A valuable peroxidase activity from the novel species *Nonomuraea gerenzanensis* growing on alkali lignin

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**Abstract**

Degradation of lignin constitutes a key step in processing biomass to become useful monomers but it remains challenging. Compared to fungi, bacteria are much less characterized with respect to their lignin metabolism, although it is reported that many soil bacteria, especially actinomycetes, attack and solubilize lignin. In this work, we screened 43 filamentous actinomycetes by assaying their activity on chemically different substrates including a soluble and semi-degraded lignin derivative (known as alkali lignin or Kraft lignin), and we discovered a novel and valuable peroxidase activity produced by the recently classified actinomycete *Nonomuraea gerenzanensis*. Compared to known fungal manganese and versatile peroxidases, the stability of *N. gerenzanensis* peroxidase activity at alkaline pHs and its thermostability are significantly higher. From a kinetic point of view, *N. gerenzanensis* peroxidase activity shows a $K_{m}$ for H$_2$O$_2$ similar to that of *Phanerochaete chrysosporium* and *Bjerkandera* enzymes and a lower affinity for Mn$^{2+}$, whereas it differs from the six *Pleurotus ostreatus* manganese peroxidase isoenzymes described in the literature. Additionally, *N. gerenzanensis* peroxidase shows a remarkable dye-decolorizing activity that expands its substrate range and paves the way for an industrial use of this enzyme. These results confirm that by exploring new bacterial diversity, we may be able to discover and exploit alternative biological tools putatively involved in lignin modification and degradation.

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the lignin polymer is highly resistant to chemical and biological degradation. Therefore, removing and using lignin constitute a central issue for industrial exploitation of plant biomasses to produce second-generation biofuels, chemicals, and new bio-based materials [4,5]. Since most of the available chemical methods for fractionating and degrading lignin generate poisonous side-products, the development of a sustainable and ecologically favorable technology, based on the use of enzyme cocktails for breaking down this polymer (and for its valorization), represents a great biotechnological challenge. The list of benefits using bio-treatment methods includes energy savings during defibration/refining steps, increase in delignification rate and facilitated access of hydrolytic enzymes to carbohydrates moieties, decrease of alkali consumption, decrease in hexenuronic acid content in Kraft pulps, removal of inhibitory phenolic compounds and other toxic intermediates in lignocellulosic hydrolysates for biofuel production [4,5]. Additionally, microbial fermentation/enzymatic degradation of lignin fractions may funnel lignin-derived aromatics to products having potential of industrial applications as: (i) vanillic acid (then converted into vanillin flavor) [6], or (ii) medium chain-length (C6-C14) polyhydroxyalkanoates (used as plastics or adhesives, or depolymerized and converted to chemical precursors or methyl-ester–based fuels) [7] or (iii) adipic acid (a polymer precursor for nylon, plasticizers, lubricants, and polyester polyls) via muconic acid [8].

Ligninolytic microbes have developed a unique strategy to circumvent the natural resistance of lignin and to degrade and mineralize the polymer. They secrete an array of oxidative enzymes, such as laccases (EC 1.10.3.2), lignin peroxidases (LiP, EC 1.11.14), manganese peroxidases (MnP, EC 1.11.13), and versatile peroxidases (VP, EC 1.11.16) [1.9–12]. Laccases are copper-containing enzymes that catalyze the oxidation of various phenolic and non-phenolic compounds and concomitantly reduce molecular oxygen to water. LiPs, MnPs, and VPs are structurally related enzymes, belonging to class II peroxidases within the heme peroxidase superfamily, which use hydrogen peroxide as electron acceptor to catalyze multi-step oxidative reactions and hydroxylat- tion. Notably, LiPs use H2O2 as the co-substrate in addition to a mediator such as veratryl alcohol to oxidize soluble semi-degraded lignin derivatives and other phenolic compounds, while MnPs oxidize Mn(II) to Mn(III), thus enhancing the degradation of phenolic compounds. VPs are hybrids of LiPs and MnPs, with bifunctional characteristics (thus being capable of using both Mn (II) and veratryl alcohol) and a broad substrate preference [10–12].

