar cosolvents such as ethanol, DMSO, 1-octanol, and ethyl acetate. Above that value, laccase activity decreased in a concentration dependent manner. Water-immiscible solvents did not significantly affect residual laccase activity after incubation. These findings are in accordance with previous findings that quite frequently solvents exhibiting a log *P* value above 4 do not affect enzyme activity or stability.[15]

Laccase stability in the presence of substrates particularly acrylamide was more problematic. Increasing the substrate concentration above 0.5 M decreased the overall amount of product formed. Above 2 M substrate, no product formation was observable. The extend of inactivation was also time-dependent for example, polymerization activity of laccase decreased with preincubation time in the presence of 0.7 M acrylamide, after 2 h no more laccase activity was detectable. With poly(acrylamide) no such inhibitory effect was observed. We attribute this effect to the Michael acceptor reactivity of acrylamide and hypothesize that nucleophilic residues in the enzyme (such as cysteins and lysines) form Michael adducts thereby destroying activity-essential H and salt bridges in the enzyme.[16,17]

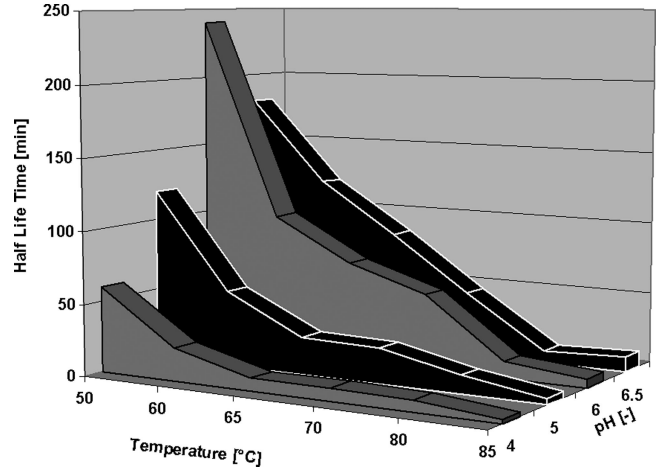


Figure 1. Laccase stability at different pH and temperature. General conditions: laccase (0.2 μM) was incubated for 1 h with buffer at different pH (at room temperature) or at different temperature (at pH 6). Samples were withdrawn at intervals and residual activity was determined by adding 10 μL of the incubated laccase to 990 μL phosphate buffer (0.1 M, pH 6), *c*[ABTS] = 18.84 μM.

Characterization of the Chemoenzymatic Polymerization System. A typical time course of a LMS-polymerization of acrylamide is shown in Figure 3, the polymerization proceeded smoothly to full conversion of the substrate within less than 2 h. The polymer's average molecular weight varied between 250000 g/mol at the beginning of the reaction and 150000 g/mol after full conversion; thus, on average, 2100-mers were formed. Apparently decreasing average weight can most easily be explained by a decreasing chain length of lately formed new chains due to depleting substrate pool. Consequently, the viscosity of the reaction medium steadily increased from 3 cSt (typical starting value) to approximately 230 cSt after full conversion. From these data, a biocatalyst turnover frequency of 1.3 min^{-1} was calculated thus falling behind the catalytic activity e.g. with ABTS by 4 orders of magnitude (vide infra).

Laccase-catalyzed chain initiation was necessary throughout the course of the reaction as shown by enzyme inactivation at intervals (Table 1). This intrinsic limitation of radical chain growth may most likely be explained by a constant inhibition by molecular oxygen (vide infra) on the one hand and by chain growth inhibition due to radical recombination.

A polymer's properties are determined by its chemical composition and by its molecular weight and polydispersity. Therefore, we systematically examined the influence of various reaction parameters on the polymer weight and polydispersity (Table 2).

