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Development of an efficient biocatalytic system based on bacterial laccase for the oxidation of selected 1,4-dihydropyridines

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ABSTRACT

Biocatalytic oxidations mediated by laccases are gaining importance due to their versatility and beneficial environmental effects. In this study, the oxidation of 1,4-dihydropyridines has been performed using three different types of bacterial laccase-based catalysts: purified laccase from *Bacillus licheniformis* ATCC 9945a (*Bli*Lacc), *Escherichia coli* whole cells expressing this laccase, and bacterial nanocellulose (BNC) supported *Bli*Lacc catalysts. The catalysts based on bacterial laccase were compared to the commercially available *Trametes versicolor* laccase (*Tv*Lacc). The oxidation product of 2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate was obtained within 7–24 h with good yields (70–99%) with all three biocatalysts. The substrate scope was examined with five additional 1,4-dihydropyridines, one of which was oxidized in high yield. Whole-cell biocatalyst was stable when stored for up to 1-month at 4 °C. In addition, evidence has been provided that multicopper oxidase CueO from the *E. coli* expression host contributed to the oxidation efficiency of the whole-cell biocatalyst. The immobilized whole-cell biocatalyst showed satisfactory activity and retained 37% of its original activity after three biotransformation cycles.

1. Introduction

Regarded as a green and sustainable technology, biocatalysis has found numerous applications in organic synthesis [1]. Whether in pure form, as part of a cell lysate, or whole cells, enzymes are increasingly being developed as biocatalysts in industrial settings [2]. As such, they can provide substantial reduction of the cost of goods, number of synthetic steps and environmental impact, as well as improved safety and selectivity [3].

Laccases (EC 1.10.3.2, benzenediol: oxygen oxidoreductases) belong to the multicopper oxidase family, and catalyze the oxidation of a wide array of compounds coupled to the four-electron reduction of molecular oxygen to water [4]. Due to the fact that these enzymes only require oxygen as a co-substrate, and they release water as the only by-product laccases have received much interest as suitable biocatalysts in various

waste valorization efforts [5] and other biotechnological applications [6,7]. Numerous reports point out the utility of laccases in water remediation, including the biodegradation of dyes and pharmaceuticals [8–10]. Laccases have a wide substrate range, which can be further extended by the use of laccase–mediator systems. The most common substrates of laccases are phenols, however aromatic amines can also be oxidized by laccases [11]. The substrate scope of laccase is expanded by the use of mediators such as 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or 1-hydroxybenzotriazole (HBT), which act as diffusible electron carriers [12]. Some notable laccase-catalyzed transformations include oxidations of C–O [13–15] and C–N bonds [16–18], aminations [19,20], oxidative dimerizations [21,22] and rearrangements [23,24]. Laccases are present in higher plants and fungi, as well as in some bacteria and insects. Compared with fungal enzymes, which are frequently used as biocatalysts, bacterial laccases are often more

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active and stable against temperature and pH variations [11]. Additionally, bacterial laccases are routinely expressed in prokaryotic hosts unlike fungal laccases due to the need of post-translational glycosylation [25,26].

Due to growing environmental concerns, along with recent advancements in the field of biocatalysis, enzymes are being used increasingly in many industrial production processes. However, the purification of enzymes on a larger scale is often deemed resource intensive and expensive [27]. One method to ameliorate this is via enzyme immobilization where novel and sustainable biomaterials, such as bacterial nanocellulose (BNC) are coming into focus [28]. Another approach towards the reduction of the overall costs of the biocatalytic processes is the use of whole-cell catalysts, as it omits expensive purification steps and it also allows cofactor regeneration, if needed [27].

The pyridine moiety represents an important heterocyclic scaffold for numerous natural products, pharmaceuticals, dyes, as well as ligands used in organic chemistry [29]. The oxidation of 1,4-dihydropyridines, obtained by Hantzsch synthesis [30] is one of the most straightforward methods for the synthesis of pyridines. Although a number of catalytic oxidative aromatizations of 1,4-dihydropyridines using molecular oxygen as the oxidant have been reported, many of these methods rely on transition metals, employ organic solvents and require high reaction temperatures [31,32]. Thus far, two reports on the enzyme-mediated oxidation of 1,4-dihydropyridines are available, using purified *Trametes versicolor* laccase (TvLacc) and aerial oxygen, under mild conditions [33,34].