The best characterized peroxidases are those secreted by white-rot families, such as Phanerochaete chrysosporium [13]. Nevertheless, the large-scale applications of fungal enzymes have been limited by the challenge of producing these post-translationally modified proteins in commercially viable amounts [14]. By contrast, bacterial peroxidases should be much easier to produce. Additionally, bacterial enzymes might offer advantages such as better stability and activity under conditions compatible with industrial applications, as already reported in the case of bacterial laccases and dye-decolorizing peroxidases (DyPs) [15–19]. Peroxi-dase activities were identified within members of different bacterial taxa, especially Proteobacteria, Firmicutes, Acidobacteria, and Actinobacteria [1,15,17]. Particularly, filamentous actinomycetes, which are mycelial, multicellular soil bacteria that grow similarly to fungi and share the same ecological niche, represent an attractive group for isolating novel peroxidase enzymes putatively involved in lignin degradation [16,19]. Actinomycetes are aerobic, chemoorganotrophic, Gram-positive bacteria that play an important role in degrading organic polymers in nature, including lignin [20–22]. The first secreted peroxidase enzyme reported to be produced by a filamentous actinomycete was the extracellular LiP from Streptomyces viridosporus T7A [23]. Since then, it was reported that Streptomyces spp. produced a few laccases [24,25] and most recently peroxidase activity [26].

There are also reports of peroxidase secretion by other soil (not-filamentous) actinomycetes (i.e., Nocardia and Rhodococcus) [19,27]. Among the filamentous actinomycetes, streptomycetes can be easily isolated and cultivated by the commonly and traditionally used microbiological methods, but increasing evidence is showing that other less known genera of filamentous actinomycetes might be widespread in specific environments, where they play a role in lignin degradation [28]. In the present study, we report on the screening of 43 filamentous actinomycetes belonging to different genera/families, including representatives of more difficult-to-handle actinomycetes [29,30]. Following this approach, we discovered and investigated the biochemical properties of a novel and efficient peroxidase activity produced by a Nonomuraea strain (Streptosporangiaceae family) recently classified as Nonomuraea genenensis [31] that might be involved in the natural metabolism of lignin.

2. Material and methods

2.1. Plate assays

Forty-three filamentous actinomycetes belonging to the culture collection of The Protein Factory research center [32] were used in screening. Escherichia coli DH5α (Invitrogen, Carlsbad, CA USA), cultivated according standard procedures [33], was used as a control. For primary screening, agar plates containing minimal salt medium and alkali lignin (also known as Kraft lignin, Sigma-Aldrich code 471003, St. Louis, MO USA) as sole carbon source (MM-L) were used. MM-L composition was as follows (in g/l): 0.8 alkali lignin, 1.6 KH2PO4, 0.5 KH2PO4, 0.58 MgSO4·7H2O, 0.25 NaCl, 0.013 CaCl2·2H2O, 1.25 (NH4)2SO4, 1 NH4NO3, 0.0025 FeCl3·6H2O, 0.0025 CuCl2, 0.0025 MnCl2, 20 noble agar for secondary screening, selected strains were then grown on manniol agar medium (MAM) (in g/l: 20 mannitol, 2 KNO3, 2 MgSO4·7H2O, 2 Na2HPO4, 15 agar) supplemented with 2.2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, 10 mM) or reactive black five (RB5, 20 mg/l), azure B (25 mg/l) or guaiacol (0.1% v/v). The plates were incubated at 30 °C up to 1 month.

2.2. Growth conditions for actinomycetes

Nonomuraea genenensis [31] and Streptomyces coelicolor A3 (2) working cell banks (WCBs) were prepared as previously described [29]. Preinoculum cultures were set up by inoculating 0.75 ml of the WCB into 15 ml VM liquid medium (in g/l: 24 soluble starch, 1 glucose, 3 meat extract, 5 yeast extract, and 5 tryptose) in 100 ml Erlenmeyer flasks, incubated at 28 °C and 200 revolutions per minute (rpm) for 72 h. An aliquot of 1.8 ml of these cultures was transferred into 300-ml baffled Erlenmeyer flasks containing 50 ml of two different basal media, VM and MM-L (liquid version, without agar), to which the following components could be added: 0.8 or 1.5 g/l alkali lignin, 6 or 12 g/l yeast extract, 2 mM MnCl2, 2 mM CuSO4, 0.2 mM FeSO4·7H2O, 5 mM tryptophan, 0.5 g/l mannose, 0.5 g/l glucose, 6 g/l meat extract, 1 g/l hydrolyzed casein, and 3.5 or 5.0% v/v ethanol. Flask cultures were incubated at 28 °C and 200 rpm, up to 480 h and regularly sampled. The growth curves were determined by collecting 5 ml of the culture, centrifuged at 1900 × g for 10 min at room temperature: on the supernatant, pH and residual glucose were measured with pH Test Strips 4.0–10.0 (Sigma-Aldrich, St. Louis, MO USA) and Diastix strips (Bayer, Leverkusen Germany), respectively; biomass was measured as wet weight on the pellet.
2.3. Enzyme assays

