Efficient secretion of a true laccase by the acidophilic green microalga *Chlamydomonas pitschmannii* switched to alkaline pH

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Abstract

Laccases are extracellular multicopper oxidases that react with a broad range of aromatic substrates and are used for many biotechnological applications. Recently, microalgal laccases were discovered and started to be characterized in some Chlorophyceae (*Tetracystis aeria* and a few other species). A volumetric activity of maximum 27 U L^{-1} could be reached with *Chlamydomonas moewusii*, which is largely insufficient to compete with fungi and heterologous expression systems. Here, the acidophilic green microalga *Chlamydomonas pitschmannii* was shown to be a new promising source of laccase competing with fungi in terms of secretion yields. By modulating culture pH and CO_2 supply, laccase volumetric activity in the supernatant could be increased from 12 to 459 U L^{-1}. Laccase-containing supernatants were found to oxidize many phenolic and non-phenolic substrates with an efficiency that was pH-dependent. 1D SDS-PAGE was achieved and lighted up that *C. pitschmannii* laccase is a glycosylated protein. Functional measurements pointed out the participation of glycosylation in functional enzymatic regulation and integrity. Two bands of about 50 and 45 kD were detected that presumably corresponded to laccase. In agreement with previous studies in *T. aeria*, we hypothesize that *C. pitschmannii* laccase is a heterodimer, a feature that denotes with most known laccases, essentially monomeric. Altogether, those results demonstrate that efforts of discovery and characterization of microalgal laccases must be continued with regards to the amazing potential they offer for biotechnological applications.

Keywords

Laccase; phenol oxidase; bioremediation; cell culture

Abbreviations

2,6-DMP	2,6-dimethoxyphenol
ABTS	2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
BPA	Bisphenol A
DW	Dry weight
KL	Kraft lignin
PAS	Periodic acid Schiff
RBBR	Remazol Brilliant Blue R

SGD Syringaldehyde

SGZ Syringaldazine

Introduction

Laccases (EC 1.10.3.2) are extracellular multicopper enzymes that catalyze the oxidation of a broad range of substrates. They are widely spread within the living kingdom and have long been known to be naturally secreted by bacteria, cyanobacteria, fungi, plants and insects. The physiological roles of laccases vary from one group to another and are too numerous to be exhaustively described here. For example, plant laccases are involved in lignin (de)polymerization and play a key role in morphogenesis, wood biosynthesis and mechanical rigidity. Insect laccases are important for cuticle sclerotization. Fungal laccases accelerate the decomposition of lignocellulose polymers, which are very resistant to chemical and biological degradation, and are thus very important for carbon cycle in the biosphere. Bacterial laccases participate in the defense against environmental stresses such as high levels of heavy metals, UV radiations and ROS exposures (Janusz et al. 2020).

A wide variety of biotechnological applications utilize laccases as essential components of industrial processes. Laccases are intensively employed in pulp, paper, food and textile industries, as well as for bioremediation, wastewater treatment, organic synthesis and biofuel refinery. They are also parts of some types of biosensors and biofuel cells (Mate and Alcalde 2017).

Native sources of laccases (i.e. organisms naturally secreting the enzyme) are insufficient to sustain the market demand. For this reason, most laccases used at the industrial scale are synthesized with heterologous expression systems (Piscitelli et al. 2010). For production purposes, bacterial and fungal laccases are expressed in prokaryotic and eukaryotic host cells, respectively. The heterologous expression of fungal laccases in yeasts (for example, *Trametes versicolor* laccase in *Pichia pastoris*) is one of the most widely spread system for large-scale laccase biosynthesis (Li et al. 2014). Plant cells can also be used for the heterologous expression of eukaryotic laccases. The expression of *Cyathus bulleri* laccase in *Escherichia coli* was achieved as the first successful attempt to produce a eukaryotic laccase in a prokaryotic host (Salony et al. 2008).

Microalgae have long been used for wastewater treatment, pollutant biodegradation and bioremediation but emerged only recently as new promising sources of laccases (Abdelfattah

et al. 2023). In 2010, the first description of a microalgal extracellular laccase was published (Otto et al. 2010). The soil green microalga *Tetracystis aeria* was demonstrated to excrete an enzyme able to oxidize a wide range of phenolic substrates, and the enzyme was partly characterized at the functional and molecular levels. This first study was followed by another that brought further insights on laccase properties (Otto and Schlosser 2014). Notably, it was shown that *T. aeria* laccase oxidizes syringaldazine but lacks tyrosinase activity. Such features make the enzyme of *T. aeria* a so-called "true laccase" (Harkin and Obst 1973).

In 2015, diverse green microalgal species were screened for their extracellular laccase activities (semi-quantitative determination) (Otto et al. 2015). A phylogenetic tree of the screened species was built and lighted up that all analyzed microalgae of the *Moewusinia* clade were secreting putative true laccases. This feature (putative true laccase activity) was restricted to the *Moewusinia* group. The latter comprised several species of the *Tetracystis* and *Chlorococcum* genus, as well as three species of the *Chlamydomonas* genus (*C. pitschmannii*, *C. moewusii* and *C. noctigama*). All of them were shown to exhibit high putative true laccase activity. The model alga *Chlamydomonas reinhardtii* was not included within the *Moewusinia* clade and did not lead to the detection of laccase activity.