The aim of this study was to develop a whole-cell catalyst and an immobilized biocatalytic system containing bacterial laccase. The biocatalysts developed during this work were applied in the oxidation of 1,4-dihydropyridines. Recombinant laccase from *Bacillus licheniformis* ATCC 9945a (BliLacc) was selected due to its high thermal stability and tolerance to organic co-solvents [35]. To further increase the economic viability of BliLacc by circumventing enzyme purification, whole cells containing this enzyme were tested for their catalytic activity. Both biocatalysts, the purified enzyme and whole cells, were immobilized onto bacterial nanocellulose (BNC) and their catalytic activity in the transformation of 1,4-dihydropyridines was also examined. Furthermore, the substrate scope of BliLacc was explored by using several substituted 1,4-dihydropyridines.

2. Materials and methods

2.1. Reagents and chemicals

Unless stated otherwise, solvents and other reagents were obtained from commercial sources and used without further purification. Compounds: diethyl-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (DHPy1) [36], diethyl 2,6-dimethyl-4-phenyl-1,4-dihydropyridine-3,5-dicarboxylate (DHPy2) [33], diethyl 2,6-dimethyl-4-propyl-1,4-dihydropyridine-3,5-dicarboxylate (DHPy3) [37], 1,1'-(2,6-dimethyl-1,4-dihydropyridine-3,5-diyl)diethanone (DHPy4) [33], 3,3,6,6-tetramethyl-3,4,6,7,9,10-hexahydroacridine-1,8(2H,5H)-dione (DHPy5) [38], and 3,3,6,6-tetramethyl-9-phenyl-3,4,6,7,9,10-hexahydroacridine-1,8(2H,5H)-dione (DHPy6) [38] were prepared according to previously reported procedures.

All media components including peptone, yeast extract, agar, sugars, citric acid, and inorganic salts were purchased either from Oxoid (Cambridge, UK), Becton Dickinson (Sparks, MD, USA), or Sigma Aldrich (Munich, Germany).

2.2. Analytical methods

Dry-flash chromatography was performed on SiO₂ (0.018–0.032 mm). Reaction progress was monitored by thin-layer chromatography (TLC Silica gel 60 F₂₅₄ (Merck, Darmstadt)). IR spectra were recorded on a Thermo-Scientific Nicolet 6700 FT-IR Diamond

Crystal instrument. ¹H and ¹³C NMR spectra were recorded on a Bruker Ultrashield Avance III spectrometer (at 500 and 125 MHz, respectively) using CDCl₃ (unless stated otherwise) as the solvent and tetramethylsilane (TMS) as an internal standard. Chemical shifts are expressed in parts per million (ppm) on the (δ) scale. Chemical shifts were calibrated relative to those of the solvent.

2.3. Origin of enzymes and microorganisms

Trametes versicolor laccase (1.07 U mg⁻¹; activity units were assessed against catechol) was purchased from Sigma Aldrich (St. Louis, MO, USA). *B. licheniformis* ATCC 9945a™ laccase (BliLacc) was recombinantly expressed and characterized as previously reported (0.8 U mg⁻¹; activity units were assessed against catechol) [35]. The gene encoding CueO (PDB: 3PAU) was amplified by using *E. coli* TOP10 (Thermo Fisher Scientific, Waltham, MA, USA) genomic DNA as template. Primers were designed with *Bsa*I sites to facilitate the Golden Gate cloning into pBAD-adapted vectors. Primers and plasmid maps are available upon request.

2.4. Expression of BliLacc and CueO

E. coli TOP10 was used as the expression host for the *Lacc* gene (*cotA*) from *B. licheniformis*

ATCC®9945a™ (American Type Culture Collection) as previously described [35]. Briefly, overnight culture was diluted (1%, v/v inoculum) into terrific broth (TB, Merck) medium containing ampicillin (100 g/ml) and CuSO₄ at final concentration of 2 mM and after reaching OD₆₀₀ = 0.5 expression was induced with 0.02% (w/w) arabinose (final concentration) and carried out for 72 h at 17 °C with shaking at 180 rpm. CueO was expressed following the same procedure as for BliLacc. Both recombinant proteins contained polyhistidine affinity tags and were purified using immobilized metal affinity chromatography (IMAC) following the manufacturer's recommendations (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Bacterial nanocellulose (BNC) production, immobilization of biocatalysts and characterization

2.5.1. BNC production procedure

BNC was produced, dried and characterized as described previously [39] using *Komagataeibacter medellinensis* ID13488 (CECT 8140 (Spanish Type Culture collection)). Briefly, the strain was cultivated in a standard Hestrin–Schramm medium (HS) containing 20 g/l glucose, 5 g/l peptone, 5 g/l yeast extract, 2.5 g/l Na₂HPO₄ and 1.15 g/l citric acid [40], at pH 4.5 under static conditions for 10 days. Bacterial nanocellulose discs were obtained from growth, which was performed in 30 ml bottles with an 18 mm diameter (approximately 2.5 cm² air-liquid surface area). These were treated with KOH (1 M), extensively washed with deionized H₂O until a pH of 7.0 was reached and were then dried under warm air at 45 °C for 6–8 h.