Control over the polymer molecular weight could be most efficiently exceeded via the ratio of enzyme to monomer (Table 2 entries 11, 12 and 13−15). Obviously, the rates of chain initiation as well as the monomer reservoir for each growing chain directly influence the final M_n. This assumption also explains the apparent decrease of M_n during the course of a polymerization reaction (Figure 3): over time the monomer concentration decreases limiting the substrate reservoir for the

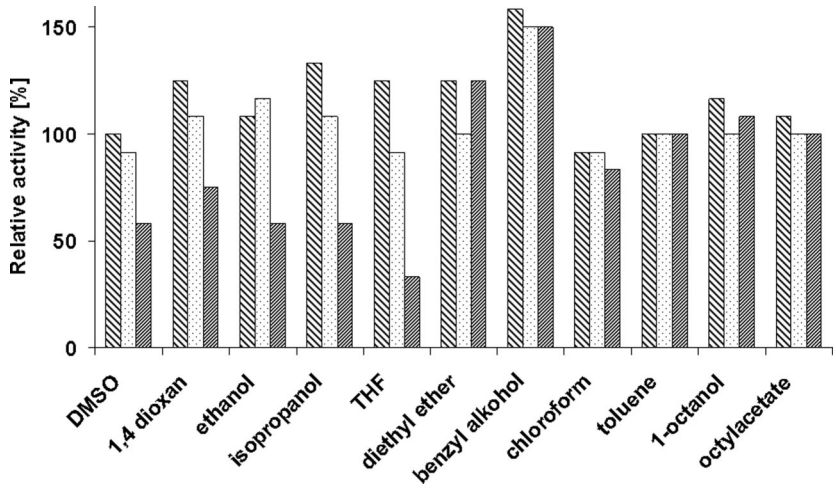


Figure 2. Residual laccase activity after incubation in the presence of various organic solvents. General conditions: laccase (0.2 μM in 0.1 M KPi pH 6) was incubated with the respective organic solvent (from left to right each: 9%, 25%, 50% v/v) for 1 h. Residual activity was determined UV-spectroscopically. For stability in the presence of some solvent: see text.

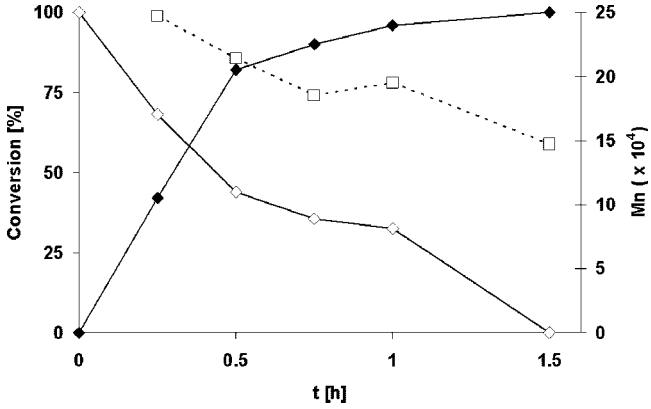


Figure 3. Representative time-course of a LMS-polymerization. General conditions: KPi buffer (0.1 M, pH 6) O_2-saturated, T = 50 °C, [acrylamide] = 0.7 M, [laccase] =5 μM, [acac] =50 mM (ratio of monomer: acac: laccase = 1.4×10^5: 1×10^4: 1) (◆) poly(acrylamide), (◇) acrylamide, and (□) average M_n.

Table 1. Influence of Enzyme Deactivation on Polymer Yield

time of enzyme inactivation after start [min]	yield [%]	M_n [$\times 10^{-4}$]
15	22	21
30	64	23
45	90	20

[a] General conditions: laccase (5 μM) was used as catalyst in acrylamide (0.7 M) polymerization in the presence of 2,4-pentandione (50 mM) at 50 °C, pH 6 (0.1 M phosphate buffer). After the times indicated the enzyme was inactivated by adding concentrated HCl (10 M). The reactions were preceded for another 2 h and neutralized with concentrated NaOH (10 M) prior analysis. M_n = average molecular weight of polymer formed.

growing chains and thereby the maximal achievable chain length. In contrast, the mediator concentration had no significant influence on M_n (Table 2 entries 6−8). Apart from that in the absence of mediator no polymerization was observed (Table 2 entry 5). Interestingly, a minimal mediator concentration was necessary to obtain polymerization activity. Above this concentration bias further increases of [2,4-pentanedione] did not significantly influence the rate of the polymerization reaction. Furthermore, the level of this bias correlated with [O_2] (Table 2 entries 6 and 17, the difference is small but significant). Currently we are lacking a plausible explanation for this behavior, maybe O_2-dependent byproduct formation (e.g., peroxo-derivates) accounts for this observation. Similar observations were made with the laccase-concentration where also a