Here, laccase secretion was characterized in the acidophilic green microalga *Chlamydomonas pitschmannii* (Pollio et al. 2005). A culture process was developed that enabled to reach a 17 folds higher volumetric activity in culture supernatant (459 U L^{-1}) compared to the maximal microalgal level known to date (27 U L^{-1} in *C. moewusii*) (Otto et al. 2015). *C. pitschmannii* laccase was shown to be a so-called "true laccase" able to oxidize many phenolic substrates.

Materials and methods

Algal strain and culture conditions

Strain 14.73 from the SAG Culture Collection (*Chlamydomonas pitschmannii*, axenic) was used. Microalgae were inoculated at 0.025 g L^{-1} dry biomass and grown photoautotrophically in 1 liter Erlenmeyer shake flasks (400 mL inside) at 25°C under 100 $\mu mol_{photons}$ m^{-2} s^{-1} continuous white light and 110 rpm orbital shaking (batch system). Culture medium was made of NH_4Cl 7.5 mM, K_2HPO_4 5.4 mM, KH_2PO_4 4.6 mM, $MgSO_4$ 1.4 mM, $CaCl_2$ 450 µM, oligo-elements (H_3BO_3 180 µM, $ZnSO_4$ 75 µM, $MnCl_2$ 25 µM, $FeSO_4$ 18 µM, $CoCl_2$ 6.8 µM, $CuSO_4$ 6.3 µM, $(NH_4)_6Mo_7O_{24}$ 890 nM) and PIPES 10 mM. For copper

induction experiments, 20 µM more $CuSO_4$ was added to reach a concentration of 26.3 µM. Algae were grown either under ambient air (CO_2 400 ppm) or CO_2-enriched air (CO_2 5%). The pH of the medium was adjusted to 3.5, 7.0 or 10.0 before culture start. When studying laccase induction, the pH of algal suspension was increased during cultivation with controlled amounts of NaOH 1 M.

Growth assessment and supernatant sampling

Algal growth was monitored as the evolution of dry biomass concentration. Culture samples (50 mL) were harvested and centrifuged at 3,000 *g* for 5 minutes. Algal pellets were washed once with deionized water, centrifuged again, dried at 70°C for 24 hours and finally weighed. Supernatants were passed through 0.2 µm PES syringe filters and secreted proteins were concentrated 50x by ultrafiltration (Amicon Ultra-15 Centrifugal Filter Units, 10 kD cutoff, Merck). 50x concentrated protein supernatants were used for all subsequent analyses related to laccase characterization.

Respiration and photosynthesis

A Clark-type electrode coupled to Oxy-Lab oxygraph and O_2view software (Hansatech) was used to measure variations of oxygen concentration in microalgal suspensions (Hunt 2003). Several steps of actinic light (0 to 1000 $\mu mol_{photons}\ m^{-2}\ s^{-1}$; 3 minutes each) were applied that enabled to build light saturation curves and to reveal 750 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ as saturating light intensity for photosynthesis (MacIntyre et al. 2002; Behrenfeld et al. 2004). Three rates of oxygen evolution were extracted from the data: at 0 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ (dark respiration), 100 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ (cultivation light intensity) and 750 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ (saturating light intensity). To calculate gross photosynthesis, dark respiration was subtracted from net photosynthesis (i.e., the "raw" oxygen evolution rate measured for each actinic light). All measurements were performed with microalgal suspensions containing 8 µg mL^{-1} total chlorophylls, and all oxygen evolution rates were normalized to intracellular contents of either chlorophylls a+b or proteins in the oxygraph cuvette. To determine protein concentration, microalgal suspensions were centrifuged at 15,000 g for 3 minutes. Pellets were resuspended in Triton X-100 1% (v/v), 0.75-1 mm glass beads were added and proteins were extracted by intensively shaking with VXR basic Vibrax (IKA) at 4°C for 15 minutes. Protein concentration was quantified with the RC/DC Protein Assay Kit (BioRad) derived from the Lowry-Ciocalteu method (Waterborg 2009). To determine chlorophyll concentration, microalgal suspensions were centrifuged at 15,000 *g* for 3 minutes. Pellets were resuspended in ice-cold methanol,

0.75-1 mm glass beads were added and pigments were extracted by intensively shaking with VXR basic Vibrax (IKA) at 4°C for 15 minutes in the dark. Pigment extracts were centrifuged again to remove any insoluble material and were passed through 0.2 µM PES syringe filters. Total chlorophyll concentration was quantified by reading absorbance at 653 and 666 nm and applying Lichtenthaler and Wellburn equations (Lichtenthaler and Wellburn 1983):

$$\text{Chlorophyll a} = 15.65\ A^{666} - 7.34\ A^{653}\ (\mu g\ mL^{-1})$$

$$\text{Chlorophyll b} = 27.05\ A^{653} - 11.21\ A^{666}\ (\mu g\ mL^{-1})$$

$$\text{Total chlorophylls} = \text{chlorophyll a} + \text{chlorophyll b}\ (\mu g\ mL^{-1})$$