2.5.2. Procedure for the immobilization of enzymes

5 mg of the corresponding laccase were added to a vial and subsequently dissolved in 0.05 ml of acetate buffer (0.1 M, pH 4.5). A dried BNC disk (average mass 10.5 mg) was immersed in this solution and kept at 30 °C for 6 h. The entire volume of the solution was absorbed by the disk, and was used without further manipulation in the oxidation of diethyl 2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (DHPy1).

2.5.3. Procedure for the immobilization of *E. coli* cells

A suspension of *E. coli* (0.6 ml; OD₆₀₀ = 150) in acetate buffer (0.02 M, pH 4.5) was added to a vial. A BNC disk (average mass 8.5 mg) was immersed in the suspension and shaken at 30 °C for 3 h at 100 rpm until the most of the volume was absorbed. The disk was removed from the suspension with tweezers, briefly rinsed with buffer and used

without further manipulation in the oxidation of diethyl 2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (**DHPy1**). The efficiency of cell immobilization was assessed by enumeration of cells (colony forming units were enumerated after plating serial dilutions on agar plates and incubation at 37 °C for 24 h) before and after incubation with nanocellulose as previously described [41].

2.5.4. BNC-immobilized catalysts characterization

Scanning Electron Microscopy (SEM). Scanning electron micrographs were obtained by a high resolution field emission Zeiss Ultra Plus-SEM (Carl Zeiss AG, Oberkochen, Germany) using an InLens detector with an accelerating voltage of 5 kV at a working distance of 5 mm. Prior to imaging, samples were sputtered with gold/palladium (80/20 ratio) for 10 s.

Fourier transform infrared spectroscopy (FTIR). The BNC, BNC-BliLacc and BNC-*E. coli* (BliLacc) samples were characterized through FTIR using a Perkin Elmer Spectrum 100 (Bruker, Germany) instrument in the wavenumber range of 650 - 4000 cm^{-1} at a resolution of 4 cm^{-1} .

2.6. Biocatalytic reactions setup

2.6.1. General procedure for the laccase-catalyzed oxidation of 1,4-dihydropyridines

In a 25 ml round-bottom flask, 1,4-dihydropyridines (final concentration 26 mM) were suspended in a mixture of acetate buffer (0.1 M, pH 4.5, 2 ml) and methanol (0.6 ml). Subsequently, ABTS diammonium salt (final concentration 0.35 mM), dissolved in 1 ml of acetate buffer, and laccase (4–5 U, see Supplementary information), dissolved in 1 ml of acetate buffer, were added to the suspension. The flask was connected to a reflux condenser and the reaction mixture was heated to the denoted temperature under constant stirring. After extraction with CH_2Cl_2 (3×10 ml), the combined organic phases were dried over anhydrous Na_2SO_4 , and the solvent was removed under reduced pressure. The crude products were purified by dry-flash chromatography (SiO_2 ; hexane/ethyl acetate, see Supporting information for details).

2.6.2. General procedure for the oxidation of 1,4-dihydropyridines catalyzed BNC-supported laccase

In a 25 ml round-bottom flask, 1,4-dihydropyridines (final concentration 26 mM) were suspended in a mixture of acetate buffer (0.1 M, pH 4.5, 3 ml) and methanol (0.6 ml). Subsequently, ABTS diammonium salt (final concentration 0.35 mM), dissolved in 1 ml of acetate buffer, and BNC-supported laccase (4–5 U, see Supplementary information) were added to the suspension. The flask was connected to a reflux condenser and the reaction mixture was heated at the denoted temperature under constant stirring. After extraction with CH_2Cl_2 (3×10 ml), the combined organic phases were dried over anhydrous Na_2SO_4 , and the solvent was removed under reduced pressure. The crude products were purified by dry-flash chromatography (SiO_2 ; hexane/ethyl acetate).

2.6.3. General procedure for the oxidation of 1,4-dihydropyridines catalyzed by the *E. coli* whole-cell suspension

In a 25 ml round-bottom flask, 1,4-dihydropyridines (final concentration 11 mM) were suspended in a mixture of acetate buffer (0.1 M, pH 4.5, 4 ml) and methanol (0.25 ml). Subsequently, 0.5 ml of an ABTS diammonium salt solution in acetate buffer (final concentration 0.17 mM), 0.5 ml of a copper(II)-sulfate solution in acetate buffer (final concentration 0.18 mM), and 0.2 ml of an *E. coli* suspension in acetate buffer ($\text{OD}_{600} = 150$), were added to the reaction mixture. The flask was connected to a reflux condenser and the reaction mixture was heated at the denoted temperature under constant stirring. After extraction with CH_2Cl_2 (3×10 ml), the combined organic phases were dried over anhydrous Na_2SO_4 , and the solvent was removed under reduced pressure. The crude products were purified by dry-flash

chromatography (SiO_2 ; hexane/ethyl acetate) (**Py1**) or their conversion was determined by NMR spectroscopy (**Py2-Py6**).

