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Kinetics of laccase-catalysed TEMPO oxidation

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Abstract

The oxidation of TEMPO (2,2,6,6-tetramethyl-piperidine-1-oxyl radical) has been studied in the presence of recombinant laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) from *Polyporus pinsitus* (rPpL), *Myceliophthora thermophila* (rMtL), *Coprinus cinereus* (rCcL) and *Rhizoctonia solani* (rRsL) in buffer solution pH 4.5–7.3 and at 25 °C. At pH 5.5 the oxidation constant calculated from the initial rate of TEMPO oxidation was 1.7×10^4, 1.4×10^3, 7.8×10^2 and 5.2×10^2 M^{-1} s^{-1} for rPpL, rRsL, rCcL and rMtL, respectively. The maximal activity of rPpL-catalysed TEMPO oxidation was at pH 5.0. The pK_a obtained in neutral pH range was 6.2. The reactivity of laccases is in a good agreement with laccases copper type I redox potential.

TEMPO oxidation rate increased 541 times in the presence of 10-(3-propylsulfonate) phenoxazine (PSPX). The model of synergistic TEMPO and PSPX oxidation was proposed. Experimentally obtained rate constants for rPpL-catalysed PSPX oxidation were in a good agreement with those calculated from the synergistic model, therefore confirming the feasibility of the model. The acceleration of TEMPO oxidation with high reactive laccase substrates opens new possibilities for TEMPO application as a mediator.

Keywords: Laccase; *Polyporus pinsitus*; *Myceliophthora thermophila*; *Coprinus cinereus*; *Rhizoctonia solani*; 2,2,6,6-Tetramethyl-piperidine-1-oxyl radical; TEMPO; 10-(3-Propylsulfonate) phenoxazine

1. Introduction

Laccases are classified as polyphenol oxidases and perform the reduction of oxygen to water while oxidizing the substrate [1]. Laccases catalyse the oxidation of lignin, inorganic and organic metal complexes, anilines, thiols and phenols [2–4]. Because of their ability catalyse the oxidation of aromatic and other various compounds, laccases are receiving increasing attention as a potential industrial enzymes in various applications.

The laccases are able to oxidize certain phenols with a redox potential (E_0) values higher than their own (from 0.5 to 0.8 V versus NHE) [2]. However, many inorganic and organic compounds with comparable E_0 values are "bad" laccase substrates due to unfavourable kinetics. Under certain conditions these compounds can be indirectly oxidized by laccase via the mediation by small redox-active substrates.

The role of mediators in an enzymatic oxidation with the laccases have been extensively discussed previously [2,3,5–12]. Different types of mediators, particularly phenothiazines, phenoxazines, N–OH compounds and others have been recognized for their mediatory function in laccase catalysis. Detailed comparative studies on the interaction between mediator and laccase remains to be reported [5–9], although various physical and chemical characterizations have been carried out on several well-known laccase mediators [7,13–16]. The discovery of new efficient laccases mediators and elucidation of mediation mechanism, in particular, could facilitate the solution of different biotechnological problems.

2,2,6,6-Tetramethyl-piperidine-1-oxyl radical (TEMPO) is one of more effective mediators. It is a stable *N*-oxyl radical and attracts a considerable amount of interest in its application in organic synthesis [5,6,17–21]. More specifically, it has been described as a highly selective catalyst in the oxidation of alcohols [5,6,11,12]. The "active" form of TEMPO is the oxoammonium ion [17,18], formed by oxidation of the *N*-oxyl radical, as it is proposed for the reaction of TEMPO-catalysed oxidation

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of alcohols [21–23]. The oxoammonium ion would be responsible for the oxidation of lignin, other organic compounds, as well as oxidation of alcohols to aldehydes. The role of laccase would be to regenerate TEMPO from the hydroxylamine form which was formed during the reaction, so that the oxoammonium ion could be restored via oxidation of TEMPO by laccase. Laccase, in turn, is re-oxidized by oxygen [23]. Marjasvaara et al. [12] determined that during the overall reaction TEMPO appears in three different forms: oxidized species, normal radical cation and reduced species. This observation confirmed an earlier proposed mechanism [11] in which actual oxidizing reagent is the oxoammonium form of TEMPO.

A better understanding of what governs mediation efficiency would require in-depths knowledge of the oxidation mechanism as well as the rates of the reactive species generated with laccase. However, to the best of our knowledge, the laccase-catalysed TEMPO oxidation rate has not been measured and oxidation constant has not been determined.