Enzyme activities were assayed spectrophotometrically at 25 °C as follows. Laccase and MnP activities were measured by monitoring the oxidation of ABTS (ε270nm = 36,000 M⁻¹ cm⁻¹) at 420 nm for 5 min. The laccase activity was assayed on 0.5 mM ABTS in 50 mM sodium acetate, pH 5.0, and the MnP activity on 0.5 mM ABTS, 0.05 mM H2O2, 0.16 mM MnCl2, in 40 mM sodium citrate buffer, pH 4.5 (100 μg protein sample in 1 ml final volume). One unit of activity was defined as the amount of enzyme that oxidized 1 μmol of ABTS per min at 25 °C. Furthermore, enzymatic activity was assayed by monitoring the enzymatic oxidation of 2,4-dichlorophenol (2,4-DCP) as substrate in the presence of H2O2 and 4-aminophenol; a 1 ml reaction mixture containing 200 μl protein sample, 5 mM 2,4-DCP (dissolved in ethanol), 3.2 mM 4-aminophenol, 10 mM H2O2 in 20 mM potassium phosphate, pH 7.0, was used. The reaction was monitored for 5 min following the absorbance change at 510 nm (ε510nm = 21,647 M⁻¹ cm⁻¹). One unit of enzyme activity corresponded to an increase of 1.0 absorbance unit per min. The presence of peroxidase activity in the broth was also detected with 0.125 mM H2O2 and 2 mM 2,6-dimethoxyphenol (2,6-DMP) in 50 mM sodium acetate buffer, pH 5.0 (1 ml final assay volume, ε468nm = 49,600 M⁻¹ cm⁻¹).

2.4. Enzyme preparation

N. gerenzanensis was grown in 500-ml baffled Erlemeyer flasks containing 100 ml VM medium supplemented with 0.8 g/l alkali lignin and 2 mM CuSO4. Cells were removed after 20 days by centrifugation at 10,000 × g for 15 min at 4 °C. Cell-free broth was filtered twice on cotton and paper filters and then concentrated by means of tangential flow microfiltration cassette Pellicon-XL (Millipore, Billerica, MA USA) with a 10-kDa cut-off membrane and washed several times, adding 50 mM sodium acetate buffer, pH 5.0. Fractional precipitation was performed at 30, 50, and 75% w/v of [NH4]2SO4 saturation. The protein precipitated at 75% w/v of [NH4]2SO4 saturation was resolubilized in 50 mM sodium acetate buffer, pH 5.0, and dialyzed against the same buffer.

2.5. Kinetic properties

The kinetic parameters of the sample obtained by 75% (NH4)2SO4 precipitation were determined at room temperature in the presence of different concentrations of H2O2 and ABTS (2–1000 μM), catechol (2–10,000 μM, ε410nm = 2211 M⁻¹ cm⁻¹), or 2,6-DMP (2–1000 μM) in 50 mM sodium acetate buffer, pH 5.0, at 25 °C. The LiP activity was assayed on 2.5 mM veratryl alcohol (ε310nm = 9300 M⁻¹ cm⁻¹) in the same buffer, at pH 5.0 or 3.0. The specific activity was expressed as unit per mg of total protein (determined by Biuret analysis). The activity on H2O2 was assayed in the presence of 2 mM 2,6-DMP; the activity on ABTS, 2,6-DMP, veratryl alcohol, and catechol was assayed in the presence of 0.125 mM H2O2. The kinetic data were fitted to the Michaelis–Menten equation or to the one modified to account for substrate inhibition [34,35].

The effect of pH on the peroxidase activity towards 2,6-DMP and H2O2 was determined in 100 mM multicomponent buffer (33 mM Tris–HCl, 33 mM Na2CO3, 33 mM H3PO4), in the 3.0–9.0 pH range [36]. The pH dependence of peroxidase activity was fitted using Eq. (1), based on two ionizations:

\[ y = \frac{[a + b \cdot (10^{pH-\text{PK2}})/(1 + 10^{pH-\text{PK2}})] + [b + c \cdot (10^{pH-\text{PK2}})/(1 + 10^{pH-\text{PK2}})]}{10^{pH-\text{PK2}}} \]  

(1)

where a is the limiting activity value at acidic pH, b is the calculated intermediate value, and c is the limiting activity value at basic pH. The effect of NaCl, dimethyl sulfoxide (DMSO), and Tween-80 concentration on the peroxidase activity toward 2,6-DMP was determined in 50 mM sodium acetate buffer, pH 5.0. Temperature dependence of peroxidase activity was determined by measuring the enzymatic 2,6-DMP oxidation in the 10–70 °C temperature range. Enzyme preparation stability was measured at 25 and 37 °C by incubating the enzyme solution in 50 mM sodium acetate buffer, pH 5.0: samples were withdrawn at different times and residual activity was determined using the 2,6-DMP assay. The peroxidase activity was also assayed in the presence of 0.125 mM H2O2 and of different concentrations of MnCl2 (2–10,000 μM) or of the dye remazol brilliant blue R (RBBR; Sigma-Aldrich, St. Louis, MO USA) (1–50 μM), in 50 mM sodium malonate buffer, pH 4.5, at room temperature. The extinction coefficients were as follows: ε270nm = 11,590 M⁻¹ cm⁻¹ for Mn3+-malonate complex, ε590nm = 8300 M⁻¹ cm⁻¹ for RBBR.