2.6.4. General procedure for the oxidation of 1,4-dihydropyridines catalyzed by the BNC-supported *E. coli*

In a 25 ml round-bottom flask, 1,4-dihydropyridines (final concentration 11 mM) were suspended in a mixture of acetate buffer (0.1 M, pH 4.5, 4 ml) and methanol (0.25 ml). Subsequently, 0.5 ml of an ABTS diammonium salt solution in acetate buffer (final concentration 0.17 mM), 0.5 ml of a copper(II)-sulfate solution in acetate buffer (final concentration 0.18 mM), and BNC-supported *E. coli* ($\text{OD}_{600} = 150$), were added to the reaction mixture. The flask was connected to a reflux condenser and the reaction mixture was heated at the denoted temperature under constant stirring. After extraction with CH_2Cl_2 (3×10 ml), the combined organic phases were dried over anhydrous Na_2SO_4 , and the solvent was removed under reduced pressure. The crude products were purified by dry-flash chromatography (SiO_2 ; hexane/ethyl acetate) (**Py1**) or their conversion was determined by NMR spectroscopy (**Py2-Py6**).

2.6.5. Recycling of the BNC-supported *E. coli* in the oxidation of diethyl 2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate

In a 25 ml round-bottom flask, diethyl 2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (final concentration 2.9 mM) was suspended in a mixture of acetate buffer (0.1 M, pH 4.5, 9 ml) and methanol (0.25 ml). Subsequently, 0.5 ml of an ABTS diammonium salt solution in acetate buffer (final concentration 0.044 mM), 0.5 ml of a copper(II)-sulfate solution in acetate buffer (final concentration 0.049 mM), and BNC-supported *E. coli* ($\text{OD}_{600} = 150$), were added to the reaction mixture. The flask was connected to a reflux condenser and the reaction mixture was heated at 50 °C for 24 h, under constant stirring. The disk was removed from the reaction mixture, washed with acetate buffer (0.1 M, pH 4.5) and stored at 4 °C before reuse. The remaining mixture was extracted with CH_2Cl_2 (3×10 ml), the combined organic phases were dried over anhydrous Na_2SO_4 , and the solvent was removed under reduced pressure. The reaction conversion was determined by NMR spectroscopy.

2.7. Molecular analysis of proteins

A homology model of BliLacc was constructed using the Phyre2 server (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>). The homology model was based on the 2 Å resolution X-ray structure of a *Bacillus subtilis* laccase (66.14% sequence identity) with a pdb code 2 × 87. Pictures were generated using PyMOL software (<http://www.pymol.org/>).

2.8. Statistical analysis

All the experiments in this study were performed in triplicates unless otherwise noted. An analysis of variance (ANOVA) was carried out using the SAS program version 8.1 (SAS Institute Inc., Cary, NC, USA) and $p < 0.05$.

3. Results

3.1. Oxidation of DHPy1 by purified laccases from *Trametes versicolor* (TvLacc) and *Bacillus licheniformis* (BliLacc)

Prompted by the successful application of TvLacc in the oxidation of 1,4-dihydropyridines [33], this study was initiated with a view to further explore this transformation in terms of enzyme and substrate scope as well as general resource and cost-effectiveness. Firstly, modification of the reported reaction conditions in the transformation of **DHPy1** (Fig. 1a) led to an improvement in terms of the amount of enzyme necessary to achieve high yield as well as shorter reaction time

Fig. 1. Chemical structure and biocatalytic oxidation of Hantzsch 1,4-dihydropyridines using bacterial laccase-based biocatalysts. Diethyl-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (**DHPy1**) was transformed into diethyl 2,6-dimethylpyridine-3,5-dicarboxylate (**Py1**) (**a**) and 1,4-dihydropyridine (**DHPy2**–**DHPy6**) substrates used in this study (**b**). All substrates except **DHPy6** gave corresponding pyridines.

Table 1

Optimization of the reaction conditions for the laccase-catalyzed oxidation of **DHPy1**. Reaction was performed in 100 mM acetate buffer (pH 4.5), in the presence of 13% MeOH (v/v) and ABTS (1.5 mol%).