The main task of our study was to study the kinetics of fungal laccases catalysed TEMPO oxidation. We have found that certain redox compounds markedly increased TEMPO oxidation rate. The kinetics of this process was studied. The recombinant laccases from *Polyporus pinsitus* (rPpL), *Myceliophthora thermophila* (rMtL), *Coprinus cinereus* (rCcL) and *Rhizoctonia solani* (rRsL) were used for the study of TEMPO oxidation.

1.1. Reagents

Recombinant laccases from *Polyporus pinsitus* (rPpL), *Myceliophthora thermophila* (rMtL), *Coprinus cinereus* (rCcL) and *Rhizoctonia solani* (rRsL) from Novozymes A/S (Copenhagen, Denmark) were additionally purified by anion exchange chromatography and were homogenous as assessed by SDS-PAGE. Concentration of laccases was determined spectrophotometrically. The extinction coefficient for rMtL is $134\,mM^{-1}\,cm^{-1}$ at 276 nm [24], whereas a coefficient of $78\,mM^{-1}\,cm^{-1}$ is used for rPpL, rCcL and rRsL at 280 nm [25]. 2,2,6,6-Tetramethylpiperidin-1-yloxy, free radical (TEMPO) was obtained from Aldrich (Germany), 10-(3-propylsulfonate) phenoxazine sodium salt (PSPX) was received from Novozymes A/S (Copenhagen, Denmark). The structures of TEMPO and PSPX are shown in Fig. 1. Sodium acetate and acetic acid, potassium dihydrogen phosphate and sodium hydroxide were "chemically pure" and were received from Reachim (Moscow, Russia).

The kinetic measurements were performed in 50 mM sodium acetate buffer solution, pH 5.5, at $25 \pm 0.1\,^{\circ}C$. All solutions were prepared in triple distilled water.

1.2. Methods

Spectrophotometric measurements were performed using computer-controlled "Nicolet evolution 300" spectrophotometer (Thermo electron Corporation, USA) in 1 cm thermostated quartz cuvette. The temperature was maintained at $25 \pm 0.1\,^{\circ}C$.

TEMPO oxidation was monitored at 245 and 300 nm in 50 mM acetate buffer solution at pH 5.5. The differential extinction coefficient of TEMPO was determined during prolonged incubation of 21–200 μM TEMPO and 100 nM of rPpL. The calculated coefficient was $2.5 \times 10^2\,M^{-1}\,cm^{-1}$ at 245 nm and $4.3 \times 10^2\,M^{-1}\,cm^{-1}$ at 300 nm.

The pH dependence of rPpL-catalysed TEMPO oxidation was studied by recording absorbance changes in the pH interval 4.5–7.3. Fifty millimolars of acetate (pH 4.5–5.8) and 50 mM phosphate (pH 5.8–7.3) buffers were used. The concentrations of TEMPO and rPpL were 240 μM and 94 nM, respectively.

1.3. Electrochemical measurements

Cyclic voltammetry (CV) was performed using an electroanalytical system (Cypress Systems, USA) equipped with a glassy carbon electrode (model CS-1087, Cypress Systems, USA), at room temperature. A saturated calomel electrode (SCE, saturated with KCl, model K-401, Radiometer, Denmark) was used as a reference electrode. Pt wire (diameter 0.2 mm, length 4 cm) mounted on the end of the reference electrode was used as an auxiliary electrode. Glassy carbon electrode was freshly polished with aluminium oxide and treated ultrasonically in water for 10 min. The electrochemical measurements were performed in 50 mM acetate buffer, pH 5.5. The concentration of compounds (TEMPO and PSPX) was 0.2 mM. Formal redox potential of compounds was calculated as the midpoint potential between the reduction and oxidation peak potentials. The potential of SCE was assumed 0.241 V versus NHE.

1.4. Calculations

Initial rate of the reaction was calculated by fitting the absorbance change (increase or decrease) by exponent with offset. The dependence of initial oxidation rate on TEMPO or PSPX concentration was approximated by Michaelis–Menten equation, producing apparent parameters K_m and V_{max}. The apparent bimolecular constant was calculated as $k_{ox} = V_{max}/K_m[E]$ or as $k_{ox} = V_o/[S][E]$.