2.6. SDS-PAGE and native-PAGE

Laemmli sample buffer was added to the proteins from the fermentation broth and the proteins were then separated by SDS-PAGE using 14% w/v acrylamide. They were visualized by staining with Coomassie Brilliant Blue R-250. Native-PAGE analysis was performed on a 14% w/v acrylamide-resolving gel without SDS. Molecular markers were from Thermo Fisher Scientific (Waltham, MA USA). Two different staining procedures were employed: a) dye decolorizing peroxidase activity was visualized by incubating the gel in 50 mM sodium acetate buffer, pH 5.0, containing 0.1 mM RBBR for 15 min; b) peroxidase activity was visualized by incubating the gel in 50 mM sodium acetate buffer, pH 5.0, containing 0.125 mM H2O2 at 25 °C.

3. Results

3.1. Screening for ligninolytic activity

A total of 43 actinomycetes belonging to different genera (Actinoplanes, Streptomyces, Nonomuraea, Microbispora, and Planomonospora, see list in Appendix A Supplementary data Table A1) were screened for their ability to grow on alkali lignin as sole carbon source in solid media. Alkali lignin is a commercially available preparation of soluble, semi-degraded (molecular mass ca. 10,000) and chemically modified derivative of insoluble high-molecular mass lignin (for its preparation see [37]). Here, 33 strains grew in the presence of alkali lignin, with two of them, i.e., Streptomyces coelicolor A3(2) and Nonomuraea gerenzanensis (former Nonomuraea sp. ATCC 39727), forming a clear degradation halo around the colony (not shown). The genome of S. coelicolor A3 (2) contains a gene for a two-domain laccase, called SLAC [25,38,39], whose role in degrading lignocellulosic biomass was recently demonstrated [16]. N. gerenzanensis is a recently classified species [31] and its genome is not yet available; to our knowledge, this is the first report on its ability to use soluble lignin derivatives for growing.

N. gerenzanensis was therefore selected for further analyses and its ability to produce laccase and peroxidase enzymes was tested on agar plates supplemented with differently colored indicator compounds (ABTS, guaiacol, and the dyes RB5 and azure B). Tests were run in parallel with S. coelicolor A3(3) and E. coli DH5α, used as positive and negative controls, respectively. MAM medium was selected since it supports growth of actinomycetes but reduces pigment production, which otherwise interferes with the detection of oxidative activity [40]. N. gerenzanensis was able to oxidize...
Table 1
Screening for oxidase activities from *S. coelicolor* A3(2) and *N. gerenzanensis* on agar plates. *E. coli* DH5α did not produce any detectable oxidase activity in the same cultivation conditions. The activity is classified on an arbitrary scale as intense (+++), medium (++), weak (+) or absent (-). The days required for the appearance of the activity are reported in parentheses.

| Substrate | pH   | *S. coelicolor* A3(2) | *N. gerenzanensis* |
|-----------|------|-----------------------|--------------------|
| ABTS      | 4.5  | +++ (3)               | + (14)             |
|           | 6.0  | ++ (3)                | + (14)             |
|           | 8.0  | ++ (6)                | + (9)              |
| Guaiacol  | 4.5  | +++ (3)               | –                  |
| 0.1% v/v  | 6.0  | ++ (14)               | –                  |
|           | 8.0  | ++ (21)               | –                  |
| RB5       | 4.5  | +++ (10)              | –                  |
| 20 mg/l   | 6.0  | +++ (10)              | –                  |
|           | 8.0  | ++ (10)               | + (10)             |
| Azure B   | 4.5  | +++ (14)              | + (14)             |
| 25 mg/l   | 6.0  | ++ (14)               | + (14)             |
|           | 8.0  | ++ (14)               | + (14)             |

ABTS and decolorize both dyes, but lacked oxidative activity on guaiacol (Table 1 and Fig. 1).

*E. coli* DH5α was not active on the indicator compounds, while the enzymes secreted by *S. coelicolor* A3(2) rapidly oxidized ABTS and guaiacol and decolorized RB5 and azure B (Table 1 and Fig. 1). For both of the selected actinomycetes, the enzymatic activity was pH-dependent: activity appeared enhanced at basic pH in *N. gerenzanensis*, that from *S. coelicolor* A3(2) in more acidic environment (Table 1).