Laccase activity

Laccase activity was routinely measured with ABTS 0.2 mM as substrate (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); ε_{420} = 36,000 M^{-1} cm^{-1}) (Childs and Bardsley 1975) in 50 mM sodium phosphate buffer, pH 4.0. ABTS oxidation was monitored for 6 minutes through elevation of absorbance at 420 nm. One enzymatic unit corresponds to the oxidation of one µmole ABTS per minute (U = μmol_{ABTS} min^{-1}). Volumetric activity is the number of U per liter of culture supernatant (U L^{-1}).

pH curves of laccase activity

Laccase activity was measured from pH 2.0 to 10.0 for 4 substrates: ABTS 0.2 mM, SGZ 0.05 mM (syringaldazine; ε_{530} = 65,000 M^{-1} cm^{-1}) (Harkin and Obst 1973), 2,6-DMP 0.2 mM (dimethoxyphenol; ε_{470} = 27,500 M^{-1} cm^{-1}) (Martínez et al. 1996) and SGD 0.2 mM (syringaldehyde; absorbance peak at 370 nm) (Pardo et al. 2013). Twenty-five fold concentrated stock solutions were prepared that were dissolved in glacial ethanol (SGZ, SGD) or deionized water (ABTS, 2,6-DMP). Assays were carried out in 50 mM sodium phosphate buffer. The reaction time was fixed to 6 minutes during which the increase of absorbance was measured. For ABTS, SGZ and 2,6-DMP, volumetric activities (U L^{-1}) were calculated and normalized with the maximal value set to 100%. For SGD, the ΔA^{370} values were used for normalization to 100%, instead of volumetric activities such as for the other substrates.

BPA, RBBR and KL oxidation by laccase

Laccase was tested for its capacity to oxidize BPA (bisphenol A), RBBR (Remazol Brilliant Blue R) and KL (kraft lignin; average $M_W \approx$ 10,000) (Tolbert et al. 2014). Reagents were from Sigma-Aldrich: 239658, R8001 and 471003, respectively. The initial concentration was 5 mM for BPA and RBBR and 10 g L^{-1} for KL. Ten-fold concentrated stock solutions were

prepared that were dissolved in glacial ethanol (BPA), deionized water (RBBR) or ethanol 60% (v/v) (KL) (Goldmann et al. 2019). Oxidation experiments were carried out at 0.2 U mL^{-1} either in the absence or in the presence of a redox mediator: ABTS 0.2 mM; 2,6-DMP 0.2 mM; SGD (syringaldehyde) 0.2 mM; SGZ 0.05 mM. The reaction mixture was buffered with sodium phosphate 50 mM pH 4.0, 7.0 or 10.0 according to the optimal operating pH of each mediator (4.0 for ABTS; 7.0 for SGZ and SGD; 10.0 for 2,6-DMP). In the absence of mediator, the three pH values were evaluated. Substrate oxidation was carried out at 25°C for 8 hours, during which samples were periodically harvested and frozen at -20°C. Each reaction mixture was paralleled by a control deprived of laccase to quantify substrate oxidation independently of the enzyme.

BPA, RBBR and KL determination

Spectrophotometric analyses were done in 96-well plates with 100 µL per well. Absorbance was read using Synergy MX plate reader (BioTek). RBBR oxidation was assessed by diluting samples in deionized water (10 folds factor) and measuring the decrease of absorbance at 595 nm (Mechichi et al. 2006) (quantitation: RBBR standards from 0.1 to 5 mM). BPA and KL oxidations were assessed by a phenol-specific colorimetric assay: 70 µL $NaHCO_3$ 0.25 M were added of 10 µL sample, 10 µL 4-AAP 20.8 mM (4-aminoantipyrine) and 10 µL potassium ferricyanide 83.4 mM (Spiker et al. 1992; Wagner and Nicell 2001; Modaressi et al. 2005). After 10 minutes, absorbance was read at 506 nm and its decrease was used to characterize the level of substrate oxidation (quantitation: BPA standards from 0.1 to 5 mM; KL standards from 1 to 10 g L^{-1}).

Protein deglycosylation

Supernatant proteins were deglycosylated with 3.5 U mL^{-1} PNGase F from *Elizabethkingia miricola* (Promega V4831). Two conditions of deglycosylation were used, i.e., "native" and "denaturing" deglycosylations: in the latter, proteins were added with SDS 0.02% (w/v) and heated at 70°C for 10 minutes prior to enzymatic digestion. Deglycosylation reaction was carried out at 37°C for 3 hours in TBS buffer (NaCl 150 mM, Tris-HCl 50 mM, pH 7.5).

1D SDS-PAGE

2 µg proteins (not including PNGase F 3.5 U mL^{-1} added in some samples) were mixed with 20 µL Laemmli buffer + DTT 10 mM, heated at 70°C for 10 minutes and loaded onto 4-15% gradient precast polyacrylamide gels (Mini-Protean TGX gel, BioRad 456-1084). Anode and cathode running buffers were made of SDS 0.4% (w/v), glycine 200 mM and Tris 25 mM.

Electrophoreses were run at 100 V (constant voltage) for 60 minutes with protein ladder (ThermoFisher Scientific 26619) as molecular size reference. BSA (0.1 µg) and PNGase F alone were used as controls. Polyacrylamide gels were fixed for 1 hour in methanol 30% + acetic acid 10% (v/v) before being silver stained or submitted to in-gel glycan detection.

