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Improving the performance of a biofuel cell cathode with laccase-containing culture supernatant from *Pycnoporus sanguineus*

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HIGHLIGHTS

- Easy to produce high redox laccase from *Pycnoporus sanguineus*.
- Using crude culture supernatant of *Pycnoporus sanguineus* in biofuel cells.
- Laccase in culture supernatant improves cathode performance under different pH values.

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ABSTRACT

Laccases are multicopper oxidoreductases that can be used in biofuel cells to improve cathode performance by cathodic oxygen reduction. Here we present a laccase from the ligninolytic white-rot fungus *Pycnoporus sanguineus* that, in contrast to the *Trametes versicolor* laccase, can be produced in the absence of inducers in a standard culture medium. After 7 days of cultivation the activity of this laccase in culture supernatant reached 2.5 U/ml, which is high enough for direct application of the supernatant in biofuel cells. The highest current density of 115.0 ± 3.5 $\mu A/cm^2$ at 400 mV vs. SCE was obtained at pH 5 with a buckypaper cathode with a laccase-containing culture supernatant. The enzyme also showed electrocatalytic activity at pH 6 and 7. These results not only present a new cost-efficient laccase for improving cathode performance, but also show that new laccases with different catalytic properties can be suitable for biofuel cells.

1. Introduction

Development of eco-friendly technologies, in particular use of enzyme biocatalysts that neither require nor produce toxic compounds, is currently of great industrial value. One of the fields, where enzymatic catalysis can be applied, is the production of electrical energy from renewable sources in biofuel cells (BFCs) (Bullen et al., 2006). In BFCs – just as in living organisms – the oxidation of organic material is coupled to the reduction of an electron acceptor. If the redox potential between the organic material and the electron acceptor is high enough and the redox reactions coupled through a pair of electrodes, the electron flow can be used to produce work. As biocatalysts, BFCs can contain microorganisms or enzymes that utilize organic substrates like lignocellulose, biowaste or glucose from body fluids, oxidize them and transfer the electrons to an anode (Kerzenmacher et al., 2008; Logan et al., 2006; Minteer et al., 2007; Pant et al., 2010). At the cathode laccase is an attractive biocatalyst with a high redox potential that can perform a direct electron transfer from the cathode to e.g., oxygen and thereby improve the BFC performance (Christenson et al., 2004; Zheng et al., 2008).

Laccases (EC 1.10.3.2) are multicopper oxidoreductases that can oxidize a variety of aromatic and non-aromatic compounds (Baldrian, 2006; Thurston, 1994). The catalytic mechanism is based on one-electron oxidation and four-electron reduction of molecular oxygen to water. The electrons are taken by type-1 (T1) copper and are transferred to one type-2 (T2) and two type-3 (T3) coppers, where oxygen reduction takes place (Baldrian, 2006). Laccases are widely distributed in bacteria, fungi and higher plants and demonstrate a variety of thermodynamic and kinetic properties depending on their function (Thurston, 1994). Fungal laccases are mostly secreted outside the cell, and can be easily harvested from the culture supernatant (Bollag and Leonowicz, 1984). However, the production levels of enzyme highly depend on cultivation conditions. Carbon and nitrogen source, micro-elements and their concentration influence the laccase yield (Gochev and Krastanov, 2007). Many studies report specific induction of laccase production

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in non-genetically modified fungal strains at reactor scale by addition of copper, aromatic compounds or detergents (Couto and Toca-Herrera, 2007). Reducing the costs by achieving high laccase production without toxic or expensive supplements is an important objective for industrial application.

Currently, laccases from the genus *Trametes* have been established as a biocatalyst for mediatorless electron transfer and oxygen reduction on the cathode in enzymatic BFCs because of their high redox potential (Christenson et al., 2004; Milton et al., 2013; Rubenwolf et al., 2010; Salaj-Kosla et al., 2013; Zheng et al., 2008). In order to produce the enzyme the fungus needs to be grown in the presence of $CuSO_4$ (Sane et al., 2013). In addition, long cultivation times are required and the overall yields are low. These are disadvantages for large-scale production. Therefore, a screen for other laccase sources is desirable. Indeed, thousands of filamentous fungi are known to date and laccases have been found in all described wood-rotting fungal species (Heinzkill and Messner, 1997). Though only the laccases from *Trametes* species have previously been characterized as high potential laccases (Christenson et al., 2004), there is a large amount of non-characterized laccases that could also be assigned to this group. It has also been shown that unpurified laccase in crude culture supernatant of *Trametes versicolor* is as efficient in increasing cathode performance as the purified enzyme in buffer (Sane et al., 2013). This fact opens an opportunity to measure the electrocatalytic activity of laccases from less characterized fungal species, by introduction of crude culture supernatant in enzymatic BFC.

Fungi of the genus *Pycnoporus* produce large amounts of biopolymer degrading enzymes like laccases and cellulases and are used for a number of applications in green and white biotechnology (Meza et al., 2006) (Teoh and Mashitah, 2010). It was previously shown that *P. cinnabarinus* constitutively expressed laccase during primary metabolism and, in contrast to *Trametes* species, no detectable amounts of lignin peroxidase or manganese peroxidase were found in the culture medium (Eggert et al., 1996). It was suggested that laccase was the main exocellular phenoloxidase in *P. cinnabarinus* and could demonstrate properties untypical for this enzyme group. The species *P. coccineus* and *Pycnoporus sanguineus* produce laccases with high redox potential and pH/temperature stability (Uzan et al., 2010) and present a potentially suitable enzymes for application in BFCs. Therefore, we tested whether the laccase-containing supernatant from *P. sanguineus* exhibits biocatalytic activity for oxygen reduction at a buckypaper cathode under conditions where no artificial mediator is added. We have also analyzed the influence of pH and temperature on the laccase activity and stability in crude culture supernatant. The electrocatalytic performance of the laccase on a cathode has been evaluated under different conditions.