3.2. Production of oxidative enzymes by *N. gerenzanensis*

*N. gerenzanensis* was cultivated in liquid VM (limpid and rich medium usually employed for growing this microorganism) and MM-L (salt minimal medium containing alkali lignin as carbon source) media supplemented with the specific inducers listed in the Material and Methods section. The addition of metal cations to cultivation media, in particular Mn²⁺, Cu²⁺ and Fe²⁺, might induce laccase and peroxidase production since metal cations represent the main cofactors of these enzymes [41]. Indeed, the use of ethanol and aromatic compounds was previously reported to stimulate laccase activity [42]. Finally, different nitrogen sources and their concentrations can influence the production of peroxidase enzymes in filamentous microorganisms and fungi [43]. For *N. gerenzanensis*, peroxidase activity was medium-dependent, whereas laccase activity was never detected (Table 2). In particular, relevant peroxidase activity (53.4 and 3.0 U/l on ABTS and 2,4-DCP as substrate, respectively) was observed in the minimal MM-L medium (containing alkali lignin) to which yeast extract and ethanol were added, whereas in the nitrogen- and carbon-rich VM medium the highest activity (65.9 and 13.8 U/l on ABTS and 2,4-DCP, respectively) was achieved by supplementing 0.8 g/l alkali lignin and 2 mM CuSO₄. Since agitation rates can influence peroxidase production in filamentous microorganisms and fungi [43], *N. gerenzanensis* was grown at different shaking conditions: without shaking, no peroxidase activity was recorded, while it increased with the shaking (the measured activity was higher at 200 rpm than at 100 rpm, data not shown).

Time courses for *N. gerenzanensis* growth and peroxidase activity production in the medium that performed better (i.e., VM added with 0.8 g/l alkali lignin and 2 mM CuSO₄) are reported in Fig. 2. In the first phase of growth, glucose was consumed, pH increased to almost 9.0, and biomass production reached its maximum of 68 g/l wet weight (Fig. 2A). Peroxidase started when cells, after 192 h from inoculum, entered into the stationary phase of growth, reaching the maximum volumetric productivity after about 20 days of growth (Fig. 2B). Peroxidase activity production during stationary phase of growth and at prolonged cultivation time is typical also for several peroxidase-producing fungi [13,14].

3.3. Enzyme preparation

The peroxidase activity in the crude broth was ca. 14, 65, and 140 U/l on 2,4-DCP, ABTS, and 2,6-DMP as substrates, respectively. The broth was clarified (to eliminate aggregates and cell residues) by centrifugation and a two-step filtration; the sample was then concentrated 10-fold. Following a fractional precipitation with ammonium sulfate, the peroxidase activity was recovered in the precipitate at 75% of saturation: this sample contained 36 mg of...
protein from 11 of fermentation broth, with a specific activity of 1.98 U/mg protein on 2,6-DMP as substrate (Table 3).

Although the enzyme preparation in SDS-PAGE showed many protein bands, the peroxidase activity was clearly observed in native-PAGE analyses (Fig. 3, lanes 3 and 4). Notably, the staining for dye-decolorizing peroxidase and classical peroxidase (on ABTS) activity co-localized, thus suggesting that both activities originated from the same enzyme.

3.4. Kinetic properties

The kinetic parameters of the peroxidase preparation from N. gerenzanensis were determined on H₂O₂, the nonphenolic ABTS, and the phenolic 2,6-DMP and catechol as substrates (Table 4). In all cases, the dependence of the activity values on the substrate concentration followed Michaelis-Menten kinetics, the only exception being H₂O₂, which showed a substrate inhibition effect (Kᵢ ≈ 340 μM). The highest activity was observed on catechol as substrate (≈ 3.8 U/mg protein).

N. gerenzanensis peroxidase preparation also possessed a dye-decolorizing activity: notably, it showed a high affinity for RBBR (Kᵢ ≈ 13 μM, significantly lower than for the other canonical substrates, Tables 4 and 5). The same preparation also showed a manganese peroxidase activity, although the catalytic efficiency was low (Table 5). Indeed, no lignin peroxidase activity was observed on veratryl alcohol as substrate.

3.5. Effect of pH and temperature

The activity of the peroxidase preparation from N. gerenzanensis on 2,6-DMP was determined at different pH and temperature values. The maximal activity occurred at acidic pH values (Fig. 4A).