In-gel silver staining

A previously described procedure was used (Yan et al. 2000). Fixed gel was washed 1 x 10 minutes in methanol 50% (v/v) and 1 x 10 minutes in deionized water. Sensibilization was done for 1 minute in a solution of methanol 20% (v/v), sodium acetate 6.8% (w/v) and sodium thiosulfate 0.02% (w/v). The gel was rinsed 2 x 1 minute in deionized water before being stained by silver nitrate 0.1% for 20 minutes. Then it was washed 2 x 1 minutes in deionized water and finally developed in formaldehyde 0.04% (v/v) + Na_2CO_3 2% (w/v). Coloring reaction was stopped by transferring the gel in acetic acid 5% (v/v).

In-gel detection of glycans

A time-saving, microwave-assisted adaptation of the PAS (Periodic Acid Schiff) method was used (Moravec and Mares 2017). Fixed gels were washed 1 x 10 minutes in methanol 50% (v/v) and 1 x 10 minutes in deionized water. For glycan oxidation, gels were covered with H_5IO_6 1% (w/v) + acetic acid 3% (v/v) solution, heated at 700 W for 1 minute and incubated in warm solution for 5 minutes at room temperature. They were washed in deionized water at 700 W for 3 x 1 minute (deionized water to be replaced from one microwave step to the other). Gels were then covered with Schiff's reagent (Sigma-Aldrich 3952016) and labeling of glycans was carried out at 700 W for 45 seconds. Incubation in warm Schiff's reagent was continued for 5 minutes at room temperature. Labeled gels were washed at 700 W for 1 minute in $Na_2S_2O_5$ 5% (w/v) solution. Warm wash solution was replaced by a less concentrated one ($Na_2S_2O_5$ 0.1% (w/v)) and gels were incubated for 5 minutes at room temperature. Every incubation step was accompanied of gentle shaking.

Datasets and statistics

Every experiment was carried out with three independent biological replicates. Each biological replicate was analyzed once. Standard deviations were calculated using the three values available, each of them obtained from one biological replicate (n = 3). Except when expressly mentioned, no hypothesis testing was undertaken to evaluate the statistical significance of the results.

Results

Autotrophic growth and laccase secretion

C. pitschmannii is an acidophilic microalga which is able to grow from acidic to neutral pH (3.0 to 8.0). The biomass evolution of *C. pitschmannii* cultures was characterized at pH 3.5 and 7.0 for 30 days. The volumetric activity of laccase in culture supernatants was also monitored, as well as whole cell dark respiration and photosynthetic efficiency at 100 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ (cultivation light intensity) and 750 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ (saturating light intensity). As shown in **Fig. 1**, the pH influences neither the rate of dry biomass accumulation (maximum 2.2 g L^{-1} at day 30) nor the photosynthetic efficiency at both light intensities. The dark respiration remains the same as well since there is no statistically significant change due to pH modification (p=0.05, Student's t-test). Remarkably, a strong dependence of laccase volumetric activity upon pH was found. In cultures started at pH 7.0, the volumetric activity was higher than at pH 3.5 all along the cultivation process. At day 14, maxima of 12 and 86 U L^{-1} were reached at pH 3.5 and 7.0, respectively. For cultures started at pH 7.0, a drop of pH was observed from day 14 that was paralleled with a decrease of laccase volumetric activity (**Fig. 1**). We hypothesized that pH could be a key factor regulating laccase secretion in *C. pitschmannii*.

C. pitschmannii growth and laccase volumetric activity in supernatants were characterized with 6.3 µM $CuSO_4$ (normal concentration) and 26.3 µM $CuSO_4$ (normal concentration + 20 µM $CuSO_4$, i.e. the level classically used to induce laccase). As illustrated in **Fig. 2**, the addition of copper exerts a negative influence on biomass accumulation and laccase volumetric activity.

Efficient process for laccase induction

Since non-acidic pH favors laccase secretion, a two-phase autotrophic culture process was undertaken to uncouple biomass accumulation and laccase secretion, as a way to trigger high laccase yields in supernatant. The following sequential procedure was applied to *C. pitschmannii* cell suspensions:

1. Cultivation at pH 3.5 with CO_2 5% to accumulate high levels of biomass (days 0 to 4)
2. Switch to atmospheric air (CO_2 400 ppm) and neutral or alkaline pH (7.0 or 10.0, respectively) to induce laccase secretion (days 4 to 7)

Fig. 3 details the evolution of dry biomass concentration, pH and laccase volumetric activity in culture supernatants. As illustrated there, the stationary phase of CO_2 5% cultures is reached in only 3 days (2.7 g L^{-1} dry biomass). During this first cultivation phase, laccase volumetric activity is undetectable. Accordingly, cultivating the green microalga *S. vacuolatus* with CO_2 5% was shown to inhibit laccase secretion (Carbone et al. 2018). The second phase was started at day 4. As expected, increasing the pH to 7.0 and 10.0 led to the detection of laccase activity in supernatants. Two controls were undertaken at pH 3.5 (CO_2 400 ppm and 5%, respectively) and did not lead to the detection of laccase activity. For both pH values, increase of laccase activity was very fast since 87% of the maximal value were reached after only 6 hours. The maximal volumetric activity was recorded 24 hours after induction (i.e. at day 5 of the process): 55 U L^{-1} at pH 7.0 and 459 U L^{-1} at pH 10.0 (**Fig. 3**). Laccase level at pH 10.0 is huge compared to the state of art of laccase secretion by microalgae. To our knowledge, the best result reported to date is 27 U L^{-1} in *C. moewusii* (Otto et al. 2015). The volumetric activity obtained here at pH 10.0 is 17 folds higher.

