Biotechnology Reports 13 (2017) 49–57

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

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

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ARTICLE INFO

Article history:
Received 26 August 2016
Received in revised form 7 December 2016
Accepted 21 December 2016
Available online 3 January 2017

Chemical compounds studied in this article:
ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (PubChem CID: 5360881)
Azure B (PubChem CID: 68275)
Cathecol (PubChem CID: 289)
2,4-DCP, 2,4-dichlorophenol (PubChem CID: 8449)
2,6-DMP, 2,6-dimethoxyphenol (PubChem CID: 78828)
Guaiacol (PubChem CID: 460)
RB5, reactive black 5 (PubChem CID: 44134920)
RBBR, remazol brilliant blue R (PubChem CID: 17409)
Veratryl alcohol (PubChem CID: 7118)

Keywords:
Alkali lignin
Kraft lignin
Peroxidases
Filamentous actinomycetes
Nonomuraea gerenzanensis

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_2O_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.

1. Introduction

Lignocellulose, consisting of a complex of three main polymers, i.e., lignin, cellulose, and hemicellulose, is the major structural constituent of plant biomass and represents the most abundant renewable carbon feedstock on earth [1,2]. Lignin, which accounts for *ca.* 20% of the lignocellulosic material, has a complex and heterogeneous molecular architecture, derived from the oxidative coupling of three main phenylpropanoid monomers (*p*-coumaryl, coniferyl, and synapyl alcohols) [3]. Due to its complex structure,

Abbreviations: LiP, lignin peroxidase; MnP, manganese peroxidase; VP, versatile peroxidase; DyP, dye decolorizing peroxidase; MM-L, minimal salt medium plus lignin; MAM, mannitol agar medium; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); RB5, reactive black 5; 2,4-DCP, 2,4-dichlorophenol; 2,6-DMP, 2,6-dimethoxyphenol; RBBR, remazol brilliant blue R.

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http://dx.doi.org/10.1016/j.btre.2016.12.005

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 lignocellulose 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 polyols) 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 1.11.1.14), manganese peroxidases (MnP, EC 1.11.1.13), and versatile peroxidases (VP, EC 1.11.1.16) [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 hydroxylation. Notably, LiPs use H_2O_2 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]. Peroxidase activities were identified within members of different bacterial taxa, especially *Proteobacteria*, *Firmicutes*, *Acidobacteria,* and *Actinobacteria* [1,15,17]. Particularly, filamentous actinomycetes, which are myceliar, 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 gerenzanensis* [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 K_2HPO_4, 0.5 KH_2PO_4, 0.58 $MgSO_4{\cdot}7H_2O$, 0.25 NaCl, 0.013 $CaCl_2{\cdot}2H_2O$, 1.25 $(NH_4)_2SO_4$, 1 NH_4NO_3, 0.0025 $FeCl_3{\cdot}6H_2O$, 0.0025 $CuCl_2$, 0.0025 $MnCl_2$, 20 noble agar. For secondary screening, selected strains were then grown on mannitol agar medium (MAM) (in g/l: 20 mannitol, 2 KNO_3, 2 $MgSO_4{\cdot}7H_2O$, 2 Na_2HPO_4, 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 gerenzanensis [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 $MnCl_2$, 2 mM $CuSO_4$, 0.2 mM $FeSO_4{\cdot}7H_2O$, 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 activity was measured by monitoring the oxidation of ABTS (ε_{420nm} = 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 H_2O_2, 0.16 mM $MnCl_2$, in 40 mM sodium citrate buffer, pH 4.5 (100 μl 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 H_2O_2 and 4-aminoantipyrine; a 1 ml of reaction mixture containing 200 μl protein sample, 5 mM 2,4-DCP (dissolved in ethanol), 3.2 mM 4-aminoantipyrine, 10 mM H_2O_2 in 20 mM potassium phosphate, pH 7.0, was used. The reaction was monitored for 5 min following the absorbance change at 510 nm (ε_{510nm} = 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 H_2O_2 and 2 mM 2,6-dimethoxyphenol (2,6-DMP) in 50 mM sodium acetate buffer, pH 5.0 (1 ml final assay volume, ε_{468nm} = 49,600 M^{-1} cm^{-1}).