2. Methods

2.1. Strains and growth conditions

The *P. sanguineus* DSM 6762 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and *T. versicolor* ATCC 32745 strains were used for laccase production in liquid culture. The growth medium (YAG) contained 0.5% yeast extract, 1% glucose, 2 mM $MgSO_4$, vitamins and trace elements (Barratt et al., 1965). For enzyme production cultures were incubated in 100 ml Erlenmeyer flasks at 28 °C and 120 rpm for 10 days.

2.2. Characterization of laccase activity

Laccase activity in culture supernatants was measured using 1 mM 2,2′-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS) as substrate in 50 mM sodium acetate buffer pH 5 at 25 °C (Eggert et al., 1996). The change in absorbance, corresponding to oxidation of ABTS, was measured at 420 nm in a volume of 1 ml over a period of 10 min with an Ultrospec III Spectrophotometer (Pharmacia). The reaction velocity was calculated with a molar absorption coefficient of ABTS of ε_{420} = 36,000 l mol^{-1} cm^{-1}. Enzyme activity was expressed in units per ml culture supernatant. One unit of laccase catalyzes the conversion of 1 μmol of substrate per min.

To determine the optimum temperature for laccase in culture supernatant of *P. sanguineus*, ABTS-Assay was performed at 30, 40, 50, 60, 70 and 80 °C in 50 mM sodium acetate buffer pH 5. The thermostability of the enzyme in culture supernatant was measured after preincubation of culture supernatant at 30, 50 and 70 °C for 10, 20, 30, 60, 90 and 120 min. The long-term stability was determined over 10 days at 30 °C.

The optimum pH for laccase was determined by performing the assay in sodium acetate buffer at pH 2.5, 3, 4, 5, 6 and in sodium phosphate buffer at pH 7. The pH stability was measured after preincubation of diluted *P. sanguineus* culture supernatant at pH 2.5, 3, 4, 5, 6 and 7 for 12 h at 4 °C.

2.3. Identification of laccase in culture supernatant of P. sanguineus

To identify the laccase in the culture supernatant of *P. sanguineus*, 12.5% SDS–PAGE was performed followed by Coomassie staining. The gel band, corresponding to the approximate molecular weight of known laccases from *P. sanguineus*, was excised and used for protein identification by peptide mass fingerprinting using MALDI-TOF/MS after protein cleavage with trypsin (TOPLAB GmbH, Martinsried, Germany).

To determine, whether several laccases were present in the culture supernatant, proteins in the sample were also separated using 7.5% native PAGE with 0.025% Nonidet P-40 (w/v) by mass and charge. Activity staining was performed with 1 mM ABTS in sodium acetate buffer pH 5.

The laccase gene was amplified by PCR from cDNA, transcribed from *P. sanguineus* mRNA, using the following primers: Lcc_AscI_fw 5′-ATG GCG CGC CAT GTC GAG GTT CCA GTC CCT C-3′, Lcc_PacI_rev 5′-CAT TAA TTA ATC AGA GGT CGC TGA GGT CAA G-3′. Three independent PCR products were sequenced using the primers: Seq_Lcc_fw 5′-CAC ACA GGG CAA GCG GTA CCG-3′, Seq_Lcc_rev 5′-CGC CGG TGG AAT CAA CTC TGC-3′.

2.4. Laccase purification from culture supernatant

For the electrochemical characterization of purified laccase, filtered culture supernatant of *P. sanguineus* was applied to a DEAE-Sepharose column (Pharmacia, Uppsala, Sweden), equilibrated in 20 mM Tris buffer pH 7. The protein was eluted with a linear gradient up to 100% elution buffer, containing 20 mM Tris pH 7 and 1 M NaCl. Laccase activity was determined using the ABTS assay. Subsequently the laccase enzyme was concentrated in dialysis tubes using polyethylene glycol (PEG-20000) and dialysed against 100 mM sodium acetate buffer pH 5. Culture supernatant, flow-through, laccase-containing fractions and laccase solution after dialysis were analyzed using SDS–PAGE. Prior to application on the cathode the protein was diluted to 1.8 U/ml and titrated to pH 5.

2.5. Electrochemical characterization

The ability of the *P. sanguineus* laccase to perform electron transfer from a buckypaper cathode in the absence of mediators was determined using both, linear sweep voltammetry and a step-wise galvanostatic technique to record quasi-steady-state polarization curves. As electrodes buckypaper made from multi-walled carbon nanotubes was prepared as described previously (Hussein et al., 2011a,b).

For all electrochemical experiments the *P. sanguineus* culture supernatant was filtered through miracloth (Merck KGaA, Darmstadt, Germany) prior to the application. Subsequently the culture supernatant was diluted to 1.8 U/ml or 3.6 U/ml laccase concentration in 100 mM sodium acetate buffer pH 5, pH 6 or 100 mM sodium phosphate buffer pH 7 and titrated to pH 5, 6 or 7.

Linear sweep experiments were performed in triplicates using 0.25 cm^2 buckypaper electrodes and a WaveNow USB Potentio-/Galvanostat (Pine Research Instrumentation Durham, USA). The electrode compartment was filled with 50 ml culture supernatant and the enzyme was allowed to adsorb to the electrode for 1 h with additional aeration under open circuit conditions. The measurements were performed from 20 mV above open circuit potential (OCP) to −244 mV vs. SCE with a rate of 3 mV/s without additional oxygen or air supply. A saturated calomel reference electrode (SCE, Sensortechnik Meinsberg, Ziegra-Knobelsdorf, Germany) was used for measurements.

Polarization curves under quasi-steady-state conditions were recorded using a step-wise galavanostatic technique (Kerzenmacher et al., 2009) and the miniature bioreactor described previously (Kloke et al., 2010). Experiments in half-cell configuration were performed in triplicates at 30 °C using buckypaper electrodes (0.9 cm^{-2}). Before recording the polarization curve, the enzyme was allowed to adsorb at the electrode for 12 h at open circuit. Then the current density was increased in steps of 5.5 $\mu A\,cm^{-2}\,h^{-1}$ until a maximum current density of 225 $\mu A\,cm^{-2}$ was reached (Sane et al., 2013). Electrode potentials were recorded vs. a SCE reference electrode.