At day 7 (i.e. 3 days after induction), dramatic drops of biomass concentrations were monitored at both pH 7.0 and 10.0. Biomass decreases were not paralleled with loss of laccase activity (no statically significant difference between days 6 and 7, $p<0.05$, Student's t-test) (**Fig. 3**). This observation indicates that laccase is alkali-stable for at least 24 hours at 25°C.

Substrate oxidation

The pH-dependence curve of laccase activity was built for 4 substrates: ABTS, SGZ, SGD and 2,6-DMP (**Fig. 4**). Concentrated crude supernatants were used. Values are expressed in terms of percentage of the maximal activity: 6176, 487 and 1212 U L^{-1} for ABTS, SGZ and 2,6-DMP, respectively; nd for SGD. Those results are in agreement with previously reported kinetic parameters of *T. aeria* laccase, in which K_m and V_{max} values pointed out a lower substrate affinity for SGZ and 2,6-DMP compared to ABTS (Otto and Schlosser 2014).

The optimal pH was found to be 4.0 for ABTS, 7.0 for SGZ and 10.0 for 2,6-DMP. For SGD, activity could not be monitored beyond pH 7.0 due to technical limitations related to interferences with the colorimetric assay; it remained stable from pH 3.5 to 7.0 (**Fig. 4**).

The ability of *C. pitschmannii* laccase to oxidize phenolic substrates of biotechnological interest was evaluated. The laccase-mediated oxidation of bisphenol A (BPA), Remazol Brilliant Blue R (RBBR) and kraft lignin (KL) was monitored by colorimetric assays (BPA and KL) and spectrophotometry (RBBR). **Fig. 5** reports the oxidation kinetics of each substrate by 0.2 U mL^{-1} laccase, in the absence or presence of a redox mediator (ABTS, SGZ, SGD and 2,6-

DMP). In the absence of mediator, the oxidation kinetics was analyzed at 3 pH values (pH 4.0, pH 7.0, pH 10.0). In the presence of mediator, the oxidation kinetics was reported at the optimal pH of each of them (pH 4.0 for ABTS; pH 7.0 for SGZ and SGD; pH 10.0 for 2,6-DMP). The initial concentration was 5 mM for BPA and RBBR, and 10 g L^{-1} for KL. Results are presented as mM or g L^{-1} of oxidized substrate.

As shown in **Fig. 5a**, maximal oxidation levels are reached within 30 minutes for RBBR and 4 hours for KL. For BPA, >90% of the maximal oxidation level are reached within 2.5 hours. In the absence of mediator, the optimal pH for laccase-mediated oxidation is 10.0 for BPA (0.9 mM oxidized), RBBR (1.8 mM oxidized) and KL (3.0 g L^{-1} oxidized) as well. A similar pH-dependence tendency was reported for RBBR in *T aeria* (Otto et al. 2010). At first glance, **Fig. 5a** seems to indicate that SGD and 2,6-DMP improve oxidation capacity. But as shown in **Fig. 5b**, while comparing oxidation with and without laccase, it appears that none of the tested mediators is actually efficient. Indeed, the increase of oxidation capability due to laccase is always similar or lower in the presence of a redox mediator compared to reaction mixtures deprived of mediator. Those results indicate that oxidation capability of BPA, RBBR and KL by *C. pitschmannii* laccase is not improved by the tested mediators.

Molecular characterization

Since laccase was secreted in the cultivation medium, attempts have been undertaken to visualize the enzyme in supernatant extracts. Crude supernatant proteins were run on 1D SDS-PAGE gels with or without previous deglycosylation with PNGase F. Deglycosylation was either performed in “native” or “denaturing” condition, depending if proteins were denatured with heat and SDS prior to enzymatic digestion. Gels were either silver stained (protein labeling) or PAS stained (carbohydrate labeling for glycoprotein detection). Electrophoretic samples came from day 6 of the two-phase cultivation process, i.e. day 2 after laccase induction (**Fig. 3**).

Stained gel profiles are illustrated in **Fig. 6** for supernatants of cultures conducted at pH 7.0 and 10.0. At pH 10.0, obviously, most supernatant proteins are glycosylated, as evidenced by the pink coloration on the PAS-stained gel and the high molecular weight smear on the silver-stained gel in the absence of PNGase F digestion. When supernatant proteins are deglycosylated (either in native or denaturing condition), the pink coloration disappears (PAS staining) and the smear is replaced by fine protein bands (silver staining). At pH 7.0, gel profiles indicate that most supernatant proteins are not glycosylated. Deglycosylation is not necessary to observe fine protein bands by silver staining, and only the appearance of a few protein bands is noticed after

deglycosylation. Without deglycosylation, no pink coloration is detected in the PAS-stained gel for pH 7.0 protein sample (**Fig. 6**).