Entry	Purified laccase	Enzyme (U)	Time (h)	Temperature (°C)	Yield (%) ^a
1	TvLacc	20	2	50	95
2	TvLacc	5	2	50	83
3	TvLacc	5	4	50	94
4	TvLacc	5	7	37	89
5	TvLacc	2.5	2	50	74
6	TvLacc	2.5	4	50	84
7 ^b	BliLacc	4	2	50	94
8 ^b	BliLacc	4	2	37	82
9 ^b	BliLacc	4	4	37	85
10 ^{b,c}	BliLacc	4	4	50	36
11 ^d	–	–	4	50	0
12 ^e	–	–	4	50	0

^a Isolated yield of the product.

^b CuSO₄ was added during the production of the recombinant protein.

^c Reaction run in the absence of ABTS.

^d Absence of enzyme and ABTS.

^e Absence of enzyme, ABTS added.

(Table 1). The enzyme activity reported as optimal in the aforementioned report afforded **Py1** in 95% isolated yield in 2 h at 50 °C (Table 1, Entry 1). Methanol was added to the reaction mixture to improve the solubility of the substrate and it was kept at 13% (v/v). A twofold reduction in enzyme amount had shown little impact on the reaction yield, and subsequently, a fourfold decrease in enzyme was shown to be possible with the retention of high yield, albeit with a reaction time of 4 h (Table 1, Entry 3). A decrease in reaction temperature to 37 °C was confirmed to be suboptimal (Table 1, Entry 4). The replacement of TvLacc by BliLacc delivered the same isolated yield of product, with 4 U being sufficient with a reaction time of 2 h (Table 1, Entry 7). Lowering the reaction temperature to 37 °C resulted in a decrease in the product yield after both 2 h and in 4 h (Table 1, Entry 8 and 9). The presence of ABTS as a mediator was proven to be

beneficial for the displayed activity of BliLacc towards **DHPy1**, with its absence causing a drop in yield by nearly 60% (Table 1, Entry 10). Thus, it was shown that bacterial laccase could successfully mediate the oxidation of **DHPy1**. The obtained results, along with the relative facility of expression of bacterial laccases in *E. coli* hosts, provided encouragement towards the development of a whole-cell catalytic system for the oxidation of 1,4-dihydropyridines.

3.2. Oxidation of **DHPy1** catalyzed by whole cells *E. coli* (BliLacc)

The catalytic activity of the *E. coli* (BliLacc) strain in the oxidation of **DHPy1** was investigated under the conditions summarized in Table 2. The cell suspension with an optical density of 150 at 600 nm (OD₆₀₀) was used in the initial reaction with the ratio of substrate to cell dry weight (CDW) being 0.57 mmol/g (6 ml of cell suspension). Under the described conditions the full conversion of **DHPy1** was obtained after 7 h at 50 °C (Table 2, Entry 1). Rather pleasingly, decreasing the

Table 2

Optimization of the reaction conditions for the *E. coli* (BliLacc)-catalyzed oxidation of **DHPy1**. Reaction was performed in 100 mM acetate buffer (pH 4.5), in the presence of 5% MeOH (v/v).

Entry	Substrate amount/CDW (mmol/g)	ABTS (mol %)	CuSO ₄ (mol %)	Time (h)	Temperature (°C)	Yield (%) ^a
1 ^b	0.57	1.6	1.7	7	50	97
2	0.57	1.6	1.7	24	37	78
3 ^b	8.55	1.6	1.7	24	50	97
4 ^b	8.55	1.6	1.7	7	50	97
5	8.55	–	1.7	24	50	65
6	8.55	1.6	–	24	50	56
7	8.55	–	–	24	50	20
8 ^c	–	1.6	1.7	24	50	0

^a Isolated yield of product.

^b Full conversion of starting material.

^c Control reaction.

amount of CDW by a factor of fifteen, compared to the initial reaction, did not lead to a loss in the reaction yield (Table 2, Entry 4). Thus, it was established that only 0.2 ml of an *E. coli* (*BliLacc*) suspension ($OD_{600} = 150$), which is equal to 7 mg of cell dry weight, was sufficient to drive the oxidation of 0.06 mmol of **DHPy1** to completion. The absence of ABTS in the reaction mixture was confirmed to be unfavorable (Table 2, Entry 5), analogous to the reaction catalyzed by the purified enzyme (Table 1, Entry 10). The reaction temperature of 37 °C was established to be suboptimal in the case of the whole-cell catalytic system, as well (Table 2, Entry 2). The presence of $CuSO_4$ was shown to have a significant impact on the catalytic activity of the whole cells expressing recombinant laccase *E. coli* (*BliLacc*). The absence of $CuSO_4$ reduced the product yield by 1.7-fold (Table 2, Entry 6). Running the reaction in the absence of *E. coli* (*BliLacc*) cells confirmed that the presence of biocatalyst was essential for the oxidation of **DHPy1** (Table 2, Entry 8).