Table 2. Influence of Various Reaction Parameters on Yield and Polymer Properties

entry	parameter	value	yield [%]	M_n [$\times 10^{-4}$]	polydispersity
		Aerated Buffers			
1	T [°C]	25	0		
2		50	100	14.5	3.2
3		65	82	18.7	2.6
4		80	72	19.5	2.8
5	[2,4-pentanedione] [mM]	0	0		
6		0.2	0		
7		5	90	18.1	2.7
8		7.5	80	18.3	2.6
9	[laccase] [μM]	0	0		
10		1	0		
11		2.5	100	18.1	2.8
12		33.3	72[a]	6.4	2.7
13	[acrylamide] [% (v/v)]	2.5	100	7.3	2.7
14		5	96	12.9	3.0
15		10	69	27.5	2.5
16		25	0		
		Degassing Shortly after the Start of the Reaction			
17	[2,4-pentanedione] [mM]	0.2[a]	4	7.7	6.9
18	[laccase] [μM]	1[a]	8	31.6	3.4

General conditions: unless indicated otherwise, [laccase] = 4.9 μM, [2,4-pentanedione] = 5 mM, [acrylamide] = 0.7 M, T = 65 °C, and reaction time = 4 h.[a] Significant amount of oligomeric compounds that were not integrated!

[O_2]-dependent bias-concentration (of appr. 2 μM in aerated media) was observed. Elevated reaction temperatures resulted in higher M_n (Table 2 entries 1−5), which we attribute to the decreasing O_2 concentration leading to decreased chain growth inhibition, increasing temperature, however, also impaired the biocatalyst's stability leading to decreasing yields. Overall, oxygen plays an interesting role in the whole reaction. On the one hand no polymerization was observed in thoroughly deareated reaction mixtures. On the other hand O_2 also inhibited the radical polymerization as shown by constantly leading air into the reaction mixture where significantly less polymer yield was obtained as compared to using air saturated media (data not shown). Figure 4 shows the effect of oxygen removal from polymerization reactions started in O_2 saturated buffers. The earlier deareation occurred the higher the polymer weight obtained and the narrower to polydispersity of the final product.

Interestingly, laccase activity observed in the polymerization reactions falls back from the activity observed with ABTS or syringaldazine by 4 orders of magnitude. For example, from

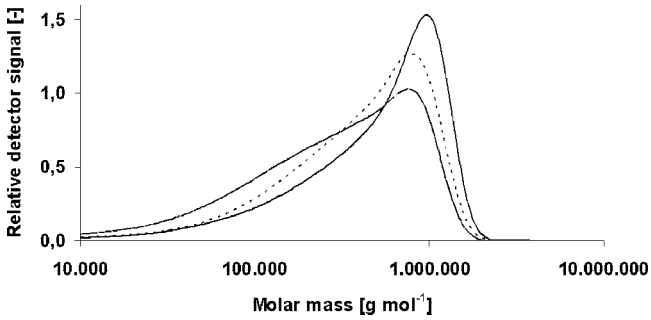


Figure 4. Effect of insetting deareation on polyacrlyamide characteristics. General conditions: KPi buffer (0.1 M, pH 6) O_2-saturated, T = 50 °C, [acrylamide] = 0.7 M, [laccase] = 5 μM, [acac] = 50 mM, deareation was started 15 (solid), 45 (dotted), and 90 min (dashed) after the start of the reaction by addition of laccase.

Figure 3, a typical laccase turnover frequency of approximately 1.3 min^{-1} can be calculated, this also suggest that the laccase-catalyzed radical formation represents the overall rate-limiting step of the polymerization reaction. In the case of phenol polymerization, this value is less dramatic accounting for "only" 2 orders of magnitude (data not shown). However, it is obvious that the catalytic potential of the biocatalyst is not exploited to its full extend. At present time, we are lacking a satisfying explanation other than that the oxidation potential of β-diketones does not allow higher turnover by laccase. ABTS was not efficient as mediator as no acrylamide polymerization was observed. The same is true for other literature-reported laccase substrates such as 2,2,6,6-tetramethylpiperidine-1-yloxy (TEMPO), *N*-hydroxybenzotriazole (HBT), and 2,6-dimethoxyphenol