The influence of deglycosylation on laccase activity was investigated in liquid concentrated supernatant from pH 10.0 cultures. Laccase activity dramatically decreases after native PNGase F digestion to reach only 13% of the value without deglycosylation (8 folds lowering). In case of denaturing deglycosylation, laccase activity drops to zero because of alteration of proteins induced by heat and SDS.

At pH 10.0 with deglycosylation, one or two intense protein bands (that were not detected at pH 7.0) appear in the silver-stained gels (**Fig. 6**). In case of native deglycosylation, there is one band of about 50 kD. In case of denaturing deglycosylation, there are two bands of about 50 and 45 kD. The absence of these bands at pH 7.0 and their presence at pH 10.0 suggested that these two signals could correspond to laccase although we cannot rule out that they could be related to other proteins expressed concomitantly.

Discussion

In this work, it is demonstrated that the acidophilic green microalga *C. pitschmannii* synthesizes a true laccase able to oxidize many phenolic substrates (**Fig. 4**, **Fig. 5**).

Non-acidic pH seems to favor laccase secretion, suggesting that environmental pH is a key factor regulating laccase synthesis and release in *C. pitschmannii* **(Fig. 1**, **Fig. 3**). A two-phase cultivation process is proposed that enables to reach 459 U L^{-1} laccase activity in the medium (**Table 1a**), a huge yield compared to those reported to date for microalgae (maximum 27 U L^{-1} in *C. moewusii*) (**Table 1b**) (Otto et al. 2015). As the decrease of biomass concentration in laccase-producing cultures (pH 10.0) is not paralleled with drop of volumetric activity in supernatant (**Fig. 3**), *C. pitschmannii* laccase appears to be alkali-stable for at least 24 hours, which is a promising feature for many biotechnological applications (Weihua and Hongzhang 2008). In most bacteria and fungi, the addition of copper to the culture medium is a common strategy to improve laccase secretion (Singh et al. 2011). Such observations were also reported in the green microalga *T. aeria* (Otto et al. 2010). Contrarily to most laccase-producing organisms, the enzyme of *C. pitschmannii* does not seem to be induced by increasing copper concentration in the culture medium (**Fig. 2**).

Remarkably, the pH-dependence profiles of ABTS, SGZ and 2,6-DMP oxidation by *C. pitschmannii* laccase (**Fig. 4**) diverge from those of the green microalgae *T. aeria* and *C. moewusii* (Otto et al. 2010, 2015; Otto and Schlosser 2014), indicating that the pH optima of

microalgal laccases exhibit a wide diversity such as in fungi (Mayer and Staples 2002; Rodríguez-Couto 2019). As well, *C. pitschmannii* laccase is shown to efficiently oxidize BPA, RBBR and KL with a pH-dependent efficiency (optimum towards alkalinity). The oxidation capability is not improved by the tested mediators (ABTS, SGZ, SGD, 2,6-DMP) (**Fig. 5**). This observation denotes from previous studies in *T. aeria*, that reported an increase of BPA and RBBR detoxification in the presence of ABTS and SGD (Otto and Schlosser 2014; Otto et al. 2015). Altogether and once again, those data point out the high functional versatility among microalgal laccases.

Importantly, SGZ was demonstrated not to be oxidized by tyrosinases and to be a specific substrate of so-called "true laccases", by opposition with "laccase-like" multicopper oxidase enzymes (Harkin and Obst 1973). Due to its ability to oxidize SGZ and various other phenolic substrates, the laccase of *C. pitschmannii* can be considered to be a true laccase, such as the enzyme of *T. aeria* and other members of the *Moewusinia* microalgal taxon (Otto et al. 2015).

Molecular characterization of *C. pitschmanni* supernatants was undertaken by 1D SDS-PAGE (**Fig. 6**). Results of gel electrophoresis and supplementary activity measurements indicate that *C. pitschmannii* laccase is a glycosylated protein and that glycosylation participates in functional enzymatic regulation and integrity. Those results and interpretations related to glycosylation are in agreement with observations reported for fungal, bacterial and plant laccases (Madhavi and Lele 2009; Glazunova et al. 2019) and emphasize the need to use eukaryotic host cells to ensure proper post-translational modifications of laccases in heterologous expression systems (Piscitelli et al. 2010).

In silver-stained gels, two bands of approximately 50 and 45 kD were detected when running samples previously deglycosylated in denaturing condition (**Fig. 6**). If these bands consist of laccase, results may indicate that *C. pitschmannii* laccase is a heterodimer such as *T. aeria* laccase. In *T. aeria*, SDS-PAGE of crude supernatants firstly suggested that laccase could be an oligomer (Otto et al. 2010). Another study by the same authors, focusing on the purified *T. aeria* laccase, finally demonstrated that the enzyme most probably is a heterooligomer AB_2 with $A \approx 110$ kD and $B \approx 71$ kD (Otto and Schlosser 2014). The discovery of heterooligomeric laccases in the *Moewusinia* clade would denote with the structural properties of fungal, bacterial and plant laccases, that were reported to be mostly monomeric and to range approximately from 50 to 90 kD (Rivera-Hoyos et al. 2013; Chauhan et al. 2017; Dana et al. 2017).