2.4. Enzyme preparation

N. gerenzanensis was grown in 500-ml baffled Erlenmeyer flasks containing 100 ml VM medium supplemented with 0.8 g/l alkali lignin and 2 mM $CuSO_4$. 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 $(NH_4)_2SO_4$ saturation. The protein precipitated at 75% w/v of $(NH_4)_2SO_4$ 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% $(NH_4)_2SO_4$ precipitation were determined at room temperature in the presence of different concentrations of H_2O_2 and ABTS (2–1000 μM), catechol (2–10,000 μM, ε_{410nm} = 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 (ε_{310nm} = 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 H_2O_2 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 H_2O_2. 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 H_2O_2 was determined in 100 mM multicomponent buffer (33 mM Tris-HCl, 33 mM Na_2CO_3, 33 mM H_3PO_4), in the 3.0–9.0 pH range [36]. The pH dependence of peroxidase activity was fitted using Eq. (1), based on two ionizations:

$$y = [(a + b\ (10^{pH-pKa})/(1 + 10^{pH-pKa}))] + [(b + c\ (10^{pH-pKa2})/(1 + 10^{pH-pKa2}))] \quad (1)$$

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 H_2O_2 and of different concentrations of $MnCl_2$ (2–10,000 μM) or of the dye remazol brillant 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: ε_{270nm} = 11,590 M^{-1} cm^{-1} for Mn^{3+}-malonate complex, ε_{595nm} = 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; the gel was then washed and incubated with 50 mM sodium acetate buffer, pH 5.0, containing 0.125 mM H_2O_2 at 25 °C; b) peroxidase activity was visualized by incubating the gel in 50 mM sodium acetate buffer, pH 5.0, containing 0.125 mM H_2O_2 and 2 mM ABTS, 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 A.1) 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)
10 mM	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 Mn^{2+}, Cu^{2+} and Fe^{2+}, 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 $CuSO_4$. 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 $CuSO_4$) 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 production 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

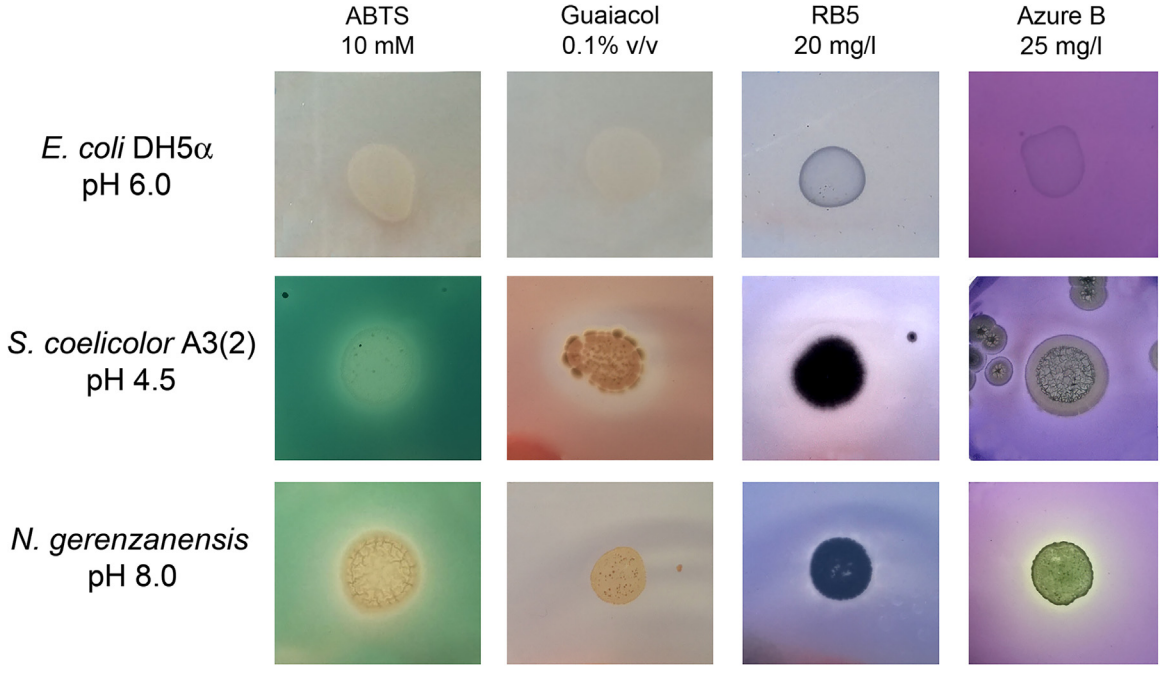

Fig. 1. Screening for ligninolytic activities in MAM agar plates supplemented with different colored indicator compounds (ABTS, guaiacol and the dyes RB5 and azure B).