The expression of initial rate of TEMPO oxidation in presence of PSPX was derived at steady state conditions for enzyme and PSPX using symbolic algebra (Mathcad 5.0 program).

2. Results

2.1. The change of absorbance during TEMPO oxidation

The lack of conjugated π-electron system in TEMPO (Fig. 1) results in a low absorbance values in a UV region. The oxidation of TEMPO was monitored measuring absorbance changes at 245 and 300 nm (Fig. 2). During oxidation of TEMPO two isosbestic points, at 233 nm and at 263 nm, were observed, indicating single product formation. The absorbance change in our study correlated well with the absorbance change observed during electrochemical TEMPO oxidation [26], confirming the oxoammonium ion formation. Initial rate of TEMPO oxi-

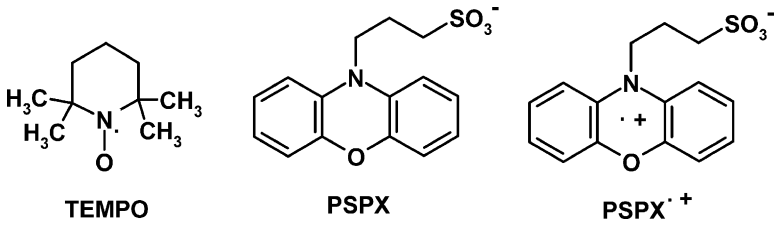

Fig. 1. Structure of 2,2,6,6-tetramethyl-piperidine-1-oxyl radical (TEMPO), 10-(3-propylsulfonate) phenoxazine (PSPX) and radical cation of 10-(3-propylsulfonate) phenoxazine ($PSPX^{\bullet+}$).

dation rate was linearly dependant on rPpL concentration. TEMPO oxidation rate increased from 1 to 22.5 μM/min with increase in rPpL concentration from 10 to 200 nM. The oxidation constant calculated from the dependence of the initial rate on enzyme concentration was equal to $(1.7 \pm 0.1) \times 10^4\ M^{-1}\ s^{-1}$.

Other laccases, i.e. rMtL, rCcL and rRsL, can also catalysed oxidation of TEMPO. However, the observed rates were considerably lower compared to that of rPpL. The apparent oxidation constants calculated from the initial rate of absorbance change were $(1.4 \pm 0.6) \times 10^3$, $(7.8 \pm 2.5) \times 10^2$ and $(5.2 \pm 1.7) \times 10^2\ M^{-1}\ s^{-1}$ for rRsL, rCcL and rMtL, respectively. Due to low reactivity of the laccases, studies of TEMPO oxidation were carried out using rPpL.

2.2. The dependence of initial rate of TEMPO oxidation on TEMPO concentration

The dependence of initial rate on TEMPO concentration showed saturation shape (Fig. 3). The data were approximated with Michaelis–Menten equation and consequently yielding $K_m = (391 \pm 97)$ μM and $V_{max} = (49 \pm 9)$ μM/min. The calculated apparent oxidation constant for TEMPO oxidation was $(1.9 \pm 0.2) \times 10^4\ M^{-1}\ s^{-1}$.

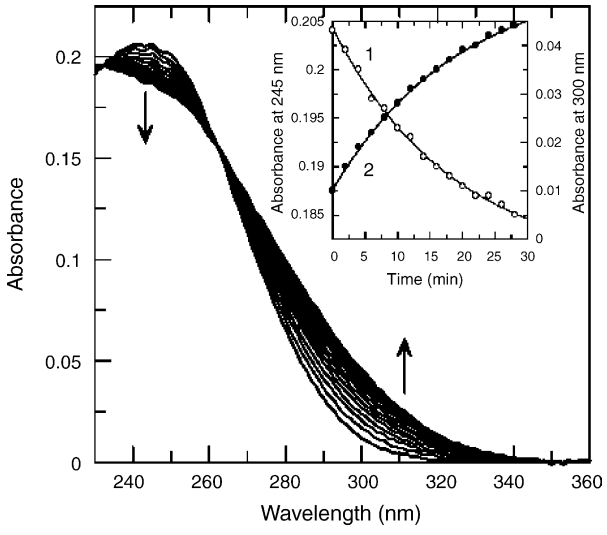

Fig. 2. The change of TEMPO absorbance during laccase action. The spectra were recorded every 2 min. The inset shows kinetics of absorbance change at 245 nm (1) and 300 nm (2). The curves in the insert were produced by fitting experimental data using exponent with offset. One hundred micromolars of TEMPO, 50 nM rPpL, 50 mM acetate buffer, pH 5.5, 25 °C.