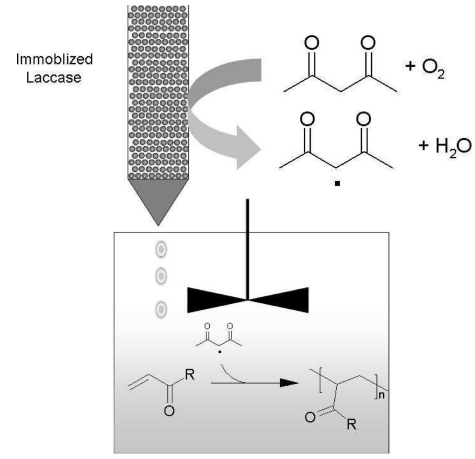
Overall, laccase-initiated polymerization of acrylamide appears to be feasible only using β-diketones as mediators. As a consequence of their low conversion rate with laccase, high enzyme concentrations are necessary to obtain significant polymerization activity. At present state-of-the-art this corresponds to an enzyme's cost contribution of more than 10 Euro per kg of polymer which exceeds the economically acceptable range by 2−3 orders of magnitude. Major limitations are the low laccase-activity with β-diketones as efficient radical starters as well as poor laccase stability toward acrylates preventing reuse of the expensive biocatalyst. Enzyme engineering to increase the oxidation potential of laccase-bound copper is one option to overcome the low polymerization initiation rate. Immobilization of the enzyme may be a suitable option to overcome at least the latter limitation. A heterogenized laccase may be physically separated from the reaction mixture containing noxious substrates (Scheme 2)

The increased laccase stability will allow multiple reuse of the enzyme thereby reducing its cost-contribution. Furthermore, such a reactor setup will overcome the oxygen dilemma by degassing the bulk reaction. A further advantage of the proposed setup over the current batch reactions might be that the amount of radical starter added to the reaction mixture can be easily controlled.

To demonstrate the feasibility of this concept a range of commercially available resins was evaluated for immobilization of laccase. Among the resins tested, Amberlite XAD 7 HP & XAD 1180, Lewatit VP OC 1600, and Eupergit C 250 L showed satisfactory properties with respect to immobilization efficiency and leaching stability of the immobilized biocatalyst.

To test our immobilization hypothesis, we applied a mixture of the aforementioned immobilizates in a frit mounted to a flask containing deareated acrylamide solution. In fact, polymer formation was observed ($M_n = 36 \times 10^4$; $M_w/M_n = 3.08$; 68% yield) in this setup. The average polymer weight obtained under these conditions is the highest observed for the LMS further approving our oxygen hypothesis. Thus, we envision a reactor setup with which the polymer properties can be controlled via oxygen content in the polymerization solution. Comparative experiments using the same enzyme preparations in the bulk solution yielded no product formation, demonstrating the importance of physical separation of the enzyme from the reactive Michael acceptors to maintain high stability. Other advantages of using immobilized enzyme are increased thermal stability as well as facile recycling of the catalysts and pure polymer products. This is further corroborated by the fact that the enzyme preparation could be reused. Investigation of the scope of this improved polymerization setup is currently under investigation in our laboratories. We expect that further development of such a process will eventually enable LMS-polymerizations that are environmentally benign and economically feasible.

Scheme 2. Schematic Representation of the Proposed Laccase-Initiated Polymerization Process[a]



[a] The polymerization initiator (e.g. acac radicals) is generated by treatment of immobilized laccase with an aerated solution of mediator. The resulting solution containing the activated initiator will be lead into the polymerization vessel.

Conclusions

Laccase-initiated polymerization reactions bear the promise of being mild and environmentally benign alternatives to established chemical procedures. However, at present stage the LMS not (yet) fulfils the requirements of economic feasibility on industrial scale. On the one hand, the laccase's catalytic activity with β-diketones needs to be improved significantly. Enzyme engineering, particular directed evolution of structure-guided site-directed mutagenesis will result in laccase variants exhibiting increased oxidation potentials and, consequently higher rates. On the other hand, in situ application of the soluble biocatalyst results in too fast biocatalyst inactivation. However, physical separation of the biocatalyzed initiation reaction from the polymerization reaction has been demonstrated to be feasible. Thus, not only enzyme inactivation can be overcome, but also control over the polymer properties can easily be exceeded by control over the oxygen content in the polymerization reaction.

Overall, we are convinced that biocatalytic polymerization reactions bear a great potential for a greener and more sustainable production of polymers also on industrial scale.

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