Experiments with complete fuel cells were performed using a graphite felt electrode (0.9 cm^2 projected area, 1 cm thickness, supplied by Morgan AM&T, USA) in the anode compartment, together with a buckypaper electrode (0.9 cm^2) in the cathode compartment. As anode enzyme, glucose oxidase (10 U/ml, Sigma–Aldrich, Taufkirchen, Germany) was diluted in acetate buffer (100 mM, pH 5), whereas glucose monohydrate (0.7% (w/v)) served as fuel and (Dimethylaminomethyl)ferrocene (0.2% (w/v)) as mediator. Fuel cell polarization curves were recorded by increasing the current density in steps of 5.5 $\mu A\,cm^{-2}\,h^{-1}$ until a maximum current density of 444 $\mu A\,cm^{-2}$ was reached. Before recording the polarization curve, the enzyme was allowed to adsorb at the electrode until a stable open circuit potential appeared.

3. Results and discussion

3.1. Laccase production in P. sanguineus

Laccases in fungi are often produced only under certain growth conditions. The white-rot fungus *T. versicolor* needs to be cultivated in the synthetic complete laccase medium with 1 mM $CuSO_4$ supplement to produce sufficient amounts of laccase for application in BFCs (Sane et al., 2013). The long incubation period of the culture (2.0 U/ml after 40 days) is also a problem for large-scale production, because filamentous fungi grow in agglomerates and prolonged cultivation can be comparably inefficient.

In comparison to *T. versicolor*, *P. sanguineus* cultures showed high laccase production in a short time period in the absence of inducers. In liquid culture of *P. sanguineus* laccase activity in culture supernatants reached 3.33 U/ml after 10 days of cultivation in standard growth medium (Fig. 1). The activity was 60 times higher than in the culture supernatant of *T. versicolor* after incubation under the same conditions. It was previously shown, that laccases from *T. versicolor* and *P. sanguineus* have similar catalytic

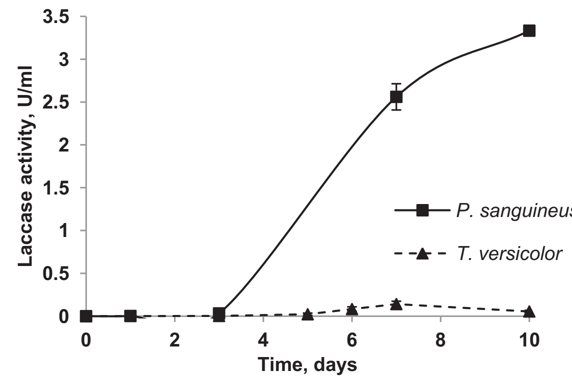

Fig. 1. Laccase activity in liquid culture of *P. sanguineus* compared to *T. versicolor*. Laccase activity in *P. sanguineus* (squares) and *T. versicolor* (triangles) liquid cultures was measured with ABTS assay at pH 5, 25 °C. Standard deviations from three independent experiments for each data point are indicated by error bars.

activity towards ABTS, as indicated for example by K_m values of app. 37.3 μM for *T. versicolor* laccases (Baldrian, 2006) and of 32–33 μM towards ABTS for laccases from *P. sanguineus* (Uzan et al., 2010). Therefore, the higher laccase activity, observed in *P. sanguineus* culture, was the result of the higher laccase production rate by *P. sanguineus* in a non-synthetic cultivation medium. Whereas *T. versicolor* was not able to produce large amount of laccase under these growth conditions.

3.2. Identification of laccase

The high activity of the *P. sanguineus* supernatant in BFCs could be due to a single laccase enzyme or a mixture of several enzymes and/or components. In order to test the two possibilities, we aimed at identifying the specific laccase produced by *P. sanguineus*. The culture supernatant of *P. sanguineus* was electrophoretically analyzed after 3, 5 and 9 days of incubation using SDS–PAGE, and several secreted proteins were detected (Fig. 2a). However, activity staining of the SDS gel with ABTS in an acetate buffer did not indicate any laccase activity (not shown). Therefore native PAGE in the absence of SDS was used for activity staining. A single laccase band was detected under these conditions (Fig. 2b). In the absence of SDS, proteins run in gels according to their pI and mass, which renders protein markers useless to determine protein molecular masses. However, the size of the most prominent protein band in the SDS gel corresponded to the sizes of the known laccases in *Pycnoporus* species and was excised for identification via peptide mass fingerprinting. Using the masses of eight peptides, obtained by trypsin proteolysis, a single protein was detected and identified, with 26% sequence coverage, as a laccase BRFM 902 from *P. sanguineus* (GenBank accession number ACZ37083) (Uzan et al., 2010). The BRFM 902 laccase was obtained from a wild isolate of *P. sanguineus* and had a high redox potential of 729 mV vs. NHE (Uzan et al., 2010), which is comparable with redox potentials of *Trametes* laccases (Christenson et al., 2004). However, in contrast to *Trametes* species, *Pycnoporus* was previously shown to primary expressed laccase with no detectable amounts of lignin peroxidase or manganese peroxidase in the culture medium (Eggert et al., 1996). This fact suggests that laccase could be the main or even only enzyme responsible for electron uptake from a cathode in *Pycnoporus* culture supernatant.