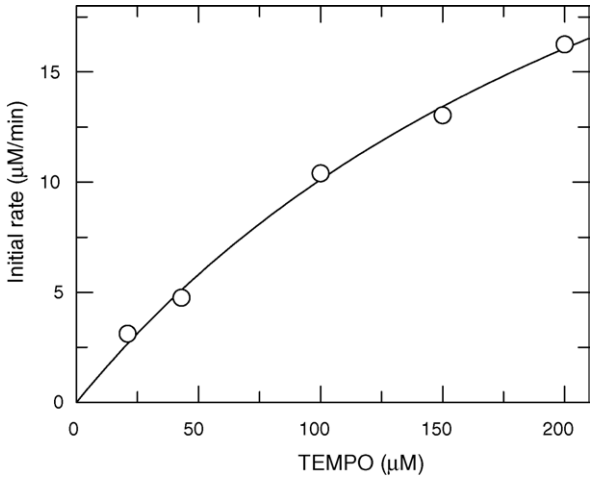

Fig. 3. The dependence of initial rate of rPpL-catalysed TEMPO oxidation on substrate concentration. The curve was drawn using Michaelis–Menten equation. Fifty millimolars of acetate buffer, 100 nM rPpL, pH 5.5, 25 °C.

2.3. The dependence of initial rate of TEMPO oxidation on solution pH

The dependence of TEMPO oxidation rate was investigated in pH range 4.5–7.3. The data exhibited bell-shaped dependence with the maximum rate at pH 5.0 (Fig. 4). The fitting of experimental data gave pK_a value 6.2 ± 0.1. The pH dependence of TEMPO oxidation rate is associated with pH dependence of laccase activity: the maximum activity of rPpL for other substrates is at pH 4.5–5.5 [27].

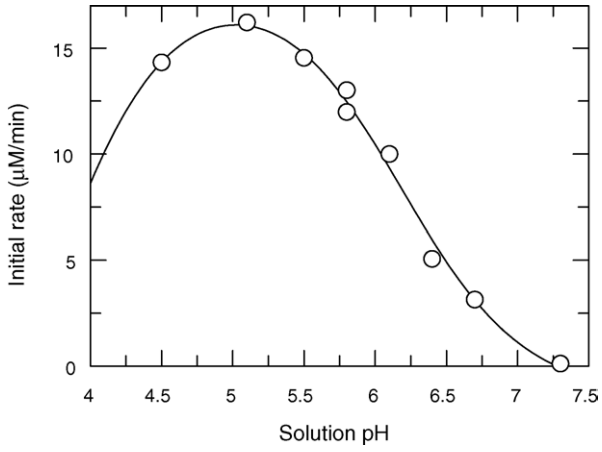

Fig. 4. The dependence of rPpL-catalysed TEMPO oxidation rate on solution pH at 25 °C. Two hundred and forty micromolars TEMPO, 90 nM rPpL.

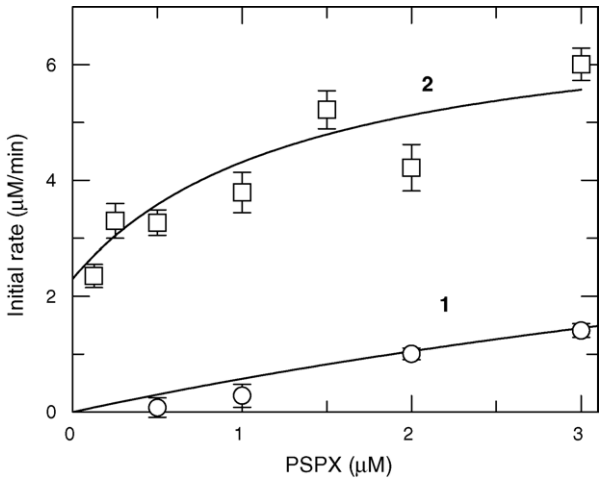

Fig. 5. The dependence of rPpL-catalysed PSPX (1) and TEMPO (2) oxidation rate on PSPX concentration. Curve (2) is a result of data fitting with proposed synergistic model. Two hundred and forty micromolars of TEMPO (2), 1.4 nM (1) rPpL and 14 nM (2) rPpL.