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]		4.4	0
	6 g/l yeast extract	5.0	1.6
	12 g/l yeast extract	4.1	3.9
	6 g/l yeast extract + 2 mM $CuSO_4$	9.0	4.4
	6 g/l yeast extract + 2 mM $MnCl_2$	1.8	1.6
	6 g/l yeast extract + 0.2 mM $FeSO_4$	1.1	0
	6 g/l yeast extract + 5 mM tryptophan	1.8	2.0
	6 g/l yeast extract + 0.5 g/l glucose	2.5	2.0
	6 g/l yeast extract + 0.5 g/l mannose	4.0	1.7
	6 g/l yeast extract + 6 g/l meat extract	1.1	1.7
	6 g/l yeast extract + 1 g/l hydrolyzed casein	2.5	1.2
	6 g/l yeast extract + 3.5% v/v ethanol	53.4	3.0
	6 g/l yeast extract + 5.0% v/v ethanol	42.2	2.6
	6 g/l yeast extract + 3.5% v/v ethanol + 2 mM $CuSO_4$	15.2	5.6
VM[b]		1.4	2.5
	0.8 g/l alkali lignin	3.1	9.3
	1.5 g/l alkali lignin	0	7.3
	2 mM $CuSO_4$ + 2 mM $MnCl_2$ + 0.2 mM $FeSO_4$	0	0
	0.8 g/l alkali lignin + 2 mM $CuSO_4$	65.9	13.8
	0.8 g/l alkali lignin + 2 mM $MnCl_2$	0	7.4
	0.8 g/l alkali lignin + 0.2 mM $FeSO_4$	0.6	7.9
	0.8 g/l alkali lignin + 5 mM tryptophan	0	7.2
	0.8 g/l alkali lignin + 3.5% v/v ethanol	5.8	0
	0.8 g/l alkali lignin + 5.0% v/v ethanol	16.3	13.8
	0.8 g/l alkali lignin + 3.5% v/v ethanol + 2 mM $CuSO_4$	13.2	7.9

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

protein from 1 l 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 H_2O_2, 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 H_2O_2, which showed a substrate inhibition effect ($K_i \approx 340\ \mu M$). 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 ($K_m \approx 13\ \mu M$, 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)

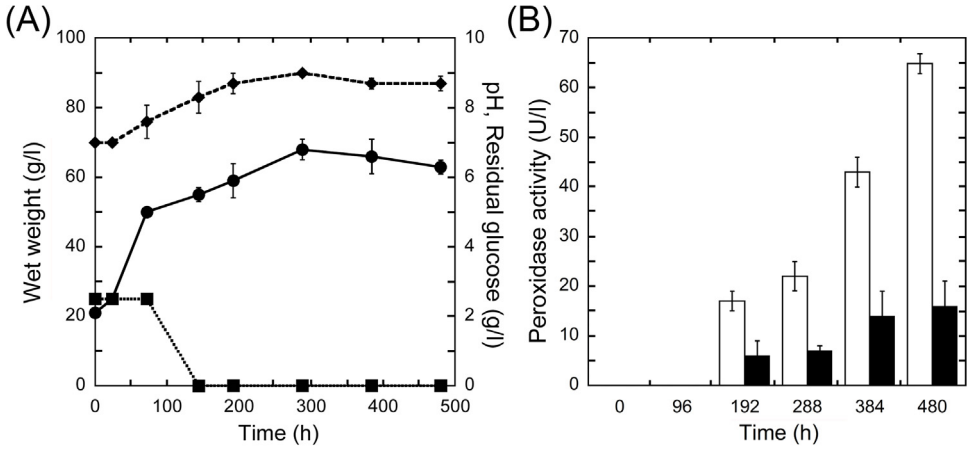

Fig. 2. Fermentation of *N. gerenzanensis* in VM medium supplemented with 0.8 g/l alkali lignin and 2 mM $CuSO_4$. (A) Growth curve: wet weight (●, continued line), pH (◆, dashed line) and residual glucose (■, dotted line). (B) Time course of peroxidase activity in *N. gerenzanensis* fermentation broth 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[a] (U/mg protein)	Purification (-fold)	Yield (%)
Crude broth	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

[a] Activity was assayed on 0.125 mM H_2O_2 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.