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**Table 2**

Peroxidase activity production by N. gerenzanensis in different liquid media. Volumetric activities are reported after 480 h from the inoculum.

| Basal medium | Additions | Activity (U/l) on: |
|--------------|-----------|-------------------|
|              |           | ABTS              |
|              |           | 2,4-DCP           |
| MM-L[^a^]    | 6 g/l yeast extract | 4.4 | 0 |
|              | 12 g/l yeast extract | 5.0 | 1.6 |
|              | 6 g/l yeast extract + 2 mM CuSO₄ | 4.1 | 3.9 |
|              | 6 g/l yeast extract + 2 mM MnCl₂ | 9.0 | 4.4 |
|              | 6 g/l yeast extract + 0.2 mM FeSO₄ | 1.8 | 1.6 |
|              | 6 g/l yeast extract + 5 mM tryptophan | 1.1 | 0 |
|              | 6 g/l yeast extract + 0.5 g/l glucose | 1.8 | 2.0 |
|              | 6 g/l yeast extract + 0.5 g/l mannose | 2.5 | 2.0 |
|              | 6 g/l yeast extract + 6 g/l meat extract | 4.0 | 1.7 |
|              | 6 g/l yeast extract + 1 g/l hydrolyzed casein | 1.1 | 1.7 |
|              | 6 g/l yeast extract + 3.5% v/v ethanol | 2.5 | 1.2 |
|              | 6 g/l yeast extract + 5.0% v/v ethanol | 53.4 | 3.0 |
|              | 6 g/l yeast extract + 3.5% v/v ethanol + 2 mM CuSO₄ | 42.2 | 2.6 |
| VM[^b^]      | 0.8 g/l alkali lignin | 15.2 | 5.6 |
|              | 1.5 g/l alkali lignin | 1.4 | 2.5 |
|              | 2 mM CuSO₄ + 2 mM MnCl₂ + 0.2 mM FeSO₄ | 3.1 | 9.3 |
|              | 0.8 g/l alkali lignin + 2 mM CuSO₄ | 0 | 7.3 |
|              | 0.8 g/l alkali lignin + 2 mM MnCl₂ | 65.9 | 7.4 |
|              | 0.8 g/l alkali lignin + 0.2 mM FeSO₄ | 0 | 7.4 |
|              | 0.8 g/l alkali lignin + 5 mM tryptophan | 0.6 | 7.9 |
|              | 0.8 g/l alkali lignin + 3.5% v/v ethanol | 0 | 7.2 |
|              | 0.8 g/l alkali lignin + 5.0% v/v ethanol | 6.3 | 7.2 |
|              | 0.8 g/l alkali lignin + 3.5% v/v ethanol + 2 mM CuSO₄ | 16.3 | 13.8 |

[^a^]: MM-L contains salts and 0.8 g/l alkali lignin.
[^b^]: VM contains complex nitrogen and carbon sources.

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Fig. 2. Fermentation of N. gerenzanensis in VM medium supplemented with 0.8 g/l alkali lignin and 2 mM CuSO₄. (A) Growth curve: wet weight (●, continued line), pH (●, dashed line) and residual glucose (■, dotted line); (B) Time course of peroxidase activity in N. gerenzanensis fermentation broth assayed on ABTS (white bars) and on 2,4-DCP (black bars). Values represent the means of three independent experiments (mean ± standard error).
Table 3
Partial purification of peroxidase activity from N. gerenzanensis fermentation broth.

| Purification step                        | Volume (ml) | Total proteins (mg) | Total activity (U) | Specific activity (U/mg protein) | Purification (-fold) | Yield (%) |
|------------------------------------------|-------------|---------------------|--------------------|----------------------------------|----------------------|-----------|
| Crude broths                             | 1000        | 5000                | 140.0              | 0.03                             | 1                    | 100.0     |
| Filtration-concentration                 | 100         | 290                 | 108.0              | 0.37                             | 13                   | 77.1      |
| Ammonium sulfate precipitation (75%)    | 12          | 36                  | 70.4               | 1.98                             | 71                   | 50.3      |

* Activity was assayed on 0.125 mM H₂O₂ and 2 mM 2,6-DMP as substrate in 50 mM sodium acetate buffer, pH 5.0.

Table 4
Kinetic parameters of N. gerenzanensis peroxidase preparation on canonical substrates. The activity was assayed at pH 5.0 and 25 °C.

| Substrate | 2,6-DMP | Catechol |
|-----------|---------|----------|
| H₂O₂      | ABTS    |          |
| Vₘₐₓ      | Kₘ      | Kₘ      | Vₘₐₓ=(Kₘ/Vₘₐₓ) | Vₘₐₓ=(Kₘ/Vₘₐₓ) | Vₘₐₓ=(Kₘ/Vₘₐₓ) |
| (U/mg)    | (μM)    | (μM)    | (μM)             | (μM)             | (μM)             |
| 2.84 ± 0.17 | 28 ± 3  | 341 ± 62 | 0.101 ± 0.017   | 2.19 ± 0.05      | 842 ± 51         |
| 0.0066 ± 0.0002 | 1.98 ± 0.08 | 512 ± 23 | 0.013 ± 0.0002 | 3.81 ± 0.13 | 41 ± 6 |
| 0.007 ± 0.0002 | 41 ± 6  | 0.093 ± 0.016 |          |                    |                    |