To verify the exact protein sequence, the laccase gene was amplified from cDNA from *P. sanguineus* with specific primers and sequenced. The resulting DNA sequence was translated in a protein sequence and compared to the laccase BRFM 902

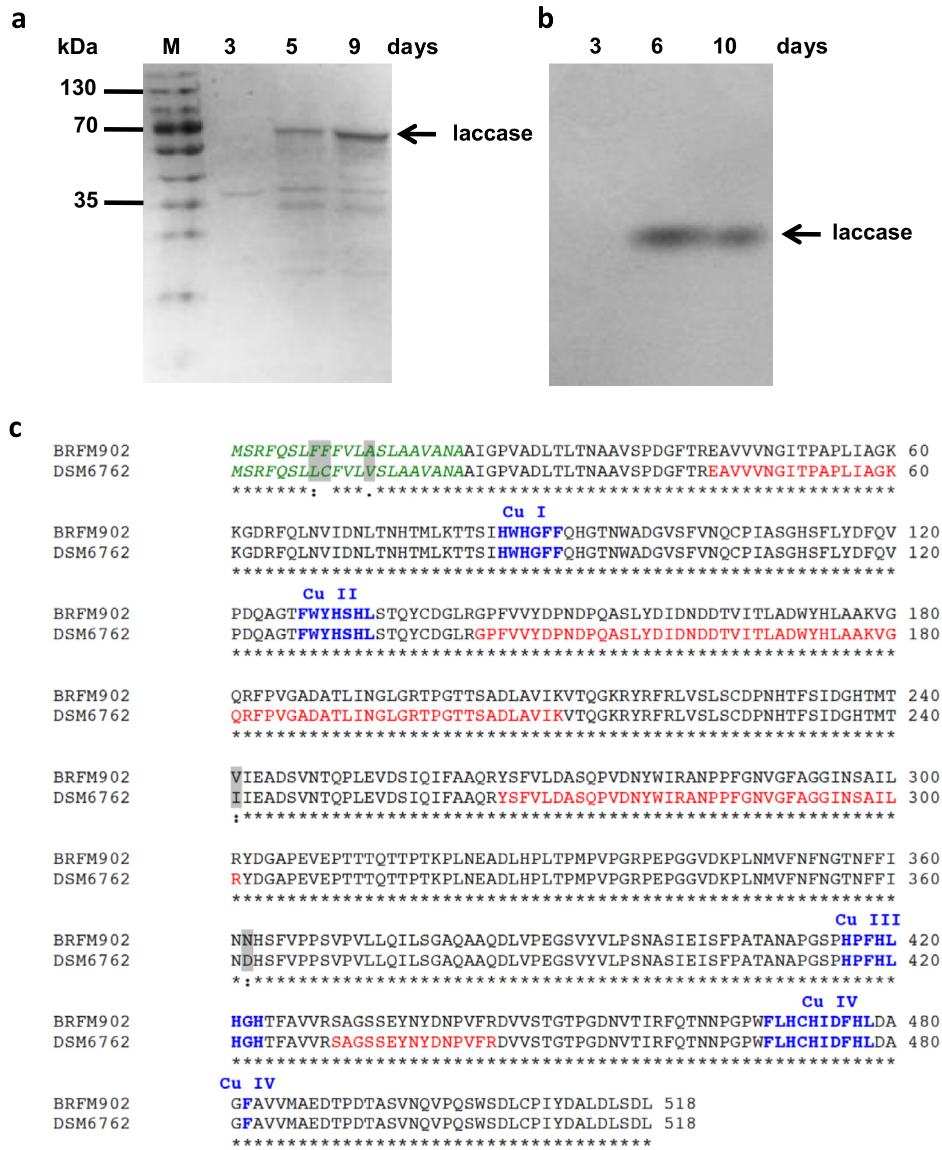

Fig. 2. SDS–PAGE followed by mass spectrometry analysis and native PAGE of crude *P. sanguineus* culture supernatant. (a) Proteins in culture supernatant were analyzed by SDS–PAGE followed by Coomassie staining. The protein band, corresponding to the known laccase size, was excised and used for protein identification by peptide mass fingerprinting. (b) Native PAGE of proteins in culture supernatant followed by activity staining of laccase with ABTS. (c) Comparison of amino sequences of laccase from *P. sanguineus* BRFM 902 (ACZ37083) with the laccase isolated from *P. sanguineus* DSM 6762. The protein sequence of the *P. sanguineus* DSM 6762 was obtained by the translation of the sequenced PCR products. The copper-binding consensus sequences are in bold blue, the signal peptide is in italic green, eight peptides identified by trypsin proteolysis are in red. The differences between sequences are highlighted.

(ACZ37083). As shown in Fig. 2c, the proteins are highly akin, with a few mismatches in the signal peptide sequence and two evolutionarily related mutations with similar residues. The *P. sanguineus* BRFM 902 strain is a wild isolate, therefore these differences to the commercially available strain could be explained by variations that happen in nature over time. This *P. sanguineus* laccase can be assigned to class 3 laccases along with the *T. versicolor* laccase (Eggert et al., 1998). Characteristic for this group is a Phe residue that is located near the carboxyl terminus of the protein (marked in Fig. 2c) and is considered important for coordination of T1 copper in the catalytic center (Xu et al., 1996). Some laccases, for example from *A. nidulans* or *P. ostreatus*, have Leu or Met residue at this position and are assigned to class 2 and 1 accordingly (Eggert et al., 1998; Xu et al., 1996). Though this residue is not solely responsible for the redox potential of T1 copper, an exchange of Met to Leu residue in a laccase from *Pseudomonas aeruginosa* increased the potential of T1 site (Karlsson et al., 1989).

3.3. Effects of temperature and pH on laccase activity and stability

Though the general biochemical characteristics of the purified BRFM 902 laccase, including molecular weight, optimal temperature and pH, stability and others, have already been determined previously (Uzan et al., 2010), proteins and other compounds from culture supernatant could influence the stability and activity of the laccase, when applying crude culture supernatant in biofuel cell. To determine the effect of these factors, ABTS-assays with the unpurified laccase were performed under different conditions.