H_2O_2				ABTS			2,6-DMP			Catechol		
V_{max} (U/mg)	K_m (μM)	K_i (μM)	V_{max}/K_m	V_{max} (U/mg)	K_m (μM)	V_{max}/K_m	V_{max} (U/mg)	K_m (μM)	V_{max}/K_m	V_{max} (U/mg)	K_m (μM)	V_{max}/K_m
2.84 ± 0.17	28 ± 3	341 ± 62	0.101 ± 0.017	2.19 ± 0.05	842 ± 51	0.0026 ± 0.0002	1.98 ± 0.08	152 ± 23	0.013 ± 0.002	3.81 ± 0.13	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.

Mn^{2+}				RBBR		
V_{max} (U/mg)	K_m (μM)	K_i (mM)	V_{max}/K_m	V_{max} (U/mg)	K_m (μM)	V_{max}/K_m
0.240 ± 0.045	531 ± 240	19.4 ± 11.3	0.128 ± 0.007	0.128 ± 0.007	13±2	$(9.8 \pm 2.1) \times 10^{-3}$

and enzymatic activity could also be detected in the 7.0–9.0 pH range (a pK_a value of 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.

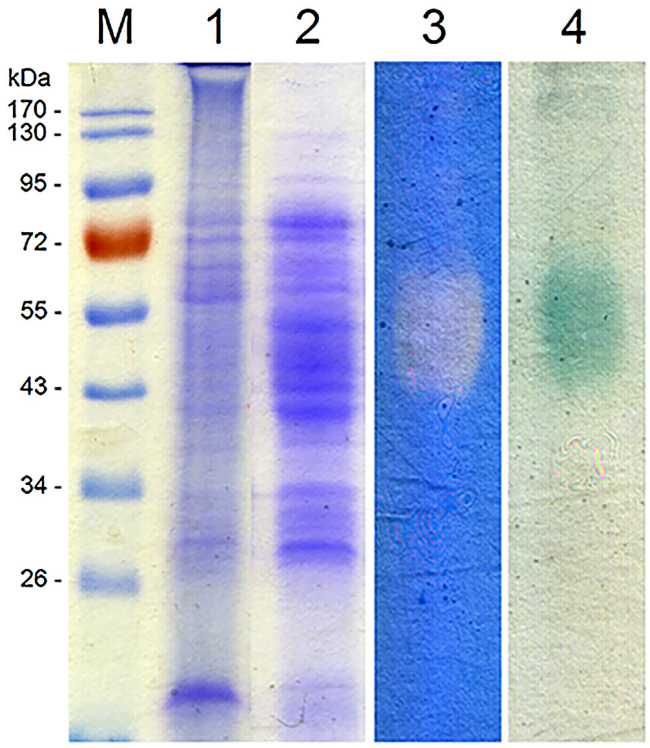

Fig. 3. Electrophoretic analysis of peroxidase from *N. gerenzanensis* broth. SDS-PAGE analysis of (1) concentrated broth and (2) sample obtained by 75% saturation of ammonium sulfate precipitation. Native-PAGE analysis of sample obtained by 75% saturation of ammonium sulfate precipitation with two different activity stainings: (3) dye-decolorizing-peroxidase staining and (4) peroxidase staining. In all lanes, 30 μg of total proteins were loaded. M: marker proteins of known molecular mass.