The observation that *C. pitschmannii* laccase is induced by non-acidic pH deserves to be highlighted. We raise two hypotheses (possibly working in synergy) to explain why medium alkalinization is a key factor for laccase induction (**Fig. 1**, **Fig. 3**):

- The pKa of the acidobasic equilibrium HCO_3^-/H_2CO_3 is 10.3. Thus, at pH 10.0, half of the available C_i (inorganic carbon) is HCO_3^-, and half is H_2CO_3. The latter is a polar molecule which cannot diffuse through cell membranes and for which no plasmatic transporter was discovered, even if thylakoid membrane transporters were found (Winck et al. 2013). In *C. pischmannii*, the occurrence of CO_2 and HCO_3^- transporters in the plasma membrane is unknown. We hypothesize that *C. pitschmannii* switched to pH 10.0 (and, to a lesser extent, to pH 7.0 where both HCO_3^- and CO_2 are available) encounters a stress due to C_i intracellular depletion and synthesizes laccase as an attempt to decompose its substrate and assimilate organic carbon heterotrophically. We found that *C. pitschmanni* cells agglomerate at pH 10.0. (data not shown), as previously reported in microalgae (Aditya et al. 2023). This phenomenon could favor the sequestration of catabolic CO_2 leaked extracellularly through its recapture by neighboring organisms, and contribute to explain why 3 days are needed for *C. pitschmannii* cultures to start declining after medium alkalinization (**Fig. 3**).

- Alkaline culture media were reported to induce cell disruption and improve protein solubilization (Phong et al. 2018). Therefore, switching *C. pitschmannii* cultures to pH 10.0 could accelerate the release of intracellular laccase and explain why most of laccase activity appears in supernatants in only a few hours (**Fig. 3**).

Conclusions

Examples of data collected in literature for bacterial, fungal and microalgal laccases are summarized in **Table 1b** and indicate that microalgal laccase now compete with fungal enzymes in terms of volumetric activity in culture supernatants (**Fig. 1**, **Fig. 3**). In *C. pitschmannii*, the unique pH-dependence curves of laccase activity (**Fig. 4**), the ability of the enzyme to efficiently remove phenolic pollutants (**Fig. 5**), the singular response of laccase secretion to copper addition (**Fig. 2**), the alkali-stability of the enzyme (**Fig. 3**) and the particular band patterns observed in silver-stained 1D SDS-PAGE (**Fig. 6**) make it interesting to further investigate microalgal laccases for biotechnological applications.

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Conflict of interest

The authors declare that they have no conflict of interest.

Availability of data and material

All materials of the present paper are commercially available. Detailed data of the experiments can be requested to the corresponding author. If this demand does not compete with privacy / confidentiality restrictions that could occur due to institutional or funding organism policies, the requested data will be made fully available by the authors.

Author contributions

SG and CR had the original idea of the work, designed the experiments and interpreted the results. SG developed lab protocols, conducted experiments, analyzed data and wrote the manuscript.

Table

Table 1 Summary of (a) laccase volumetric activities in culture supernatants obtained here, and (b) comparison with examples collected in literature for different types of organisms.

(a)

Experiment	**Max. U L^{-1}**	**Biomass at 'max. U L^{-1}'**	**U g_{DW}^{-1}**	**Conditions**	**Time**
1 phase	12 ± 2	0.65 ± 0.04 g L^{-1}	18	Inoculation at pH 3.5 & weak cellular density under AIR	14 days after inoculation
1 phase	86 ± 2	0.79 ± 0.07 g L^{-1}	109	Inoculation at pH 7.0 & weak cellular density under AIR	14 days after inoculation
2 phases	55 ± 5	2.45 ± 0.16 g L^{-1}	22	pH 7.0 & AIR (after growth with CO_2 at pH 3.5)	5 days after inoculation
2 phases	459 ± 43	2.73 ± 0.17 g L^{-1}	241	pH 10.0 & AIR (after growth with CO_2 at pH 3.5)	5 days after inoculation

(b)

Microalgae			
Species	**U L^{-1}**	**Time**	**Reference**
Tetracystis aeria	22	10 days	(Otto et al. 2010)
Tetracystis aeria	26	10 days	(Otto and Schlosser 2014)
Tetracystis aeria	18	6 weeks	(Otto et al. 2015)
Chlamydomonas moewusii	27	3 weeks	(Otto et al. 2015)
Scenedesmus vacuolatus	16	1 week	(Otto et al. 2015)
Scenedesmus vacuolatus	4	1 month	(Carbone et al. 2018)
Cyanobacteria			
Species	**U L^{-1}**	**Time**	**Reference**
Spirulina platensis	85	4 days	(Afreen et al. 2017b)
Arthrospira maxima	55	10 days	(Afreen et al. 2018)
Several species	5 to 67	10 days	(Afreen et al. 2017a)
Several species	9 to 27	7 days	(Afreen and Fatma 2013)
Several species	4 to 34	3 days	(Afreen and Fatma 2013)
Fungi			
Species	**U L^{-1}**	**Time**	**Reference**
Trametes versicolor	180	4 days	(Bucić-Kojić et al. 2017)
Pleurotus pulmonaris	2860	10 days	(dos Santos Bazanella et al. 2013)
Funalia trogii	1375	9 days	(Qiu et al. 2014)
Marasmiellus palmivorus	667	5 days	(Chenthamarakshan et al. 2017)
Trametes hirsuta	542	12 days	(Dhakar and Pandey 2013)
Bacteria			
Species	**U L^{-1}**	**Time**	**Reference**
Streptomyces cyaneus	50	9 days	(Margot et al. 2013)
Serratia proteamaculans	74	4 days	(Ali et al. 2022)
Bacillus pumilus	9	3 days	(Liu et al. 2021)
Pantoea ananatis	614	3 days	(Shi et al. 2015)
Bacillus licheniformis	410	nd	(Koschorreck et al. 2009)