The laccase in the culture supernatant showed the highest activity at 60 °C (Fig. 3a) and remained stable at 30° and 50 °C after 2 h incubation (Fig. 3b). At 70 °C it lost its activity almost completely after 2 h of incubation (Fig. 3b). Since the BFCs experiments are run at 30 °C, the long-term stability of the laccase in culture supernatant was measured (Fig. 3c). After over night incubation at 30 °C the laccase activity in filtered culture supernatant increased compared to the freshly harvested supernatant. This effect may be explained by the presence of unstable compounds in culture supernatant that hinder the laccase activity and degrade after prolonged incubation. The laccase activity in culture supernatant slowly decreased over time, reaching approximately 50% activity after 7 days. This result is comparable to the half-life of purified laccase from *T. versicolor* dissolved in citrate buffer of pH 5 at room temperature (Rubenwolf et al., 2012).

The optimal pH for most fungal laccases is acidic, though it depends on the substrate (Baldrian, 2006). Laccase from *P. sanguineus* showed highest activity at pH 2.5 and lowest activity at pH 7, which is normally observed in laccase enzyme assays, if ABTS is used as substrate (Heinzkill et al., 1998). The optimal pH of laccases depends on both the difference in redox potentials of the substrate and T1 copper and the binding of a hydroxide anion to T2/T3 coppers, which inhibits the laccase at higher pH (Xu, 1997). At pH 5 the negative effects of hydroxide anion binding did not influence the laccase activity, however at neutral pH values the balance seemed to be shifted towards inhibition of the laccase.

The stability of the laccase from *P. sanguineus* under different pH conditions was tested over night (Fig. 4b). Laccase solution retained 60% of its activity even after incubation at pH 2.5. The highest stability was observed at pH 5 with almost 100% of activity after 12 h of incubation.

3.4. Potentiodynamic electrode characterization

The ability of the laccase from *P. sanguineus* to interact with a buckypaper (BP) cathode was evaluated using linear sweep voltammetry. To compare the cathode performance of the laccase in culture supernatant with the purified enzyme, filtered culture supernatant of *P. sanguineus* was applied to a DEAE-Sepharose column. Laccase was eluted with a linear sodium chloride gradient and detected via the ABTS-assay (not shown). Proteins in the laccase-containing fractions were concentrated, dialysed against 100 mM sodium acetate buffer pH 5 and diluted to 1.8 U/ml. Culture supernatant, flow-through, laccase-containing fractions and laccase solution after dialysis were analyzed using SDS–PAGE (Fig. 5a). Experiments with 1.8 U/ml purified laccase and crude

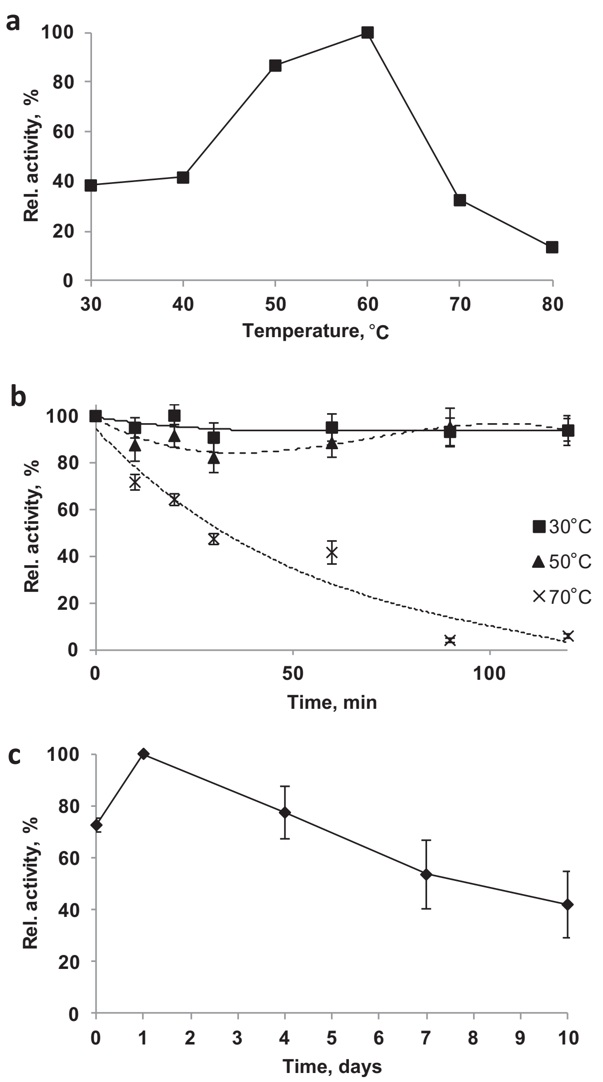

Fig. 3. Effect of temperature on stability and activity of laccase in *P. sanguineus* culture supernatant. (a) Laccase activity in culture supernatant was assayed at 30, 40, 50, 60, 70 and 80 °C. The percentage of laccase activity, using the reaction velocity at 60 °C as 100%, was plotted against the respective temperature values (standard deviations from three independent experiments for each data point are indicated by error bars). (b) Thermostability of the laccase in culture supernatant was determined under standard conditions after incubation at 30, 50 and 70 °C for 0, 10, 20, 30, 60, 90 and 120 min, as indicated. The percentage of laccase activity, using the reaction velocity at time zero as 100%, was plotted against the incubation time (standard deviations from three independent experiments for each data point are indicated by error bars). (c) Long-term stability of the laccase in culture medium at 30 °C was measured after 0, 1, 4, 7 and 10 days. The percentage of laccase activity, using maximal measured reaction velocity after 1 day as 100%, was plotted against the incubation time (standard deviations from three independent experiments for each data point are indicated by error bars).