Table 5
Kinetic parameters for manganese oxidation and dye decolorization activity of N. gerenzanensis peroxidase preparation. The activity was assayed in 50 mM sodium malonate, pH 4.5, at 25 °C.

| Substrate | Mn²⁺ | RBBR |
|-----------|------|------|
| Vₘₐₓ      | Kₘ   | Vₘₐₓ=(Kₘ/Vₘₐₓ) | Vₘₐₓ=(Kₘ/Vₘₐₓ) |
| (U/mg)    | (μM) | (μM)             | (μM)             |
| 0.240 ± 0.045 | 531 ± 200 | 19.4 ± 11.3 | 0.128 ± 0.007 |
| 0.128 ± 0.007 | 13 ± 2 | (9.8 ± 2.1) × 10⁻³ |

and enzymatic activity could also be detected in the 7.0–9.0 pH range (a pKᵢ value 5.8 for the second ionization was determined based on a two-ionizations equation). Peroxidase preparation from N. gerenzanensis possessed a good stability in the 3.0–7.0 pH range following incubation for 24 h at 25 °C (Fig. 4B), showing the highest residual activity at pH 4.0–5.0. The trend of pH stability resembled the one observed for pH activity.

The N. gerenzanensis peroxidase preparation is quite thermophilic, showing an optimum at around 60 °C (Fig. 4C), and is quite stable: after 24 h incubation at 25 and 37 °C, peroxidase maintained ca. 90% of its initial activity.

3.6. Effect of NaCl, solvents, and detergents

A further main issue affecting peroxidase applications in decolorizing dye effluents is the presence of halide ions. Accordingly, the effect of sodium chloride concentration on enzymatic activity was investigated. Interestingly, the enzymatic activity of N. gerenzanensis peroxidase increased at increasing NaCl concentration, reaching a 1.6-fold increase in the presence of 1 M NaCl (Fig. 5A).

In order to verify the potential for using the peroxidase in processes requiring solvents, the effect of DMSO on enzymatic activity was also investigated. In the presence of 30% v/v DMSO, N. gerenzanensis peroxidase retained ca. 20% of the activity value assayed in the presence of buffer only (Fig. 5B). Indeed, the enzymatic activity was strongly affected by the presence of Tween-80 in the reaction medium; in the presence of 1% v/v of the detergent, the activity was halved (Fig. 5C).

4. Discussion

Peroxidases represent one of the main components of the ligninolytic system and comprise several members, namely LiPs, VPs, and MnPs. These enzymes are oxidoreductases that utilize hydrogen peroxide for catalyzing oxidation of structurally diverse substrates. Since a single peroxidase can act on a wide range of substrates by employing different modes of oxidation, peroxidase classification based on structure-function relationships is not simple. In addition, peroxidases having diverse molecular structures may catalyze the same reaction.

The white-rot fungus P. chrysosporium secretes an exceptional array of peroxidases, which act synergistically during ligninolysis;
these enzymes are currently used in other biotechnology applications including transformation of environmental pollutants and biobleaching of pulp water. Production of differently-composed peroxidase cocktails was also reported in other white-rot fungi, especially in Pleurotus spp.; recently, sequencing of the Pleurotus ostreatus genome revealed a comprehensive picture of the peroxidase gene family, consisting of three VPs and six short-MnPs [44,45]. Notably, the production of MnP is apparently limited to certain basidiomycetous fungi [44], whereas P. chrysosporium wild-type does not produce VPs [46]. Indeed, white-rot fungi produce diverse patterns of LiPs, and/or VPs, and/or MnPs depending on the species, medium composition and cultivation period [47]. Genes encoding such enzymes were found to be differentially regulated in response to a wide variety of environmental signals such as the concentration and origination of bioavailable nitrogen and carbon [13]. Several fungal species such as P. chrysosporium produce LiPs and MnPs in liquid media under nutrient-limited conditions; in contrast, for other species such as P. ostreatus and Trametes trogii, MnPs are preferentially produced in the presence of high concentration of nutrient nitrogen [13]. Additionally, P. ostreatus, Pleurotus eryngii and T. trogii were found to produce relatively low amounts of peroxidases when grown on solid wheat straw medium [47].