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;

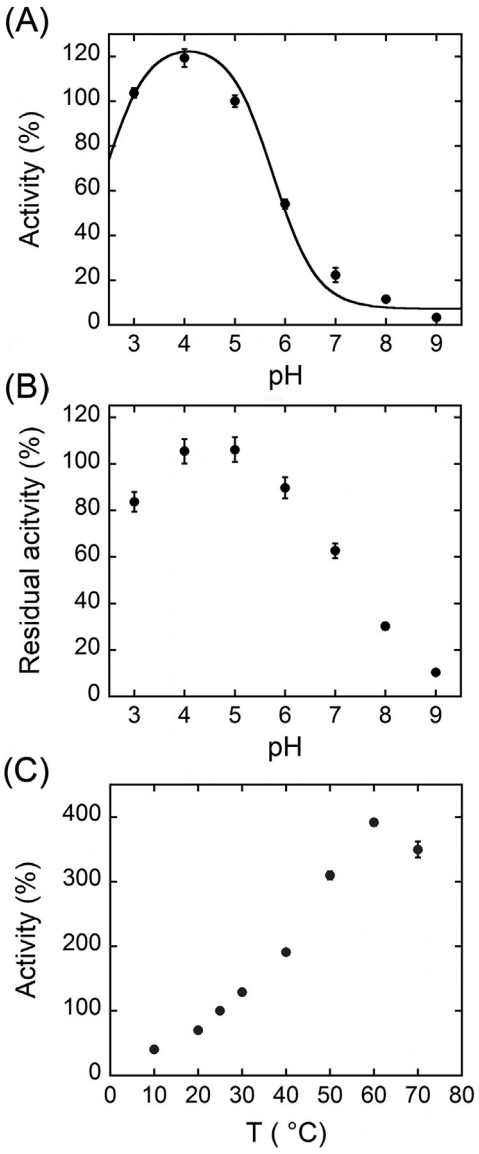

Fig. 4. Effect of pH and temperature on the activity and stability of *N. gerenzanensis* peroxidase preparation. (A) pH effect on the enzymatic activity assayed on 2 mM 2,6-DMP and 0.125 mM H_2O_2 as substrates and at 25 °C. The value at pH 5.0 was taken as 100%. The data were fitted using Eq. (1), based on two ionizations: pK_{a2} is 5.8 ± 0.1 (and pK_{a1} 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 24 h 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).

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 environments. From a kinetic point of view, *N. gerenzanensis* peroxidase activity shows a K_m for H_2O_2 similar to that of *P. chrysosporium* and *Bjerkandera* MnPs (≈ 30–55 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_2O_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,

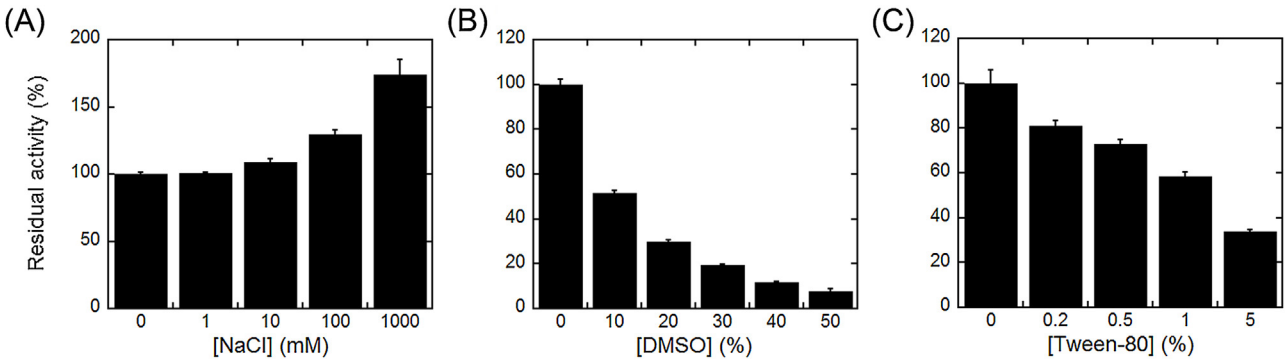

Fig. 5. Effect of NaCl (A), DMSO (B) and Tween-80 (C) concentration on the peroxidase activity, determined by measuring 2,6-DMP oxidation, at pH 5.0, 25 °C. The value in absence of the different compounds was taken as 100%. Values represent the means of three independent experiments (mean ± standard error).

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 Chirurgiche" 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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