As a means of determining the stability of *E. coli* (*BliLacc*) as a catalyst over time, the effect of storage time of the cell suspension on the yield of the oxidation of **DHPy1** was examined. The cell suspension was kept at the temperature of 4 °C. It was established that *E. coli* (*BliLacc*) retained its activity after 30 days, providing full conversion in the oxidation of **DHPy1** (Fig. 2).

3.3. Oxidation of **DHPy1** catalyzed by *E. coli* TOP10 whole cells

In order to test the possible background reaction by the host enzymes, a control reaction was run using *E. coli* TOP10 cells without any plasmid for the expression of additional genes. Notably, *E. coli* cells displayed activity towards the conversion of **DHPy1** (39% in 7 h, 99% in 24 h, Table 3, Entry 1). Although requiring significantly longer reaction time, this method avoids the entire process of recombinant protein production, including the addition of antibiotics in the production medium. The dependence of reaction yield on the presence of ABTS and $CuSO_4$ was investigated for this catalytic system, as well. Although a decrease in yield was observed upon the removal of ABTS, as well as $CuSO_4$ (Table 3, Entry 4 and 5), in the absence of both simultaneously, a fair yield of 58% was observed (Table 3, Entry 6). This compares favorably with the result presented in Table 2 (Entry 9), where *E. coli* (*BliLacc*) provided a 20% yield under the same conditions.

A genome search of the *E. coli* TOP10 strain revealed the presence of multicopper oxidase CueO involved in copper homeostasis and tolerance in this bacterium [42]. This enzyme was reported to have a functional role as a cuprous oxidase [43]. To test if CueO could indeed be the active species, the gene for CueO was cloned in the pBAD vector, expressed and purified using IMAC chromatography. The expressed CueO multicopper oxidase was isolated, purified and tested as the

Table 3

Oxidation of **DHPy1** using *E. coli* TOP10 cells. Reaction was performed in 100 mM acetate buffer (pH 4.5), in the presence of 5% MeOH (v/v).

Entry	Substrate amount/CDW (mmol/g)	ABTS (mol %)	$CuSO_4$ (mol %)	Temperature (°C)	Time (h)	Yield (%) ^a
1	8.55	1.6	1.7	50	7	39
2 ^b	8.55	1.6	1.7	50	24	95
3	8.55	1.6	1.7	37	24	67
4	8.55	–	1.7	50	24	72
5	8.55	1.6	–	50	24	60
6	8.55	–	–	50	24	58

^a Isolated yield of product.

^b Full conversion of starting material.

catalyst in the oxidation of **DHPy1**, utilizing optimal conditions displayed in Table 1 for *BliLacc*. Surprisingly, quantitative conversion of the starting material to **Py1** was observed by NMR in the same time-frame (results not shown). Additionally, *E. coli* TOP10 whole cells expressing CueO were also tested as a catalyst under optimal conditions displayed in Table 2. Again, quantitative conversion of the starting material to the desired product was confirmed by NMR within shorter time-frame in comparison to *E. coli* TOP10 cells without the plasmid (7 h in comparison to 24 h), indicating that indeed CueO multicopper oxidase contributes to the catalytic activity of the expression host in the oxidation of **DHPy1**. The general protein structure fold is conserved among laccases from *T. versicolor*, *B. licheniformis* and *E. coli* CueO and the overall structure of three proteins appears to be similar (Fig. 3).

3.4. Substrate scope

Encouraged by the success of the three catalytic systems (*BliLacc*, *E. coli* (*BliLacc*), *E. coli* TOP10) in the oxidation of **DHPy1**, the investigation was extended to include an additional five different 1,4-dihydropyridines, **DHPy2**–**DHPy6** (Fig. 1b). It was shown that substrates possessing bulky substituents in the 4-position (**DHPy2**, **DHPy3**) and overall sterically demanding substrates (**DHPy5**, **DHPy6**) were not transformed to the corresponding products, or their transformation provided poor yield (Table S1). Possessing greater steric resemblance to **DHPy1**, substrate **DHPy4** was oxidized quantitatively. To establish if the possibility of diminished substrate diffusion into the cell contributed to low conversion of bulky substrates, the transformation of **DHPy2**–**6** was attempted with purified *BliLacc* with higher conversions of **DHPy2**, **DHPy3**, and **DHPy5** to the corresponding substituted pyridines noted with the purified enzyme (Supporting information), though still remaining far under those obtained in the oxidation of **DHPy1** and **DHPy4**. The previously described trend in reactivity of substrates **DHPy2**–**DHPy6** was also obtained with *E. coli* TOP10 as the catalyst. Taken together, the substrate scope was better for the purified *BliLacc* in comparison to whole-cell biocatalyst.