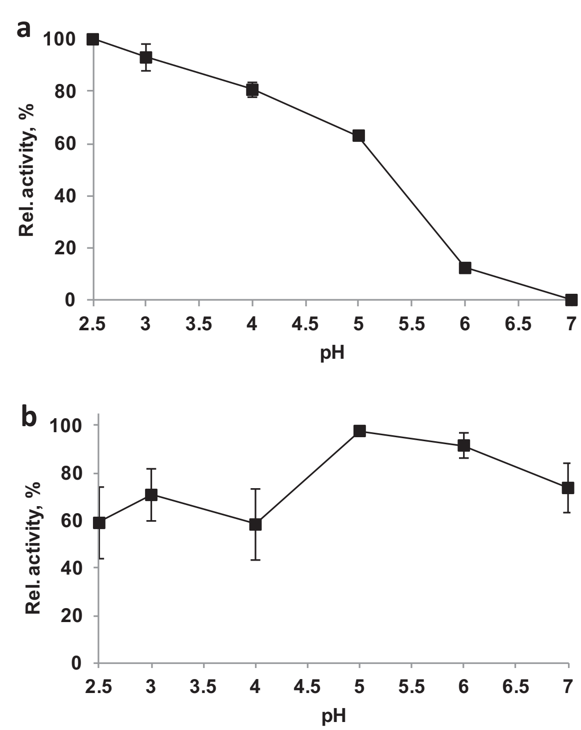

Fig. 4. Effect of pH on the stability and the activity of laccase in *P. sanguineus* culture supernatant. (a) Laccase activity in culture supernatant was assayed in sodium acetate buffer pH 2.5, 3, 4, 5, 6 and sodium phosphate buffer pH 7. The percentage of laccase activity, using the reaction velocity at pH 2.5 as 100%, was plotted against the respective pH values (standard deviations from three independent experiments for each data point are indicated by error bars). (b) Stability of the laccase in culture supernatant under different pH conditions was determined in sodium acetate buffer pH 5 after 12 h incubation of culture supernatant at pH 2.5, 3, 4, 5, 6 and 7. The percentage of laccase activity, using reaction velocity at pH 5 before incubation as 100%, was plotted against the respective pH values (standard deviations from three independent experiments for each data point are indicated by error bars).

laccase-containing culture supernatant at pH 5 showed that the presence of the enzyme raised the open circuit potential of the cathode more than three times compared to the control with medium (Fig. 5b). Laccase was also capable to perform electron transfer from the BP cathode and the oxygen reduction on the cathode, as indicated by the increasing current densities above 400 mV vs. SCE. At 400 mV vs. SCE (644 mV vs. NHE) the current density with laccase-containing culture supernatant was -135.6 ± 6.2 μA/cm^2. Though the onset potential with purified laccase was higher than with culture supernatant, the current density at 400 mV vs. SCE was significantly lower (-61 μA/cm^2) (Fig. 5b). Similarly, it was also previously demonstrated that culture supernatant from *T. versicolor* shows improved performance on the cathode, compared to the purified laccase (Sane et al., 2013). The culture supernatant probably creates a more suitable environment for the laccase electrocatalysis than buffer. For example natural electron mediators (Astolfi et al., 2005) could be secreted by *P. sanguineus* into the culture medium and support electron transfer from the cathode to laccase. At potentials lower than 400 mV the drop in current density could be explained by the electrode reaction being increasingly dominated by masstransfer limitation (Bron, 2008; Hussein et al., 2011a).

Increasing the pH by one unit caused a decrease in the onset potential of oxygen reduction in the range of 30–80 mV, which is close to the theoretically expected value of 59 mV per pH unit (Fig. 5b). This shows that the laccase was still interacting with BP cathode at pH 6 and 7. However, the current density at 400 mV vs. SCE was relatively low (-37.0 ± 8.1 μA/cm^2 and -28.6 ± 4.5 μA/cm^2, respectively). Doubling the amount of laccase in the solution (3.6 U/ml) caused a significant increase in current densities only at pH 5, at pH 6 and 7 the electrochemical activity of the laccase has not improved (Fig. 5c). High pH partially inhibited the activity of the enzyme, probably due to the binding of a hydroxide anion to T2/T3 coppers, which disturbed the electron transfer inside the catalytic center of the laccase (Xu, 1997). The electrocatalytical activity of the laccase in culture supernatants was further characterized by galvanostatic studies.