2.4. TEMPO oxidation in presence of PSPX

A typical Michaelis–Menten dependence with the apparent $K_m = (9.5 \pm 1.7)\,\mu M$ and $V_{max} = (6.1 \pm 0.5)\,\mu M/min$ was observed when rPpL-catalysed PSPX oxidation was investigated (Fig. 5). PSPX oxidation constant calculated from K_m and V_{max} values was equal to $(7.6 \pm 0.8) \times 10^6\ M^{-1}\ s^{-1}$.

Addition of PSPX to the reaction mixture increased TEMPO oxidation rate (Fig. 5): the initial rate of TEMPO oxidation increased from 1.5 μM/min in the absence of PSPX up to 6.0 μM/min at 3 μM of PSPX.

2.5. Electrochemical oxidation of TEMPO and PSPX

The electrochemical oxidation of TEMPO and PSPX was investigated using glassy carbon electrode. TEMPO is oxidized at higher potential than PSPX (Fig. 6). The oxidation of both compounds proceeded at diffusion limiting conditions

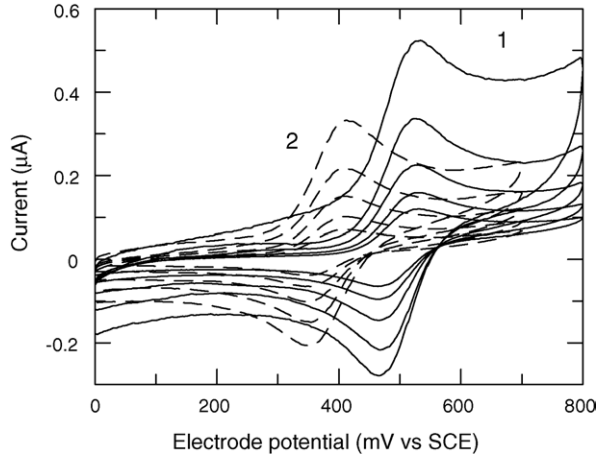

Fig. 6. Cyclic voltamogram of 0.2 mM TEMPO (1) and 0.2 mM PSPX (2). Fifty millimolars of acetate buffer, pH 5.5, 25 °C; potential scan rate 6, 12, 25, 50 and 100 mV/s.

since the peak current of anodic oxidation was proportional to square root of the potential scan rate. The electrochemical oxidation of both compounds was quasi reversible; the difference between anodic and cathodic peak potential was 62 and 56 mV for TEMPO and PSPX, respectively, at scan rate 100 mV/s. At pH 5.5 formal redox potential calculated as a mean of anodic and cathodic peak potential was 495 ± 5 mV for TEMPO and 382 ± 4 mV for PSPX. The estimated redox potential of TEMPO is comparable to a value of 482 mV versus SCE [28].

3. Discussion

Several microbial laccases are used to catalyse TEMPO oxidation in presence of oxygen. However, TEMPO oxidation is markedly slower with rMtL, rCcL or rRsL in comparison to rPpL. In the case of rPpL the mean value of the constant calculated from different experiments was $(1.7 \pm 0.2) \times 10^4\ M^{-1}\ s^{-1}$. The model of TEMPO oxidation can be written as follows:

$$\text{laccase(red)} + O_2 + 4H^+ \rightarrow \text{laccase(ox)} + 2H_2O \quad (1)$$

$$\text{laccase(ox)} + \text{TEMPO(red)} \rightarrow \text{laccase(red)} + \text{TEMPO(ox)} + H^+ \quad (2)$$

The catalytic activity of laccases from different sources may be ranked in the order of their decreasing redox potential: 0.78, 0.71, 0.55 and 0.47 V versus NHE for rPpL, rRsL, rCcL and rMtL, respectively [24,29]. The k_{ox} ranked in the same order for the respective laccases: 1.7×10^4, 1.4×10^3, 7.8×10^2, $5.2 \times 10^2\ M^{-1}\ s^{-1}$.