3.5. Oxidation catalyzed by BNC-supported biocatalysts

The possibility of immobilization of purified laccases and whole cells utilized in this study on BNC was investigated. The straightforward physical adsorption approach has been applied for immobilization of the biocatalysts.

BliLacc and *E. coli* (*BliLacc*) were immobilized on BNC, and the morphology of the obtained materials was analyzed by scanning electron microscopy (SEM) (Fig. 4). The SEM images confirmed a change in surface morphology upon adsorption (Fig. 4). This was further corroborated by FTIR spectra of BNC, BNC - *BliLacc* and BNC - *E. coli* (*BliLacc*) shown in Fig. 5. The characteristic absorption bands appearing at 3345 cm^{-1} and 2895 cm^{-1} can be attributed to O–H stretching and C–H stretching vibrations, respectively. The absorption band around

Fig. 2. The effect of storage time of *E. coli* (*BliLacc*) at 4 °C on the yield of oxidation of **DHPy1**. The reactions were run with 0.06 mmol of the substrate, at 50 °C for 7 h. ^a Full conversion of starting material.

Fig. 3. Comparison of the structures of laccases from *Bacillus subtilis* (CotA), *E. coli* (CueO) and *Trametes versicolor*. The conservation of the fold in all three enzymes is evident in both overall structure (left panel) and in the active site architecture (b). PDB codes: CueO (3PAU) – red, BliLacc model based on CotA (2×87) – green, *Trametes versicolor* laccase (1KYA) – blue, in complex with xylydine (blue spheres); Copper ions are presented as orange spheres (right panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

1050 cm^{-1} can be attributed to the C–O stretching vibration. In the BNC - BliLacc sample, bands observed at 1640 cm^{-1} and 1530 cm^{-1} were assigned as Amide I and Amide II bands, indicating the presence of a protein on the BNC surface. Purified TvLacc as well as expression host, *E. coli* TOP10 have also successfully been immobilized on BNC (results not shown) and all immobilized biocatalysts were subsequently used as catalysts in the oxidation of DHPy1 under conditions outlined in Table 4.

The immobilization of purified enzymes was shown to have a negative impact on the activity, which is reflected in a longer reaction time, although good product yields were attainable (Table 4, Entry 1 and 2). Likewise, the immobilization of bacterial cells enabled good conversion of the starting material after 24 h (Table 4, Entry 3 and 4). Providing the highest yield, without the need for enzyme purification, BNC-supported *E. coli* (BliLacc) was selected as the optimal catalytic system and its reusability was subsequently investigated. The initial reaction run afforded quantitative conversion of the substrate (Fig. S1). However, after the first recycling, the conversion dropped to 69%, and after the second recycling, to 37% of the original activity (Fig. S1). Notably, biocatalyst detachment during the reaction was observed (results not shown).

4. Discussion

In this study, recombinant bacterial laccase, BliLacc from *B. licheniformis* was successfully applied in the preparative oxidation 1,4-dihydropyridines. Given the superior thermal stability as well as solvent tolerance of BliLacc [35] this was an important finding, having in mind the amenability of bacterial enzymes for further recombinant tailoring. The fact that the expression of bacterial laccases in *E. coli* hosts is also less difficult and more cost-effective, provided encouragement towards the development of a whole-cell catalytic system for the oxidation of 1,4-dihydropyridines based on the bacterial laccase with a view to further lower the resource intensity of the process.

For the first time whole-cell biocatalysts were efficiently employed for the oxidation of 1,4-dihydropyridines under relatively mild conditions (Table 2), thus surpassing the chromatographic protein purification step. Furthermore, the *E. coli* (BliLacc) whole-cell biocatalyst

Fig. 5. FTIR spectra of BNC, BNC-supported BliLacc, BNC-supported *E. coli* (BliLacc).

proved to be stable upon prolonged storage. Generally, the applicability of the robust whole-cell biocatalyst is recognized as advantageous in the design of sustainable bio-based processes [44]. During the study, it was also found that the *E. coli* expression host, without the BliLacc, could oxidize these substrates. The genome search of the *E. coli* TOP10 strain revealed CueO as Cu^{2+} dependent phenol oxidase [43]. Our results provided evidence that CueO contributes to the catalytic activity of the expression host in the oxidation of DHPy1, based on the demonstrated activity of the pure enzyme and on the similar general protein structure fold to laccases from *T. versicolor* and *B. licheniformis* (Fig. 2). Nevertheless, significant differences among laccases exist in terms of pH optimum, stability, redox potential and rate of catalysis [45,46]. While one may attempt to generalize that any enzyme able to oxidize ABTS could perform this reaction, it is now clear that the requirements are more complex. CueO shows moderate activity on ABTS under tested conditions and yet it efficiently facilitates the oxidation of the tested compound. In addition, intracellular conditions may be significantly different from known *in vitro* optimal conditions for BliLacc and CueO

Fig. 4. SEM images of the surfaces of BNC, BNC-supported BliLacc, BNC-supported *E. coli* (BliLacc).

