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Cell thermolysis – A simple and fast approach for isolation of bacterial laccases with potential to decolorize industrial dyes

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ABSTRACT

Laccases belong to multicopper oxidases and are widespread in nature. Currently, mainly fungal laccases are applied in biotechnological processes. One reason for this is that fungal laccases are much better studied. Compared to fungal laccases, bacterial laccases possess some advantageous characteristics like high stability at elevated temperatures and alkaline pH values. Intracellular recombinant expression of bacterial laccases in *E. coli* makes however downstream processing more complex and time-consuming compared to extracellular expression of fungal enzymes. Here, we demonstrate that cell disruption by cell thermolysis is an efficient and simple method for the isolation and partial purification of recombinant bacterial laccases. Three different laccases, Tth from *Thermus thermophilus*, CotA from *Bacillus subtilis* and Ssl1 from *Streptomyces sviceus*, were used to compare cell disruption by cell thermolysis with sonication and high-pressure homogenization, with and without subsequent heat treatment. Cell thermolysis resulted in high laccase activities per gram of cell wet weight and in the highest specific activities of the laccases. For example, specific activity of Tth laccase after cell thermolysis was 469-fold higher than after sonication. Furthermore, high decolorization activity towards indigo carmine and alizarin red S of these laccases, isolated via cell thermolysis, demonstrate their potential for technical applications.

1. Introduction

Laccases [E.C. 1.10.3.2] belong to the family of multicopper oxidases. They catalyze the oxidation of a broad range of substrates, including mono-, di- and polyphenols, aromatic and aliphatic amines, benzenethiols as well as organic and inorganic metal compounds coupled to the four electron reduction of molecular oxygen to water. Laccase-generated radicals are either further oxidized by laccases or undergo non-enzymatic reactions to form dimers, oligomers or polymers or lead to ring cleavage of aromatic compounds or degradation of polymers. Laccases are widespread in nature and are found in fungi, plants, insects and bacteria [1,2]. Numerous studies demonstrated their potential for biotechnological application in the pulp and paper industry, food industry, biosensors, bioremediation and textile industry, and particularly in the treatment of dye containing wastewater [2–5]. A number of laccases have shown to efficiently decolorize and degrade a broad variety of industrial dyes [6–8]. Today more than 100,000 dyes are commercially available and about 500,000 tons of textile dyes are produced annually [9,10]. Approximately 10–15% of the total dyes are estimated to be released to the environment every year during dye manufacture and textile dyeing [11]. Some of these dyes are highly toxic and mutagenic and can adversely affect photosynthetic activity [9]. Unfortunately, physical and chemical treatment methods of textile wastewater like adsorption, photocatalysis or oxidation processes are quite expensive, generate a significant amount of sludge, due to the incomplete removal of recalcitrant azo dyes, and often involve complicated procedures [12,13]. In contrast, biological treatment methods are less expensive, environmentally-friendly and produce no or less sludge [13]. The use of fungi, yeasts, plants or isolated enzymes for biodegradation of dyes from industrial wastewater has therefore attracted considerable attention [13–15]. However, application of isolated enzymes in effluent treatment might suffer from increased toxicity of reaction products, which should be investigated. Among others, white-rot fungi and their extracellular enzymes such as laccases have been intensively investigated for treatment of dye containing wastewater. However, fungal laccases require mild acidic conditions at temperatures between 30 and 55 °C for optimal activity [15], while industrial wastewater usually have neutral to alkaline

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pH values. Bacterial laccases have some advantageous characteristics, which make them interesting for biotechnological application as well: They can easily be expressed in prokaryotic expression systems because of lacking glycosylation, and are more active and stable at alkaline pH values and higher temperatures than fungal laccases [15]. For example, laccase Tth from *Thermus thermophilus* shows the highest activity at about 92 °C with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate and a thermal half-life of >14 h at 80 °C [16]. Interestingly, bacterial laccases originating from mesophilic microorganisms are also quite thermotolerant. For instance, laccase CotA from *Bacillus subtilis* demonstrates maximal activity at 75 °C with ABTS and has a half-life of 2 h at 80 °C [17], while laccase Ssl1 from *Streptomyces sviceus* has a half-life of 88 min at 60 °C [18]. Both, CotA and Ssl1, show the highest activity towards syringaldazine as substrate at neutral to alkaline pH values (pH 7.0 and 8.0, respectively), while the pH optimum of Tth laccase with syringaldazine is at pH 5.5 [16–18].

Most bacterial laccases could be produced intracellularly in recombinant *E. coli* after truncation of signal sequence to improve their expression [16,18,19]. However, downstream processing of intracellularly produced enzymes includes an additional step of cell disruption and the isolated enzyme preparations contain more foreign proteins as compared to the proteins, for example fungal laccases, isolated after secretory production [20,21].

Herein, we present a new approach based on cell thermolysis enabling simultaneous cell disruption and partial purification of thermotolerant bacterial laccases that were intracellularly expressed in *E. coli*. By using this method, laccases from *T. thermophilus*, *S. sviceus* and *B. subtilis* were successfully isolated from recombinant *E. coli* cells. Furthermore, decolorization of indigo carmine and alizarin red S by the laccases was investigated. Both dyes are used for dyeing textiles. The laccases were shown to efficiently catalyze the decolorization of both dyes under alkaline conditions in the presence of a redox mediator.