The reaction of oxidized laccase with PSPX(red) and radical cation (PSPX(ox)) with TEMPO may be added to describe PSPX-assisted TEMPO oxidation:

$$\text{laccase(ox)} + \text{PSPX(red)} \rightarrow \text{laccase(red)} + \text{PSPX(ox)} + H^+ \quad (3)$$

$$\text{PSPX(ox)} + \text{TEMPO(red)} \rightarrow \text{PSPX(red)} + \text{TEMPO(ox)} \quad (4)$$

To obtain k_{ox} for PSPX during TEMPO oxidation the scheme (Eq. (1)–(4)) was analysed at quasi steady state conditions for the enzyme and the PSPX. Expression of initial rate of TEMPO (S) oxidation is rather complex due to the presence of enzyme as well as PSPX (M) which exists in both oxidized and reduced forms:

$$\begin{aligned} V_{st} = {} & k_2[S]_0(1/(2(k_1[O_2]k_3 + [S]_0k_2k_3))(-k_1[O_2]_0k_4[S]_0 \\ & -[S]_0^2k_2k_4 - k_4[M]_0k_3[S]_0 + [E]_tk_1[O_2]_0k_3 \\ & +(k_1^2[O_2]^2k_4^2[S]_0^2 + 2k_1[O_2]_0k_4^2[S]_0^3k_2 \\ & +2k_1[O_2]_0k_4^2[S]_0^2M]_0k_3 + 2k_1^2[O_2]_0^2k_4[S]_0[E]_tk_3 \end{aligned}$$

$$+[S]_0^4k_2^2k_4^2+2[S]_0^3k_2k_4^2[M]_0k_3+2[S]_0^2k_2k_4[E]_tk_1[O_2]_0k_3$$
$$+k_4^2[M]_0^2k_3^2[S]_0^2-2k_4[M]_0k_3^2[S]_0[E]_tk_1[O_2]_0$$
$$+[E]_t^2k_1^2[O_2]_0^2k_3^2)^{1/2}))$$
$$+k_4[S]_0[M]_0/(1+k_4[S]_0/(k_3(1/(2(k_1[O_2]_0k_3$$
$$+[S]_0k_2k_3))(-k_1[O_2]_0k_4[S]_0-[S]_0^2k_2k_4-k_4[M]_0k_3[S]_0$$
$$+[E]_tk_1[O_2]_0k_3+(k_1^2[O_2]_0^2k_4^2[S]_0^2+2k_1[O_2]_0k_4^2[S]_0^3k_2$$
$$+2k_1[O_2]_0k_4^2[S]_0^2[M]_0k_3+2k_1^2[O_2]_0^2k_4[S]_0[E]_tk_3$$
$$+[S]_0^4k_2^2k_4^2+2[S]_0^3k_2k_4^2[M]_0k_3+2[S]_0^2k_2k_4[E]_tk_1[O_2]_0k_3$$
$$+k_4^2[M]_0^2k_3^2[S]_0^2-2k_4[M]_0k_3^2[S]_0[E]_tk_1[O_2]_0$$
$$+[E]_t^2k_1^2[O_2]_0^2k_3^2)^{1/2}))))$$

where k_1, k_2, k_3 and k_4 are the rate constant for corresponding reactions 1, 2, 3 and 4. The concentration of oxygen ($[O_2]_0 = 0.25$ mM [30]) was assumed to be constant during the determination of the initial rate, $[M]_0 = [PSPX(ox)] + [PSPX(red)]$.

The fitting of experimental data (Fig. 5) was performed at fast cross reaction (Eq. (4)): the rate of this reaction did not limit process. The precision (coefficient of variation) of approximation was 4.9%. The calculations gave $k_1 = 3.3 \times 10^4\ M^{-1}\ s^{-1}$, $k_2 = 1.7 \times 10^4\ M^{-1}\ s^{-1}$ and $k_3 = 9.2 \times 10^6\ M^{-1}\ s^{-1}$. The calculated k_2 and k_3 values were similar to the independently determined TEMPO $(1.7 \pm 0.2) \times 10^4\ M^{-1}\ s^{-1}$ and PSPX oxidation rate ($(7.6 \pm 0.8) \times 10^6\ M^{-1}\ s^{-1}$) which supports proposed model of synergistic TEMPO oxidation. The ratio of k_3/k_2 indicates an increase in TEMPO oxidation rate in the presence of synergistic substrate PSPX.

The comparison of PSPX and TEMPO oxidation rates suggests that TEMPO belongs to a group of low reactivity substrates like other N–OH substrates [10]. The acceleration of TEMPO oxidation with PSPX or other highly reactive laccase substrates [31] opens new possibilities in TEMPO application. The use of TEMPO/mediator system for water soluble cellulose oxidation is under investigation.

Acknowledgments

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