Table 4

Oxidation of **DHPy1** catalyzed by BNC-supported laccase biocatalysts. Reaction was performed in 100 mM acetate buffer (pH 4.5), in the presence of 5% MeOH (v/v) at 50 °C.

Entry	Biocatalyst	Enzyme (U)	Substrate amount/CDW (mmol/g)	ABTS (mol%)	CuSO ₄ (mol%)	Time (h)	Yield (%) ^a
1	TvLacc	5	–	1.5	–	20	87
2	BliLacc	4	–	1.5	–	20	70
3	<i>E. coli</i> (BliLacc)	–	7.12	1.6	1.7	24	87
4	<i>E. coli</i> TOP10	–	9.49	1.6	1.7	24	75

^a Isolated yield of product.

therefore this should be taken into account when designing a whole-cell biocatalyst. Recently, a set of CueO mutants, including G304 K, with increased laccase and the reduced cuprous oxidase activity have been generated [47]. The crystal structure mutated versions of *E. coli* CueO revealed the importance of methionine-rich helix and the relative regulatory loop and their interaction and movements when the enzyme is Cu-soaked for the access of the substrate, opening the possibility to further design specific substrate-binding mutants [47,48].

As a method of increasing stability and commercial viability of biocatalysts, various methods of immobilization have been developed [49,50], with numerous recent reports describing the immobilization of laccases [51–54]. Displaying unique structural and mechanical properties such as three-dimensional nanostructure, high porosity, high water absorption capacity, and excellent wet mechanical strength, as well as convenient biotechnological route of production [55,56], BNC was chosen as a suitable candidate for the immobilization of BliLacc and *E. coli* (BliLacc) biocatalysts. BNC has been found to have a structure that could efficiently entrap enzymes and cells without covalent binding, while still allowing substrate to diffuse easily with only a minor loss in activity [55,57]. However, the immobilization of both biocatalysts was shown to have a negative impact on the activity which was reflected in longer reaction time (Table 4). Previous reports on BNC-immobilized purified laccases also showed decline of the activity of the immobilized biocatalysts in comparison to free enzyme that was pH dependent [58,59]. On the other hand, an example of no significant change in activity after laccase immobilization has also been reported [60]. However, the efficient reusability of the BNC-immobilized laccases of up to 7 cycles retaining between 60 and 70% of the original activity using ABTS as substrate has been reported. To the best of our knowledge, this is the first study of the reusability of a whole-cell BNC-immobilized laccase biocatalyst that has been used in the oxidation of diethyl-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (**DHPy1**) in three consecutive runs with the retention of 37% of the original activity. Low recyclability of immobilized *E. coli* cells is likely a result of their non-specific, non-covalent binding to the nanocellulose surface. Although a near-linear decline in reaction yield was noted after three reaction cycles coupled with biocatalyst detachment during the reaction, partial retention of bacterial cells on BNC provides a basis for further optimization in order to increase the binding affinity of *E. coli* to the surface of cellulose. In some cases, cross-linking with glutaraldehyde prior to immobilization had a beneficial effect on the efficiency of the immobilized laccase based biocatalysts [16,58]. The surface display of the cellulose binding domain on the expression host would possibly prove beneficial for the tighter attachment of the whole-cells onto BNC as previously described for cellulose matrix [61].

5. Conclusion

In this study, recombinant bacterial laccase, BliLacc from *B. licheniformis* was successfully applied in the preparative oxidation of **DHPy1** and **DHPy4**, while the oxidation of bulkier substrates resulted in poor yields of the corresponding products. In the case of **DHPy6**, no conversion of the starting material was observed. The bioprocess was optimized to lower the resource intensity by applying a whole-cell approach. It was found that multicopper oxidases, such as CueO from the

E. coli expression host have a synergistic effect with the recombinantly expressed laccase. The convenient biotechnological methods for the production of BNC and its remarkable mechanical properties were employed to generate immobilized purified laccases and whole-cell laccase-expressing biocatalysts to further improve the positive environmental aspects of the selected transformation. The immobilization of both purified enzymes as well as whole-cell catalysts resulted in a lower catalytic activity in the oxidation of **DHPy1**. The recycling of *E. coli* (BliLacc) on BNC was shown to be feasible, albeit with a near-linear drop of reaction yield after three reaction cycles. Different biocatalytic systems developed in this study may be applied to other laccase-mediated transformations.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.enzmtec.2019.109411>.

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