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Figure legends

Fig. 1 Influence of the pH (initial value and its variations) on algal growth, laccase secretion and photosynthetic efficiency in photoautotrophic batch cultures of *C. pitschmannii* grown under ambient air (CO_2 400 ppm) (*n*=3). (a) Evolution of dry biomass concentration, pH and laccase volumetric activity in culture supernatant. Laccase activity was determined with ABTS 0.2 mM as substrate in sodium phosphate buffer 50 mM, pH 4.0. (b) Dark respiration and gross photosynthesis measured under 100 and 750 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ (cultivation and saturating light intensities, respectively) by Clark-type oximetry at day 15. Oxygen evolution rates were normalized to intracellular contents of either chlorophylls a+b or proteins in the oxygraph cuvette (upper and lower graph, respectively)

Fig. 2 Influence of adding $CuSO_4$ 20 µM to the initial medium (total concentration: 26.3 µM) on algal growth and laccase secretion in the supernatant of *C. pitschmannii* photoautotrophic batch cultures started at pH=7.0 and grown under ambient air (CO_2 400 ppm). The graph presents the values obtained for Cu-supplemented cultures as % of the control ($CuSO_4$ 6.3 µM at start). Laccase activity was determined with ABTS 0.2 mM as substrate in sodium phosphate buffer 50 mM, pH 4.0 (*n*=3)

Fig. 3 Two-phase cultivation of *C. pitschmannii* cells in photoautotrophic batch system (*n*=3). Step 1: biomass accumulation under CO_2 5%, pH 3.5 at start (days 0 to 4). Step 2: induction of laccase secretion by switching to air (CO_2 400 ppm) and increasing pH to 7.0 or 10.0 with NaOH 1 M (days 4 to 7). Laccase activity in culture supernatants was determined with ABTS 0.2 mM as substrate in sodium phosphate buffer 50 mM, pH 4.0. In pH and volumetric activity graphs, the blue line superimposes to the black line, thus it is not visible

Fig. 4 pH-dependence curves of laccase activity for 4 phenolic substrates (*n*=3). Laccase assays were performed with ABTS 0.2 mM, SGZ 0.05 mM, 2,6-DMP 0.2 mM and SGD 0.2 mM in sodium phosphate buffer 50 mM, pH 2.0 to 10.0. Results were normalized to the maximal value for each substrate. 100% = 6176, 487 and 1212 $U\ L^{-1}$ for ABTS, SGZ and 2,6-DMP, respectively (unavailable for SGD)

Fig. 5 Oxidation of bisphenol A (BPA), Remazol Brilliant Blue R (RBBR) and kraft lignin (KL) with or without mediator (ABTS 0.2 mM, SGZ 0.05 mM, 2,6-DMP 0.2 mM or SGD 0.2 mM). Incubation was carried out at the optimal pH of each mediator (4.0 for ABTS; 7.0 for SGZ and

SGD; 10.0 for 2,6-DMP). In the absence of mediator, the 3 pH values were tested. (a) Substrate oxidation in the presence of laccase 0.2 U mL^{-1}, with grey zone referring to the max. oxidation value obtained at pH 10.0 without mediator, i.e. after 2.5 hours for BPA, 30 minutes for RBBR and 4 hours for KL. (b) Comparison of substrate oxidation in the presence and absence of laccase (full and dotted lines, respectively), only for the mediators that improved oxidation yield relatively to digestion without mediator at pH 10.0 (black traces), i.e. the mediators that found their trace in the grey zone of panel (a). Standard deviations (n=3) are not shown for graph readability; they range from 1 to 29% of the corresponding value

Fig. 6 1D SDS-PAGE profiles of *C. pitschmannii* secretome. Microalgae were grown at pH 3.5 with CO_2 5% for 4 days, and then switched to air at either pH 7.0 or 10.0. Supernatant was harvested at day 6, i.e. 2 days after laccase induction. Protein deglycosylation was carried out at 37°C for 3 hours with 3500 U/mL PNGase F, either in « native » condition (no denaturation prior to deglycosylation) or « denaturing » condition (denaturation with heat and SDS 0.02% prior to deglycosylation). 4-15% gradient polyacrylamide gels were run in reducing condition (DTT 10 mM). Proteins were silver stained (upper gel) and the PAS method was used to label glycoproteins (lower gel). In both gels, PNGase molecular weight is highlighted by a horizontal dotted line referring to PNGase control well on the right