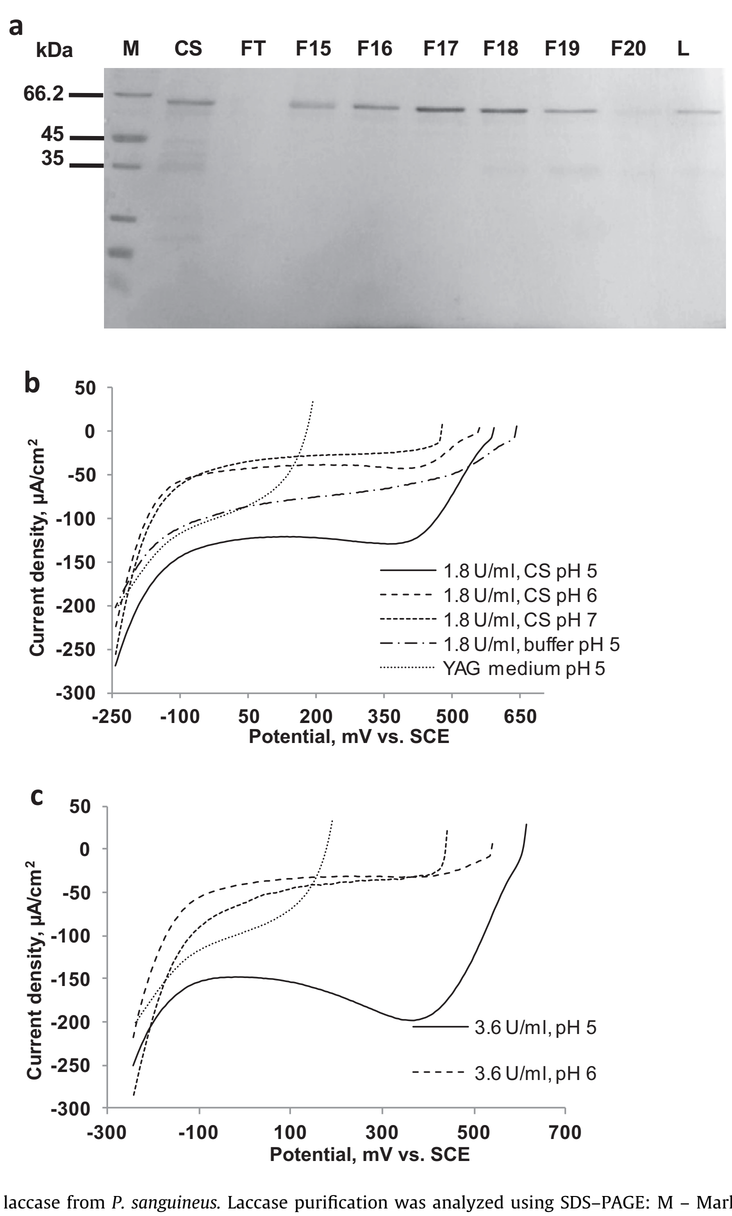

Fig. 5. Linear sweep voltammetry with laccase from *P. sanguineus.* Laccase purification was analyzed using SDS–PAGE: M – Marker, CS – culture supernatant, FT – flow-through, F15–F20 – laccase-containing fractions, L – laccase solution after dialysis (a). The LSV experiments were performed with 1.8 U/ml purified laccase solution in sodium acetate buffer pH 5 (b) and crude culture supernatants containing 1.8 U/ml (b) and 3.6 U/ml (c) laccase at pH 5, 6 and 7, as indicated. YAG culture medium at pH 5 was used as a control. LSV was measured from 20 mV above OCP to -244 mV vs. SCE with a rate of 3 mV s^{-1}. Potentials are shown vs. a SCE reference electrode.

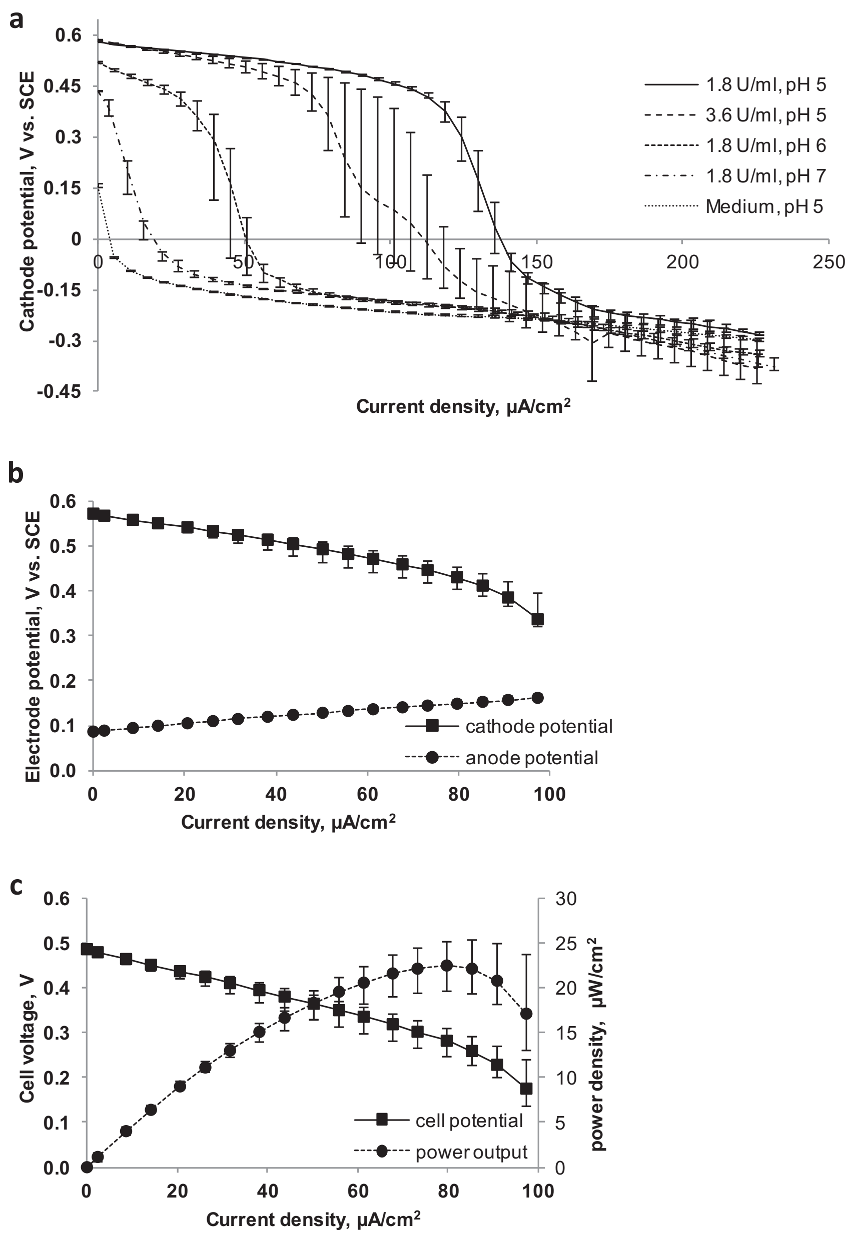

Fig. 6. Polarization curves with laccase-containing crude culture supernatant of *P. sanguineus*. (a) The experiments in half-cell configuration were performed at 30 °C with culture supernatants containing 1.8 U/ml or 3.6 U/ml laccase at pH 5, 6 and 7, as indicated. YAG medium at pH 5 was used as a control. The current density was increased in steps of 5.5 μA cm^{-2} h^{-1} till a maximum current density of 225 μA cm^{-2} was reached. (b and c) Fuel cell polarization curves were measured at pH 5 with 3.6 U/ml culture supernatant. The current density was increased in steps of 5.5 μA cm^{-2} h^{-1} until a maximum current density of 444 μA cm^{-2} was reached. The anode and cathode potentials (b) and power output (c) of the fuel cell were measured. Shown are the average values of triplicate experiments, bars represent maximum and minimum values.