2. Material and methods

2.1. Materials and strains

All reagents were of analytical grade or higher and were purchased from commercial sources. Enzymes for molecular cloning were obtained from Fermentas (St. Leon-Rot, Germany). *Bacillus subtilis* 168 (DSM 402) and *Thermus thermophilus* HB27 (DSM 7039) were obtained from DSMZ (Braunschweig, Germany). *E. coli* DH5α (Clontech, Saint-Germain-en-Laye, France) was used for cloning. *E. coli* BL21(DE3) (Novagen, Darmstadt, Germany) was used for expression of CotA and Tth, and *E. coli* BL21-CodonPlus(DE3)-RP (Stratagene, Waldbronn, Germany) for expression of Ssl1.

2.2. Cloning of laccase genes

The *cotA*-gene (NC_000964) was amplified by PCR with the primers CGGGATCCA**AGGAGA**TATACAATGACACTTGAAAAATTTGTGGATG and CCCAAGCTTTTATTTATGGGGATCAGTTATATCC from genomic DNA of *B. subtilis* 168. *Bam*HI and *Hind*III restriction sites introduced via primers are underlined, the ribosomal binding site is shown in bold letters. The purified and restricted PCR product was cloned into pET22H [22]. The *tth*-gene (AE017221.1) was amplified by PCR with the primers GCAACTCCATATGCAAGGCCCTTCCTTCCCCG and CGGGATCCTTAACCCACCTCGAGGACTCCC using genomic DNA of *T. thermophilus* HB27. *Nde*I and *Bam*HI restriction sites are underlined. The *tth*-gene was amplified without its twin arginine translocation signal peptide sequence. After purification and endonuclease treatment the PCR product was cloned into pET-22b(+). The sequences of the inserted genes were verified by sequencing (GATC, Konstanz, Germany).

2.3. Expression and cell harvest

Expression of CotA and Tth was conducted as described earlier [23]. For expression of laccase Ssl1 *E. coli* BL21-CodonPlus(DE3)-RP cells were transformed with pET22-ssl1 [18] and grown in 5 ml LB medium supplemented with ampicillin (100 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}) at 37 °C and 180 rpm for 4 h. 200 ml TB medium supplemented with ampicillin and chloramphenicol was inoculated with 2 ml of the pre-culture and grown at 37 °C and 180 rpm. At an OD_{600} of 1.9, expression was induced by adding 20 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 2 mM copper sulfate and cells were incubated at 19 °C and 140 rpm for 18 h. Afterwards, cells were harvested by centrifugation at 11,325*g* and 4 °C for 20 min. Cell pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.5, supplemented with 0.3 mM copper sulfate and 0.1 mM PMSF.

2.4. Cell disruption

Cell thermolysis was performed in 2 ml reaction tubes in a thermo-shaker. Samples were incubated at 65–95 °C for 20 min at 300 rpm. Cell lysates were cleared by centrifugation at 39,191*g* and 4 °C for 30 min.

Cell disruption by sonication was performed on ice with a Branson Sonifier 250 (3 cycles at Duty Cycle 4, Output Control 40% for 1 min with at least 1 min chilling on ice in between). Cell debris was removed by centrifugation as described above.

Cell disruption by high-pressure homogenization was conducted with a TS Series Bench Top Disruption System 0.75 kW (Constant Systems Ltd., Warwickshire, UK). Samples were processed twice at 1.7 kbar and centrifuged as described above.

Heat treatment was carried out by incubating cell lysates for 15 min at 70 °C (CotA and Tth) and 20 min at 65 °C (Ssl1), respectively. Denatured proteins were removed by centrifugation at 12,300*g* for 10 min.

Protein concentration was determined by using the Bradford method with BSA as standard. Laccase solutions were stored at −20 °C.

2.5. Activity assay

The activity of laccases towards 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 0.5 mM) was determined in 0.1 M sodium acetate buffer, pH 5 with or without externally added Cu^{2+} (2 mM copper sulfate). The oxidation of ABTS was monitored at 420 nm (ε = 36,000 M^{-1} cm^{-1}) and room temperature. One unit is defined as the amount of enzyme that oxidizes 1 μmol of substrate per minute.

Specific activity of laccases (Units (μmol converted substrate per min) per mg of total protein) was determined with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate.

2.6. Dye decolorization

Enzymatic treatment of indigo carmine (50 μM, 23.33 mg L^{-1}) and alizarin red S (100 μM, 34.43 mg L^{-1}) was performed in 50 mM potassium phosphate buffer, pH 7.5 with 10 μM acetosyringone (1.96 mg L^{-1}) and 10 mU ml^{-1} of laccase (determined with the ABTS assay). Optionally, 2 mM copper sulfate was added. Reaction mixtures were incubated at 40 °C and 500 rpm for up to 4 h. Decolorization was determined spectrophotometrically at 608 nm for indigo carmine and at 513 nm for alizarin red S as the relative

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