Here we demonstrated that a novel bacterial species belonging to Nonomuraea genus produces during stationary phase (when grown in liquid media containing alkali lignin) a peroxidase activity, whose features favorably compare with the fungal enzymes. In fact, when compared to P. chrysosporium MnP, our N. gerenzanensis peroxidase preparation shows a significantly higher stability at pH >6.5 and a higher thermostability (P. chrysosporium MnP is fully inactivated in ≈ 3 min at 55 °C) [46,48]. Although more active at acidic than at basic pHS, N. gerenzanensis peroxidase activity is more stable at higher pHS than the fungal counterparts. This finding is coherent with the ecological niche from which this actinomycete was isolated: it was demonstrated that it grows easily at pHs of 10.0 and 11.0 [31], whereas fungi usually prefer acidic conditions. From a kinetic point of view, N. gerenzanensis peroxidase activity shows a $K_{m}$, for H$_2$O$_2$ similar to that of P. chrysosporium and Bjerkandera MnPs (≈ 30–50 μM), and a lower affinity for Mn$^{2+}$ ($K_{m}$ = 50–80 μM) [46,49]. Our preparation also differs from P. ostreatus MnPs: the six known MnP isoenzymes significantly differ in $K_{m}$ for H$_2$O$_2$ (23–530 μM) and for Mn$^{2+}$ (≈ 7–101 μM) [50]. Indeed, in contrast to the Bjerkandera MnP, no activity was apparent for the N. gerenzanensis peroxidase on veratryl alcohol (although both enzymes were able to oxidize ABTS and 2,6-DMP), even when the activity was assayed at pH 3.0. This result demonstrates that N. gerenzanensis does not produce LiP or VP-like activities. Furthermore, N. gerenzanensis peroxidase preparation shows a dye-decolorizing activity that expands its substrate range and paves the way for using this enzyme in industrial sectors, including the textile (for bleaching) and dye industry. Indeed, dye-decolorizing peroxidases show activity on lignin model compounds [19], a further valuable field of application.

In conclusion, we discovered a valuable peroxidase activity produced by a novel species belonging to the Nonomuraea genus by screening 43 filamentous actinomycetes from different genera/families that are considered a yet-poorly-exploited promising source for enzymes involved in lignin modification and degradation [28]. Successful ingredients for such screening were (i) assaying enzyme activity on diverse compounds that act as preferential substrates for different families of oxidases (laccases, LiP, MnP and VP peroxidases), and (ii) exploiting a novel bacterial group that is involved in lignin degradation and resembles fungal life style. To our knowledge, this is the first report on a secreted peroxidase activity from a microorganism belonging to Nonomuraea taxon. Further studies will be devoted to the purification to homogeneity of the enzyme(s) responsible of the peroxidase activity detected in N. gerenzanensis. We are aware that understanding the role of such enzyme(s) in lignin degradation in nature is not straightforward since the model compounds (such as the commonly used alkali lignin) are water soluble and can be degraded by substrate-specific enzymes that could differ from those attacking complex, water-insoluble lignin. Moreover, in white-rot fungi growing on wheat straw medium the rate of lignin degradation was found as not necessarily correlated with the level of laccase and peroxidase activities, suggesting that additional activities should be involved [47]. Although we cannot simply correlate peroxidase production with lignin degradation, we are confident that genome sequencing of this novel microbial species,

![Fig. 4. Effect of pH and temperature on the activity and stability of N. gerenzanensis peroxidase preparation. (A) pH effect on the enzymatic activity assayed on 2 mM 2,6-DMP and 0.125 mM H$_2$O$_2$ as substrates and at 25 °C. The value at pH 5.0 was taken as 100%. The data were fitted using Eq. (1), based on two ionizations: $pK_{a1}$ is 5.8 ± 0.1 (and $pK_{a2}$ is estimated <3.0). (B) Effect of pH on the stability of peroxidase activity determined by measuring 2,6-DMP oxidation. The residual activity was assayed after 24h of incubation at 25 °C: the activity value at time = 0 at each pH value was taken as 100%. (C) Effect of temperature on the peroxidase activity determined as in panel (A). The value at pH 5.0 and 25 °C was taken as 100%. Values represent the means of three independent experiments (mean ± standard error).](image-url)
able to grow on alkali lignin as unique carbon source, will contribute to understand ligninolytic system in actinomycetes. Additionally, production of a peroxidase cocktail in a single bacterial strain clearly remains a desirable trait for degrading a complex substrate as lignin and for converting chemically diverse compounds in biotechnological applications.

Acknowledgements

Authors acknowledge Ilaria Presti for her early work in screening actinomycetes. This work was done as part of the EU ValorPlus project (FP7-KBBE-2013-7-613802) and of BioRefill grant (Project ID 42611813) to LP. CC and FT are PhD students of the “Dottorato in Biotecnologie, Bioscienze e Tecnologie Chimiche” at Università degli Studi dell’Insubria. The support of Consorzio Interuniversitario per le Biotecnologie (CIB) is also acknowledged (FB).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2016.12.005.

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