3.5. Cathode polarization curves

The electrochemical activity of the laccase-containing supernatant was analyzed in detail by recording polarization curves of culture supernatant in half-cell configuration with an enzyme activity of 1.8 U/ml and 3.6 U/ml. At pH 5 the culture supernatant from *P. sanguineus* with laccase activity of 1.8 U/ml showed similar results as the culture supernatant of *T. versicolor* (OCP of 612 mV vs. SCE) with the enzyme activity of 3.6 U/ml (Sane et al., 2013). Cathode with *Pycnoporus* laccase showed an OCP of 582 ± 1.5 mV vs. SCE, compared to OCP of 156 ± 6.2 mV vs. SCE without the laccase (Fig. 6a). The electrocatalytic activity of the laccase was high at pH 5, the potential slightly decreasing with increasing current density. A drop in cathode potential occurred around 375 mV vs. SCE (619 mV vs. NHE), its shape resembling the onset of to mass-transport limitation (Hussein et al., 2011a). However, the electrode potential inflects to a value at around −200 mV vs. SCE, which corresponds to the non-catalyzed oxygen reduction at the buckypaper electrode (see the medium control without laccase in Fig. 6a). Therefore, this limitation can be attributed to a limited amount of enzymes connected to the electrode. However, increasing laccase activity to 3.6 U/ml at pH 5 in half-cell configuration did not improve cathode performance (Fig. 6a). In the case of buckypaper cathode laccase molecules are not covalently bound to the cathode material, but rather freely change their position in the solution. It is possible that other compounds in the culture supernatant can influence the ability of the laccase to approach the cathode and perform electron transfer from the cathode to its catalytic center. In the more diluted culture supernatant (1.8 U/ml laccase) the concentration of these components would be lower than in 3.6 U/ml solution, which explains the better electrocatalytic performance of the 1.8 U/ml laccase solution. This effect was not observed in the linear sweep experiments probably due to the shorter time frame of these experiments. In contrast, the step-wise galvanostatic experiments take longer and consequently these components would have more time to aggregate for example on the cathode and hinder the laccase-cathode electron exchange. Also some supernatant components, for example mediators, could degrade over time and would no longer support the laccase activity on the cathode. Therefore, the polarization curves at pH 6 and 7 were recorded with culture supernatants containing 1.8 U/ml laccase. The OCP of the cathode was increased to 521 ± 3.9 mV vs. SCE at pH 6 and 436 ± 1.3 mV vs. SCE at pH 7 in the presence of *P. sanguineus* laccase, demonstrating the interaction between the enzyme and the cathode (Fig. 6a). These values are significantly higher than previously measured OCP of 229 mV vs. SCE at pH 7.4 in the presence of multi copper efflux oxidase CueO, expressed in *Escherichia coli* (Sane et al., 2014).

Experiments with complete fuel cells were performed using the laccase-containing supernatant of *P. sanguineus* with 3.6 U/ml at the cathode and 10 U/ml glucose oxidase at the anode at pH 5. The cathode and anode potentials in the fuel cell are separately shown in Fig. 6b. According to the changes in cathode potential, the cathode showed a polarization behavior that is comparable to the half-cell experiments. The maximum power density of the complete fuel cell was 22.5 ± 2.8 $\mu W/cm^2$ at a current density of 79.7 ± 1.5 $\mu A/cm^2$ (Fig. 6c).

P. sanguineus culture supernatant showed electrocatalytic activity at pH 5, 6 and 7, though the performance was significantly lowered with increasing pH. At 400 mV vs. SCE (644 mV vs. NHE) the current density with laccase-containing culture supernatant at pH 5 was 115.0 ± 3.5 $\mu A/cm^2$, which is comparable to the values recorded for the laccase from *T. versicolor* (129 ± 19 $\mu A/cm^2$, 3.6 U/ml) (Sane et al., 2013). At pH 6 the current density in the presence of laccase-containing supernatant of *P. sanguineus* was 30.1 ± 3.5 $\mu A/cm^2$ and at pH 7 – 2.8 ± 1.5 $\mu A/cm^2$. The laccase activity was apparently partially inhibited at higher pH probably due to the binding of hydroxide anions (Xu, 1997). Polarization curves confirm the result of linear sweep voltammetry and show that in the presence of *P. sanguineus* culture supernatant the high current densities are achieved under application-relevant steady-state conditions.

Though, the electrocatalytic activity of laccase at pH 6 and 7 was low, it may be improved in the future for example by aeration with oxygen instead of air to reduce the effect of hydroxide anions by increasing the molecular oxygen concentration. Also, catalytic activity of laccases at different pH values is positively influenced by the redox potential difference of the T1 copper of laccase and its substrate (Xu, 1997). Increasing concentration of hydroxide anions has an opposite effect and inhibits the laccase activity at higher pH values. A mediator like ABTS may be introduced in BFC to improve laccase performance by improving the electron exchange between the cathode and the enzyme and possibly weakening the effect of hydroxide anions. Though no artificial mediators were added to the culture supernatant prior to the linear sweep voltammetry and the galvanostatic measurements, natural mediators could be secreted by *P. sanguineus* into the culture medium (Astolfi et al., 2005). The availability of the laccase gene opens now the possibility for its heterologous expression and e.g., a molecular evolution approach to further improve its properties.

4. Conclusions

The example of *P. sanguineus* laccase demonstrates that high redox laccases from white-rot fungi other than from the genus *Trametes* can be used in biofuel cells. The *P. sanguineus* laccase-containing culture supernatant significantly increased the cathode performance at pH 5 and showed interaction with the cathode at higher pH values. In contrast to *Trametes*, *P. sanguineus* does not require synthetic growth medium and $CuSO_4$ for induction of laccase. The possibility to use crude culture supernatant and high expression rates in liquid culture reduce the production costs even further and opens new avenues such as the application of BFCs in large-scale applications.

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